Genetic and environmental factors affecting the germination of *Eucalyptus globulus* seeds

By

Kieren David Rix

BAgrSc(Hons)
University of Tasmania

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School of Agricultural Science, University of Tasmania,

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Declaration

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Abstract

Eucalyptus globulus is widely planted in pulpwood plantations in temperate regions of the world, including Australia. Most plantations are established from seedlings rather than clones. Slow and uneven germination of *E. globulus* seed-lots has been a problem in some nurseries and has been linked with periods of high temperatures. Trials were conducted using commercial and research seed-lots to identify genetic and environmental factors affecting seed germination. Six response traits were studied: proportion germination, proportion of normal seedlings, proportion of germinated seeds which developed into normal seedlings, germination rate, normal seedling development rate, and rate of development from germination to normal seedlings.

First, the response of genetically diverse commercial seed-lots across a range of temperatures was studied. Temperatures above 30°C generally delayed germination, reduced total germination percentage, caused a high proportion of seed death and suppressed the development of normal seedlings. Low temperatures also delayed germination but caused less seed mortality. The optimum temperature for rate of normal seedling development was between 24.8°C and 25.5°C and for maximum percentage normal seedling development was between 21.2°C and 24.8°C. The cardinal temperatures for normal seedling development were between 9.1°C and 10.5°C at low temperature and between 40.3°C and 41.4°C at high temperature. Despite general consistency in responses there was evidence that seed-lots can exhibit a differential response to temperature, particularly for normal seedling development at the extremes of temperature tested.

Second, by sampling from multiple randomised ramets (trees) of maternal genotypes in grafted seed orchards across two sites and two seasons, the extent of maternal genetic regulation at the race and genotype level of seed germination responses was assessed. Maternal genotype had a significant effect on most germination traits but a differential response to temperature was only detected for percent germination and rate of development from germination into normal seedlings. The maternal genotype effect on germination traits varied with sampling season and site, suggesting

interaction with environment or harvesting factors. The maternal genotype effect could be explained by maternal race of origin for two traits relating to normal seedling development. Differences between genotypes at 25°C could be used to predict differences at 37°C and germination rate and normal seedling development rate were generally the most correlated between 25°C and 37°C.

Third, by using a small diallel crossing design it was shown that both the paternal and maternal parent can affect the germination response, arguing for at least some influence of the nuclear genotype of the embryo. However, the response to high temperature stress was more influenced by the maternal than paternal parent. These results argue that while the nuclear genotype of the embryo may cause variation in the average germination response, it is the maternal genetic effect that affects the response to high temperature stress. This shows that in addition to maternal environment and maturation effects, there may be additive genetic as well as maternal genetic effects on seed germination responses of *E. globulus* which may affect the proportion and synchrony of seed germination and development. *E. globulus* has a mixed mating system, and in a separate experiment, comparing seeds from self-pollination, open-pollination and mass-supplementary pollination the germination and development of selfed seeds were poorer than the open-pollinated and mass-supplementary pollinated seeds averaged across temperatures. There was also the suggestion that selfed seeds were more sensitive to high temperature stress.

Fourth, the effect of time of seed harvest, kilning temperature at seed extraction and irrigation treatments applied to trees on the germination of *E. globulus* seeds and the germination response of seeds was tested at 25°C, 32°C and 37°C. Seeds were harvested from three genotypes at 11 months from commencement of flowering (early), 13 months (commercial) and 15 months (late). Kilning temperatures were 30°C, 40°C and 50°C. Irrigation treatments were full irrigation, half irrigation and no irrigation. The kilning temperatures of capsules and irrigation treatments applied to trees had no effect on the germination traits studied. Time of seed harvest affected the rates of seed germination and normal seedling development. There was little delay in germination rate at 32°C in early harvested seeds compared with that experienced by the commercially and late harvested seeds, suggesting that it has a higher temperature threshold. The effect of harvest date on germination was

genotype dependent and it is suggested that variability in seed germination traits between harvest dates may be related to different heat sums which seeds are exposed to during development.

The outcomes from this work have significant industry application. Seed germination and seedling development in the nursery could be significantly enhanced by accounting for tree genetics, maternal environment and harvesting factors including seed harvest site, season and age.

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Preface

This thesis documents the research undertaken between March 2007 and April 2010. The project was initiated and funded by seedEnergy Pty Ltd, a company specialising in elite forest tree seed production through seed orchard management. The research is in preparation for publication and the thesis structure has incorporated these manuscripts as research chapters. An introduction chapter provides background on the thesis topic and an overall context for the research chapters. A general discussion expands on the discussions presented in the research chapters.

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Glossary

Maternal:	Female parent
Paternal:	Male parent
Diallel:	Reciprocal crossing of each of several individuals with two or more others in all combinations 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:	Control pollination
MSP:	Mass supplementary pollination
PRD:	Partial root zone drying
RDI:	Regulated deficit irrigation
CI:	Conventional irrigation
NI:	No irrigation

Chapter 1

Introduction: Project Background and Context

Natural Distribution and Taxonomy

Eucalyptus globulus Labill. (Tasmanian Blue Gum) is a hardwood forest tree, native to south-eastern Australia. The subspecies *globulus* is one of four taxa in the *E. globulus* complex that intergrade extensively (Jordan *et al.* 1993). The subspecies are *globulus, bicostata, pseudoglobulus* and *maidenii* (Dutkowski and Potts 1999; Jordan *et al.* 1993). The four taxa are differentiated on reproductive traits, including the number and size of flower buds per umbel. The largest, normally solitary buds and capsules are found on core *E. globulus*, although intergrade populations may have up to three buds per umbel and have smaller flowers (Jones *et al.* 2002; Jordan *et al.* 1993). This thesis deals with *E. globulus* subspecies *globulus* and where reference is made to *E. globulus* herein this is the subspecies being referred to.

E. globulus subspecies *globulus* is most preferred for pulpwood. The other three subspecies have not been planted on a large scale (Eldridge *et al.* 1993). *E. globulus* is mainly confined to the southeast coast of Tasmania but also grows in small pockets on the west coast of Tasmania, on islands in the Bass Strait north of Tasmania and on Cape Otway and Wilson's Promontory in southern Victoria, Australia (Hall *et al.* 1970). Other subspecies are found northward in Victoria and New South Wales (Kirkpatrick 1975).

E. globulus is genetically variable across its geographic range and the broad-scale, quantitative genetic variation in numerous traits has been summarised using a hierarchy of 13 races and 20 subraces (Dutkowski and Potts 1999). These genetically differentiated races form three major lineages comprising the main populations from (1) Victoria, (2) King Island and Western Tasmania, and (3) Eastern Tasmania and the Furneaux Islands (Steane *et al.* 2006). These races differ genetically in numerous characteristics including age of first flowering (Chambers *et al.* 1997; Jordan *et al.*

2000), reproductive output (McGowen *et al.* 2004a) and season of flowering (Apiolaza *et al.* 2001; McGowen 2007; Potts and Gore 1995).

Reproductive Biology

E. globulus is heteroblastic, possessing different juvenile and adult leaf forms (Jordan *et al.* 1999; Jordan *et al.* 2000; Lawrence *et al.* 2003). The onset of sexual reproduction in *E. globulus* is normally associated with adult leaves and first occurs in plantations at 3-4 years of age, although flowering levels are usually reduced in closed plantations (Barbour *et al.* 2007). *E. globulus* flower buds require approximately one year to develop from initiation to flowering (Espejo *et al.* 1996). The timing and age of flowering is genetically variable both between and within races/subraces (Potts and Gore 1995). *E. globulus* trees flower once per year and the flowers are bisexual, protandrous and relatively large (Gore *et al.* 1990). The flowers produce copious nectar and are predominantly animal-pollinated, mainly by birds and insects (Hingston and Potts 1998; Hingston *et al.* 2004). After fertilisation, seeds develop within the woody capsules and take approximately 12 months to mature (Espejo *et al.* 1996).

As in most eucalypts, *E. globulus* has a mixed mating system (Potts *et al.* 2008). In native stands, outcrossing rates range from 38 to 100% (Foster *et al.* 2007; Hardner *et al.* 1996; Jones 2005; McGowen *et al.* 2004; Patterson *et al.* 2001), increasing with degree of self-incompatibility (Patterson *et al.* 2001; Patterson *et al.* 2004b) and stand density (Borralho and Potts 1996; Hardner *et al.* 1996). Generally eucalypts are preferential outcrossers, with high levels of out-crossing maintained by protandry and various incomplete pre- and postzygotic barriers to self-fertilisation (Potts 2004). Levels of self-incompatibility vary from 0 to 100% in *E. globulus* (McGowen *et al.* 2004a; Pound *et al.* 2002) and are driven by late-acting post-zygotic mechanisms (Pound *et al.* 2002a; Pound *et al.* 2003a) and are under some degree of genetic control (McGowen 2007). Self-pollination usually results in a significant reduction in seed set, compared to outcross pollinations (Hardner and Potts 1995; Hardner *et al.* 1998; Pound *et al.* 2002) and when self-pollination is successful, severe reductions in growth and survival of offspring occur in comparison with those derived from unrelated outcrosses (Costa e Silva *et al.* 2010b; Costa e Silva *et al.* 2010a; Hardner and Potts 1995; Hardner *et al.* 1998).

Plantation Development

E. globulus is the main *Eucalyptus* species for pulpwood plantations in several temperate countries around the world including Australia, Chile, China, Columbia, Ethiopia, India, Peru, Portugal, Spain, USA and Uruguay (Eldridge *et al.* 1993; Potts 2004). In 2005 there were approximately 700 000 ha in Portugal, 500 000 ha in Spain, 320 000 ha in Chile and 268 000 ha in Uruguay (Potts *et al.* 2004). There were 454 095 ha of plantations of *E. globulus* in Australia in 2005 (Parsons *et al.* 2006).

E. globulus has superior growth rates for pulpwood compared with other eucalypts and about 50% of the plantation estate is cut on a coppice rotation of about 8-12 years, usually two or three times (Eldridge *et al.* 1993). The other major harvesting method involves clearing the plantation and replanting with seedlings. This allows capitalising on genotype improvement when replanting (Potts *et al.* 2008), which is not possible with coppicing, however it is more expensive than coppicing. The world average for growth rate of *E. globulus* is suggested to be 20 m^3 ha^{-1} $year^{-1}$ for temperate zones, but it is anticipated that this could reach 25-30 m^3 ha^{-1} $year^{-1}$ in the future with improved silviculture and breeding (Potts 2004). While *E. globulus* is mainly grown for its pulp, plantings for producing sawn timbers, reconstituted wood products and veneers are increasing (Hamilton *et al.* 2010; Raymond and Apiolaza 2004).

The expansion of plantations of *E. globulus* to meet the global demands for paper has led to increased interest in improving traits related to the quantity and quality of wood produced. There are breeding programs in at least eight countries, many of which are two or more generations removed from the landrace or native populations (Potts 2004). Breeding programs have aimed to select fast-growing trees with wood characteristics suitable to the pulping process (Eldridge *et al.* 1993). The wood properties of interest are basic density, pulp yield/cellulose content and fibre length (Raymond and Apiolaza 2004). Economic weights have been determined for the key biological traits influencing the economics of pulpwood production and these allow estimation of total breeding value (Potts 2004). Extensive research has been undertaken on the genetic control of traits affecting pulp yields (Raymond 2002). Trees can then be selected with the appropriate genes and used in breeding programs,

with the highest performing trees then being planted (or grafted) into seed orchards (Potts 2004).

There is high demand for improved eucalypt germplasm for plantation establishment. *E. globulus* does not clonally propagate well and therefore most plantations in Australia have, and continue to be established using seedlings (Potts *et al.* 2008). This is mainly due to low average rooting success for the majority of genotypes and the generally higher costs of clonal propagules compared with seedlings (Borralho 1997; Cañas *et al.* 2004; deLittle 2004; Dutkowski and Whittock 2004). For these reasons clonal propagation has only been used partially for deployment in Spain (Toval 2004), Portugal (Araújo *et al.* 1997) and Chile (Griffin 2001). Demand for improved *E. globulus* seedlings for plantation establishment has led to increased focus on seed production.

Seed Production

Early *E. globulus* plantations were established from open-pollinated seeds sourced directly from the best native provenances (Eldridge *et al.* 1993; Potts *et al.* 2004). Improved germplasm is now mainly derived from open-pollinated seedling (Griffin 2001; McGowen *et al.* 2004a; Tibbits *et al.* 1997) or grafted (Patterson *et al.* 2004b) seed orchards. Traditionally, seed production in orchards was obtained by open-pollination (Griffin 2001), however large scale manual pollination is beginning to replace open-pollinated seed in many countries (Callister and Collins 2007; Harbard *et al.* 1999; Leal and Cotterill 1997; Patterson *et al.* 2004b; Patterson *et al.* 2004a). Within the seed orchard, controlled pollinations can be undertaken to achieve full-sib deployment (Patterson *et al.* 2004a).

Controlled pollination in *E. globulus* has traditionally involved three visits to the flower. These are emasculation and isolation at operculum shed, pollination at sigma receptivity and then removal of isolation bags several weeks later (Moncur 1995). This has been termed three visit pollination (TVP) (Venter and Silval 2007). This is a time consuming approach and while it has been used for producing full-sib seeds in Portugal (Leal and Cotterill 1997), it has not been adopted in Australia due to the high labour cost associated with this method. The major breakthrough for improved

breeding and deployment of *E. globulus* seeds was development of what is termed single visit pollination (SVP) (Williams *et al.* 1999) or one stop pollination (OSP) (Harbard *et al.* 1999). SVP involves cutting the style transversely, whereas OSP uses a longitudinal cut. Development of this technique was based on the finding that the stigma is not necessary for pollination in some eucalypts. It has been shown that pollen will germinate on the cut surface of the style at (Harbard *et al.* 1999; Williams *et al.* 1999), or even prior to (Trindade *et al.* 2001) operculum shed. Stigma receptivity in *E. globulus* usually occurs 5-7 days after operculum shed (Patterson *et al.* 2004a).

The development of OSP/SVP has increased the efficiency of breeding and deployment programs in *E. globulus*. Similar pollination techniques have been used by some organisations in Australia, however high labour costs have led to further refinements being necessary. seedEnergy Pty Ltd, a company specialising in elite forest seed production, for example, now routinely uses the cut style technique without emasculation, flower isolation or labelling. This operational system is termed mass-supplementary pollination (MSP) (Patterson *et al.* 2004a). MSP is now routinely used for the mass production of *E. globulus* seeds for the plantation industry in Australia (Callister and Collins 2007; Potts *et al.* 2007).

Some level of self-pollination or out-crossing with pollen other than the target would be expected with the MSP technique. A study by Patterson *et al*. (2004a) showed that the outcross pollinations are likely to comprise 87% of the seeds obtained, the remainder being self-pollinated (4.6%) and presumably high quality outcrosses from other selected genotypes in the orchard (8.4%). By selecting female genotypes that are self-incompatible, selfing can be completely eliminated. Contamination levels of approximately 10% are considered acceptable by Harbard *et al*. (2000) for commercial seed production.

Control pollination allows full-sib deployment, so that the genetic gain can be maximised by controlling both parents. This offers two advantages: firstly, it allows capturing of specific combining effects and, secondly, it avoids inbreeding (Patterson *et al.* 2004a). The accuracy of breeding and deployment value prediction is enhanced by full pedigree control (Dutkowski *et al.* 2006). Due to the mixed mating system in

E. globulus, there can be some self-fertilisation when seeds are open-pollinated, which makes *E. globulus* susceptible to inbreeding depression (Hardner and Potts 1995). Inbreeding depression can lead to selfed progeny having a 48% reduction in the volume growth of trees, compared with fully out-crossed progeny (Hardner and Potts 1995). This reduction can adversely affect the productivity of plantations (Costa e Silva *et al.* 2010a). Seeds obtained from open-pollination can contain self-pollinated individuals and are therefore sub-optimal for deployment purposes (Patterson *et al.* 2004b), as they may negatively affect plantation productivity.

The majority of *E. globulus* plantations are now established from genetically improved seeds derived from seed orchard systems (Potts 2004). The production of seeds from seed orchards is labour intensive and therefore the final cost of the seeds is relatively high (Suitor *et al.* 2010). High labour costs are attributable to pruning of trees, hand picking of capsules, cleaning of samples to 100% purity and germination testing of seeds prior to dispatch. Control pollination requires large scale hand-pollination of flowers to allow full pedigree control and while this increases seed yield and improves capsule retention (Suitor *et al.* 2008) it involves higher cost than open-pollination. The high cost of the seeds increases the significance of losses suffered at the nursery.

Deployment of Seedlings and Nursery Problems

Deployment of seedlings for plantation establishment relies on successful germination of seeds in nurseries. Success is measured in terms of germination rate and total germination percentage. Nurseries purchase genetically elite seeds from specialist seed production companies, germinate it and grow seedlings out to very specific guidelines. Forestry companies which deploy seedlings send nurseries seedling specifications that outline desirable physical, chemical and/or physiological characteristics (Close *et al.* 2003).

Some forestry companies in Australia provide contracted nurseries with a specification for *E. globulus* seedlings, based on seedling height, collar diameter, number of leaf pairs, root system development, foliar nutrient concentrations and disease status (Close *et al.* 2003). This is done to ensure that nurseries satisfy

expectations of the final product. For eucalypts, the specifications may differ within regions (Close *et al.* 2000) and between regions (Knight and Nicholas 1996), depending on the prevailing environmental factors that limit the growth of the species at the planting site (Close *et al.* 2003). Low seed germination percentages and uneven seedling emergence rates resulting from slow seed germination rates have created problems for seed nurseries producing *E. globulus* seedlings (Lopez *et al.* 2000; Potts *et al.* 2008). Variable germination of *E. globulus* seeds can result in significant variation in seedling size. This can result in a large proportion of seedlings being discarded or requiring re-grading as they do not meet planting specifications. In some cases, low or asynchronous germination can reduce nursery output of specification seedlings by up to 25% (Potts *et al.* 2008). In large commercial nurseries that may sow up to 10 million seeds annually, this amounts to significant loss in production. There is some evidence to suggest that this problem may be linked to periods of high temperature exposure during germination (C. Spurr unpubl. data).

The location of the largest seed nurseries across southern, south-eastern and Western Australia leaves them vulnerable to high temperature conditions. This is exacerbated by the requirement for seedling supply to plantations in the winter, meaning that seed-lots have to be germinated during the hottest summer months to allow sufficient time for seedling development to reach planting specification by winter. Eucalypt plantations in South Australia are generally planted in winter, using seedlings approximately 6 months old (Sasse *et al.* 2003b). Planting during winter is necessary to provide sufficient soil moisture to aid seedling establishment. Nurseries therefore normally begin sowing seeds in December and seeds are germinated during a time of significant risk of high temperature exposure. Given the high-value nature of improved *E. globulus* seeds, losses suffered during germination in seed nurseries represent a significant economic hurdle. Nurseries operate with tight profit margins and must maintain a high turnover of seeds to seedlings to remain viable.

The optimum temperature for germination rate of *E. globulus* seeds has been reported to be 28°C (Lopez *et al.* 2000) but is given as 25°C by the International Seed Testing Association (ISTA 1999). Temperatures in nurseries often exceed these temperatures and during summer temperatures can reach as high as 40°C. Seed-lots

are tested for germination performance prior to dispatch to nurseries. This testing is performed at 25°C and total germination of above 90% is common (C. Spurr unpubl. data). The conditions used for testing do not reflect the nursery environment and as such may not be indicative of a seed-lot's performance in the nursery. This has been found with some nurseries reporting slow, uneven and reduced overall germination from seed-lots which performed highly in the testing prior to sale.

Management

Most nurseries rely on the use of shade cloth to moderate temperatures within seedling trays, however this has been shown to be only marginally effective. Temperature monitoring within seed trays has shown that temperatures can still reach as high as 40°C. Preliminary work has shown that temperature varies little with tray type, colour and position within tray (C. Spurr unpubl. data). Overhead irrigation has been shown to be relatively effective in reducing temperature, however data suggest that the reduction is only transient and the temperature quickly rises after the irrigation ceases (C. Spurr unpubl. data).

Uniformity of seed germination and seedling growth is important in large-scale commercial nurseries as seeds are mechanically sown into fixed celled containers (Potts *et al.* 2008). The only effective method to reduce the temperature within nurseries, such that it does not negatively impact on seed germination and seedling development, is to use air-conditioning. This is a very high-cost measure and due to the very large area many nurseries cover, is not a practical or cost-effective solution. Maintaining seeds in individual tree seed-lots when grading by density and size may help nurseries achieve more synchronous and uniform germination of seeds (Potts *et al.* 2008). The vast majority of variation, however still occurs within graded seed-lots (Watson *et al.* 2001) and this may reflect numerous factors including maternal and direct environmental effects. Identifying and understanding the factors in the nursery related to the problems of reduced and uneven germination of *E. globulus* seeds is important in tackling this problem.

Scope of the Project

This PhD project seeks to characterise the response of genetically diverse commercial seed-lots to a range of temperatures, ranging from sub to supra-optimal. This characterisation will allow definition of the cardinal upper, lower and optimum temperatures for germination in this species. This project also aims to determine the extent of maternal genetic regulation at the race and genotype level of seed germination responses. It will also be determined whether the level of tolerance of seeds to high temperature stress is inherited predominantly from the maternal or the paternal parent. Further to this, the germination and development of seeds from self-pollination, open-pollination and mass-supplementary pollination will be compared.

There is always pressure on seed orchard managers to harvest elite seeds early for dispatch to nurseries, to ensure that seedlings are ready for planting during autumn however this risks reducing the quality of the seed crop with immature seeds (Potts *et al.* 2008). It is unknown what effect the time of seed harvest would have on the germination of *E. globulus* seeds and germination response to high temperature stress. This study will seek to determine the effect of agronomic factors such as time of seed harvest, kilning temperature used for seed extraction and irrigation inputs to maternal trees on the sensitivity of seeds to high temperature exposure during germination.

This PhD project seeks to (i) define the temperature response of genetically diverse commercial *E. globulus* seed-lots, (ii) determine the extent of maternal genetic regulation at the race and genotype level of seed germination responses and (iii) determine whether agronomic factors including time of seed harvest, kilning temperature used for seed extraction and maternal tree irrigation inputs affect the germination response.

Chapter 2

Germination response of *Eucalyptus globulus* seeds exposed to low and high temperature stress

Introduction

The rapid expansion in *Eucalyptus globulus* forestry plantations in Australia (Parsons *et al.* 2006) has created a high demand for genetically improved seeds. Early plantations were established from open-pollinated seeds sourced directly from the best native provenances (Eldridge *et al.* 1993; Potts *et al.* 2004). Improved germplasm is now derived from open-pollinated seedling (Griffin 2001; McGowen *et al.* 2004a; Tibbits *et al.* 1997) or grafted (Patterson *et al.* 2004b) seed orchards. Traditionally, seed production in such orchards was through open-pollination (Griffin 2001), however in recent years an increasing proportion of seeds are produced from manual pollination of flowers in grafted seed orchards (Potts *et al.* 2008). This allows the production of polymix or full-sib families to maximise genetic gain through avoiding inbreeding as well as exploiting both additive and, in the case of full-sib families, non-additive genetic effects.

The production of such seeds from seed orchards is still labour intensive and therefore the final cost of the seeds is relatively high. The greater cost of manually pollinated elite seeds and the high demand for elite open-pollinated seeds has increased the focus on production efficiency in nurseries where elite seeds are used. Uniformity of seed germination and seedling growth is important in large-scale commercial nurseries as seeds are mechanically sown into fixed celled containers (Potts *et al.* 2008). Variability in emergence patterns between seed-lots and with changing environmental conditions in nurseries will reduce the efficiency of nursery production.

Seasonal fluctuations in the success of seedling establishment in nurseries in Australia have been noted. Seed-lots displaying high germination percentage under standard laboratory germination tests have been observed to vary in emergence percentage and uniformity when sown at different times of the year, and differences

in seed-lot response to these seasonal changes have also been noted. Variability in germination rate both within and between seed-lots is an additional problem that causes greater variation in seedling size and increases nursery costs through the necessity to regrade seedlings. Eucalypt seed germination in the nursery is quite unpredictable, and poor emergence together with long periods for full emergence are limitations to achieving efficient seed usage (Humara *et al.* 2000).

Anecdotal evidence from key nursery personnel raised the possibility that poor seedling establishment and high variation in seedling size in nurseries may be linked to periods of high temperature during seed germination (C. Spurr unpubl. data). It was postulated that high temperature conditions may result in slower and more uneven germination and seedling emergence. Published data on *E. globulus* seed germination at high temperatures however are scarce, and no detailed profiling of germination response with temperature has been published.

Temperature has a significant influence on plant growth and development, and the effects of high temperature on seed germination have been widely studied in crop species. The failure of seeds to germinate at high temperature is termed thermoinhibition, if germination proceeds immediately when the temperature is reduced below a certain threshold, or thermodormancy, if some form of dormancy-breaking treatment is required before germination can proceed at the favourable temperature (Corbineau *et al.* 2002; Hills and van Staden 2003; Kepczynski and Bihun 2002).

The cardinal temperatures (minimum, maximum and optimum) at which germination occurs, and the nature of the germination response, vary between species (Hills and van Staden 2003). For example, cool season and temperate crops often have lower minimum temperature compared to tropical crops. The maximum temperatures can also differ for different plant species and genotypes within species (Wahid *et al.* 2007).

Determining the maximum temperature for germination in a species would be useful for predicting when nurseries are likely to have problems due to high temperatures. Germination models indicate that the timing of germination is closely linked to physiologically determined temperature and water potential thresholds for radicle

emergence (Bochenek *et al.* 2010; Welbaum *et al.* 1998). These models can be used to predict germination patterns in response to temperature (Finch-Savage *et al.* 1998). Description of the germination response of a species across temperatures ranging from minimum to optimum and maximum is necessary to build these models and gauge the accuracy of their outputs.

The optimum temperature for germination of *E. globulus* seeds is reported to be 25°C (ISTA 1999), however very little published information exists on the effect of low and high temperature exposure on germination in this species. Evidence from Lopez *et al*. (2000) suggested that germination rate and percentage germination of *E. globulus* seeds decreased at temperatures above 28°C. This response is consistent with the observations of nursery operators of impaired germination at high temperatures. The study by Lopez *et al*. (2000) focused on a single seed-lot from the Flinders Island provenance and did not define the upper or lower temperature thresholds for germination in this species.

The present study aimed to determine: (i) the germination response of *E. globulus* seeds exposed to a range of temperatures; (ii) the upper, lower and optimum temperatures for rate of normal seedling development in this species; and (iii) if the response to high temperature stress varies between commercial seed-lots.

Materials and Methods

Seed-lots

Four broad genetic based commercial seed-lots were used in this study. All seed-lots were supplied by seedEnergy Pty Ltd and were from two open-pollinated multi-provenance seedling seed orchards (Bawdens and Heath). The seed orchards were established as genetics trials in 1988 using 197 open-pollinated families from range-wide seed collections made by CSIRO in 1987 (Dutkowski and Potts 1999). The trials each consisted of 3940 trees in four replicates of five-tree family plots. The Southern Tree Breeding Association (STBA) converted the trials into seed orchards in 1997 by culling genetically inferior trees using a selection index which was based on a breeding objective to maximise profit from plantations grown for kraft pulp production (McRae *et al.* 2004; Raymond 2002). In recent years the genetic worth of the seed orchards has been improved by further culling of inferior trees from the

orchard based on the STBA selection index. The origin of the parent trees of the four commercial seed-lots used was quite diverse, with parent trees originating from a broad genetic background.

Seed-lot „Glob 185' was harvested in November 2006. The seed was a mix of open-pollinated seeds from 47 highly ranked trees based on the STBA's selection index that were sourced from 2 seed orchards (Bawdens and Heath). Seed-lot „Glob 228' was harvested from Bawdens seed orchard in November 2007 and was a mix of open-pollinated seeds from 44 trees that were highly ranked on the STBA selection index. Seed-lot „Glob 244' was harvested from Heath seed orchard in November 2007 and was a mix of open-pollinated seeds from 48 trees that were highly ranked on the STBA selection index. Seed-lot „KI 133' was harvested from the Heath and Bawdens seed orchards in November 2004. It comprised a mix of open-pollinated seeds from 10 trees that originated from King Island. These trees were selected as having good breeding values for volume alone.

Seed Germination

Seed germination experiments were conducted on a thermogradient table (Terratec); 2 m long by 1 m wide. The table consisted of a flat aluminium plate (25 mm thickness) along which a user-defined temperature gradient was maintained by setting temperatures for each end of the table with an Allen-Bradley panel view 600. Custom built germination cabinets (Plate 1) were positioned along the temperature gradient. Temperature was logged inside the cabinets at the seed level for the duration of the trials using Tiny Tag Data Loggers (Gemini Data Loggers UK). In all cases, seeds were germinated under continuous fluorescent light. Although it has been reported that uniformity and germination rate of *E. globulus* seeds can be improved with constant darkness (Nair *et al.* 2009; Nair *et al.* 2007) the present study used constant light because the experiments included normal seedling development which requires light.

The germination cabinets were designed with eight separate sections in each cabinet, to accommodate eight lots of 50 seeds. A single length of perspex covered the eight sections. In each section, 50 seeds were placed, evenly spaced, on separate 5cm by

12.5cm lengths of Advantec filter paper (number 1 type). These sections with 50 seeds comprised the experimental unit and the allocation of seed-lots to sections within cabinets was random. 75mL of distilled water was added to each section and wicked up to the seeds to maintain a consistent level of moisture for the duration of the experiment. Water levels within chambers were assessed daily and distilled water was added where necessary.

An experiment commenced when seeds were placed on to the moist filter paper and this point in time was considered day zero of the test. On the final day of scoring, remaining seeds were scored as abnormal, fresh-ungerminated or dead, in accordance with ISTA (2006). Tweezers were used to squash seeds which had not germinated to separate the fresh-ungerminated seeds from dead seeds (Yates *et al.* 1996). Seeds were scored as fresh-ungerminated when the embryo was firm, white and intact (Hardner and Potts 1995). Seeds were scored as dead when the embryo was very soft and discoloured. The abnormalities included stunted radicles, failure of radicles to emerge but emergence of cotyledons, and failure of seedlings to "push-off" the seed coat, in accordance with ISTA guidelines for seedling evaluation (Bekendam and Grob 1993).

Plate 1: Cabinet used to germinate *E. globulus* seeds on the thermogradient table. Each section shown contained 50 seeds and comprised the experimental unit.

Experiment 1

Characterisation of the germination and normal seedling development response of seed-lot Glob 185 to temperatures, ranging from 15.0°C to 42.7°C (15.0, 17.0, 19.9, 22.6, 25.5, 28.8, 32.9, 36.3 and 42.7°C). The trial was conducted using seeds in the 1.5 to 1.7 mm size class. Four experimental units of 50 seeds each were placed in each germination cabinet, leaving a well between each replicate. Two germination cabinets were placed at each temperature, giving 8 replicates per temperature. Seeds were placed on the moist filter paper on the 7th February 2008 (day 0).

The experiment was scored daily from day 5 through to day 22, with the exception of days 17, 19, and 21. Seeds on one side of the table, in one series of germination cabinets were scored for radicle emergence (termed ‚germination') while those on the other side of the table were scored for occurrence of normal seedlings according to ISTA guidelines (Bekendam and Grob 1993; ISTA 1999). Radicle emergence was recorded when there was visible protrusion of the radicle through the seed coat. A seedling was classified as normal and removed when it had a radicle at least one third the length of the seedling, a hypocotyl and two cotyledons which were not covered by the seed coat. The day at which a seed germinated or seedling was classified as exhibiting normal development, was recorded. The final day of scoring was undertaken as outlined above.

Experiment 2

This experiment compared normal seedling development for four commercial seed-lots (Glob 185, Glob 228, Glob 244, and King Island 133) exposed to low, optimal and high temperatures. This trial was set up on the 10th of April 2008 (day 0). Seeds from the size class 1.5 mm to 1.7 mm were used for all seed-lots, with the exception of Glob 228 where the 1.2 mm to 1.5 mm and 1.5 mm to 1.7 mm size classes were combined. This was done due to low seed numbers for this seed-lot. The Glob 185 seed-lot was used again to provide a reference to Experiment 1. The range of seed size for these seed-lots was from less than 1.2 mm to greater than 1.7 mm.

Two germination cabinets were placed at each of nine temperatures (12.2, 14.4, 18.0, 22.6, 25.1, 28.1, 33.0, 34.8 and 36.9°C) along the thermogradient table. Compared with Experiment 1, this trial extended the low temperature treatment down almost 3°C and the highest temperature treatment was reduced by 6.7°C. The four seed-lots were germinated in a single run, with four experimental units of 50 seeds of each seed-lot at each temperature. There were thus 16 lots of 50 seeds at each temperature and the allocation of seed-lots to sections within germination cabinets was random.
The trial was scored daily from day four through to day twenty seven, with the exception of days 10, 17 and 24. At each scoring, germinated seedlings were recorded as normal, as defined above, and removed. Radicle emergence was not scored. The day at which a seedling was classified as exhibiting normal development was recorded. Final scoring was undertaken as outlined above.

Statistical analysis

To estimate the response rate of each experimental unit of 50 seeds, the time taken for both 50% germination and normal seedling development (T50) were calculated. The T50 was estimated for each experimental unit (i.e. section comprising 50 seeds) by fitting the logistic function to the cumulative curves for germination or normal seedling development (Shafii and Price 2001). This was done using the Marquardt's iterative non-linear procedure PROC NLIN of SAS version 9.1 (SAS Institute Inc 2003). The logistic function was chosen because it is one of the functions most widely used to model seed germination data (Dumur *et al.* 1990; Thompson *et al.* 1994; Torres and Frutos 1990). The logistic function is sigmoidal and symmetrical and is similar to the cumulative normal distribution (Shafii and Price 2001). The logistic function was defined as:

$$y(t)= M[1+exp(-K*(t-L))]^{-1} \quad (1)$$

where $y(t)$ is the cumulative germination at time t, M is the asymptote (theoretical maximum for y(t), K is proportional to the rate of germination, and L is the time required to reach 50% maximum germination (i.e. T50). The logistic function provided a good fit to the data.

For Experiment 1, statistical testing of the effect of temperature on the proportion of imbibed seeds that germinated, the proportion of imbibed seeds that developed into normal seedlings, the proportion of germinated seeds that developed into normal seedlings and the rate (T50) of germination and normal seedling development was undertaken using the linear mixed model procedure PROC MIXED of SAS version 9.1 (SAS Institute Inc 2003). The model included temperature as a fixed effect. For Experiment 2, statistical testing of the rate of normal seedling development (T50), proportion of seeds developing into normal seedlings, and the proportion of the seeds which did not give normal seedlings which were fresh-ungerminated seeds, dead seeds and abnormal seedlings was undertaken using the linear mixed model procedure PROC MIXED of SAS version 9.1 (SAS Institute Inc 2003). Data for fresh-ungerminated seeds, dead seeds and abnormal seedlings were analysed separately, as a proportion of total seeds that did not give normal seedlings and as outright proportions of seeds sown. The model included seed-lot, temperature and seed-lot by temperature as fixed effects. The data from both experiments for T50 were log transformed and proportional data angular transformed for the analysis.

For both experiments, the predictions of the minimum, optimum and maximum temperatures were calculated for each seed-lot by fitting a parabolic curve to the inverse of the T50 or proportional data plotted against temperature (Shafii and Price 2001). The minimum and maximum temperature limits for the response traits were estimated from the temperature at which the parabola intersected the x-axis (minimum and maximum) and the optimum estimated from the temperature at which the slope of the parabola equalled zero. The mean percentage normal seedlings was correlated with rate of normal seedling development using data from all experimental units in Experiment 2 and the PROC CORR (Pearson's Correlation) procedure of SAS (SAS Institute Inc 2003).

Results

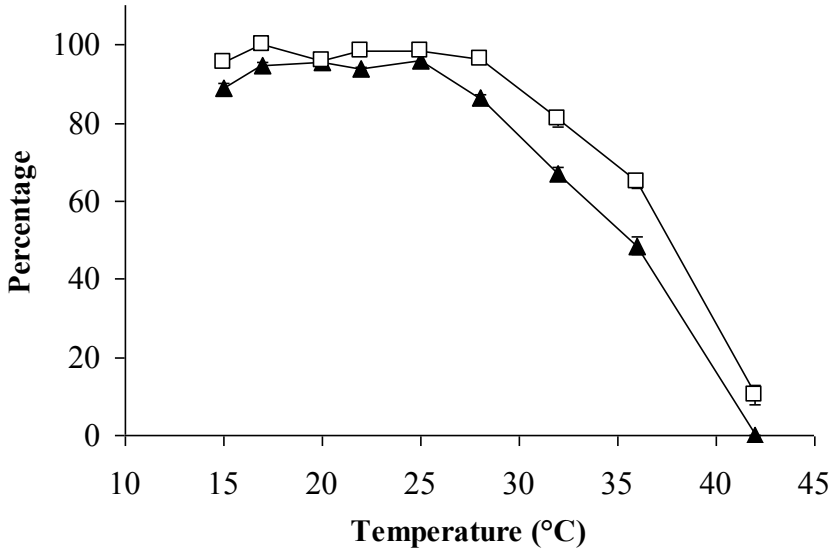
Experiment 1

Temperature had a significant ($F_{8,27}$ = 82.6; P<0.001) effect on percentage germination and percentage normal seedling development ($F_{8,27}$ = 119.6; P<0.001). Temperature also had a significant ($F_{8,24}$ = 49.8; P<0.001) effect on the proportion of

germinated seeds which developed into normal seedlings. Exposure to temperatures above 29°C had a negative effect on the percentage and rate of seed germination and normal seedling development in the seed-lot Glob 185 (Figure 1a and 1b). The calculated optimum for percentage germination was 22.2°C and for percentage normal seedlings was 21.2°C. There was, however, a broad temperature optimum for both percentage germination (15.0 - 28.8°C) and normal seedling development (17.0 - 25.5°C). Above 28.8°C, the percentage germination and normal seedlings obtained declined sharply (Figure 1a). However, there was a trend for percentage germination to be maintained higher over a greater temperature range compared to the percentage of normal seedlings obtained. There was little decline in germination and normal seedlings obtained at the low temperatures studied. At the highest temperature (42.7°C) there was still some germination, however normal seedling development was completely inhibited (Figure 1a). This result suggests that high temperature exposure has a slightly greater negative effect on seedling development than germination. The calculated upper temperature limit for percentage germination was 44.5°C and for percentage normal seedling development was 42.7°C.

Temperature also had a significant effect on the rate of germination ($F_{8,27}$ = 54.3; P<0.001) and normal seedling development ($F_{7,24}$ = 187.8; P<0.001). These rates, expressed as the time to reach 50% (T50) of maximum germination and maximum normal seedling development, respectively, were more responsive to low temperatures than the percentage germination or percentage normal seedling development (Figure 1b). The rate was fastest (T50 was lowest) in the range 19.90°C to 28.8°C for germination, and 22.6°C to 28.8°C for normal seedling development and slowed sharply (T50 increased) at temperatures above and below this range (Figure 1b). The optimum temperature for germination rate in seed-lot Glob 185 was 24.4°C and for normal seedling development rate was 25.5°C (Figure 1). Although the optimum temperature for germination rate in this seed-lot was found to be 24.4°C, the rate at the temperatures from 19.9°C to 28.8°C did not differ significantly from the temperature tested closest to this optimum. Similarly, the optimum temperature for normal seedling development rate in seed-lot Glob 185 was found to be 25.5°C, and the rate at the temperatures from 22.6°C to 28.8°C did not differ significantly from the temperature tested closest to this optimum.

a)



b)

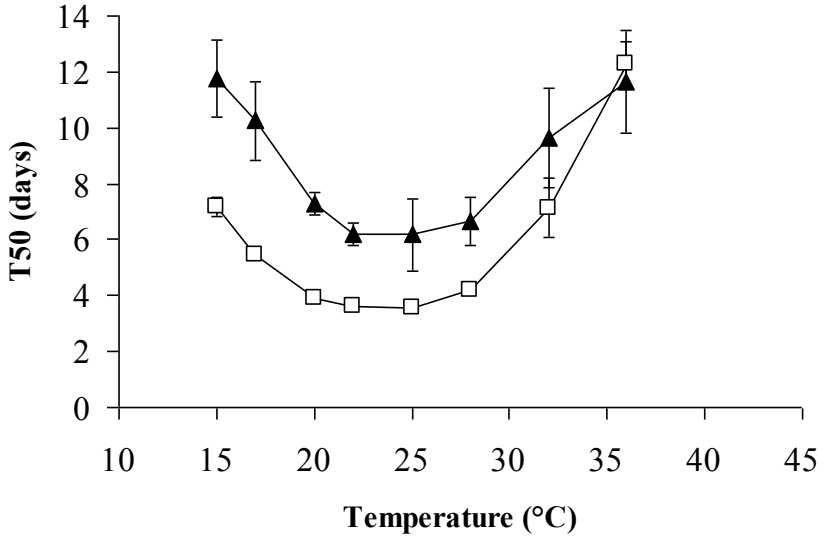


Figure 1. a) Mean (±s.e.) percentage germination (□) and percentage normal seedling development (▲) and **b)** rate (T50) of germination (□) or normal seedling development (days) (▲) (after 22 days from sowing) for seed-lot Glob 185 in Experiment 1. Each point is the mean of four replicates of 50 seeds. Where s.e. bars are not visible they are smaller than the plot symbol.

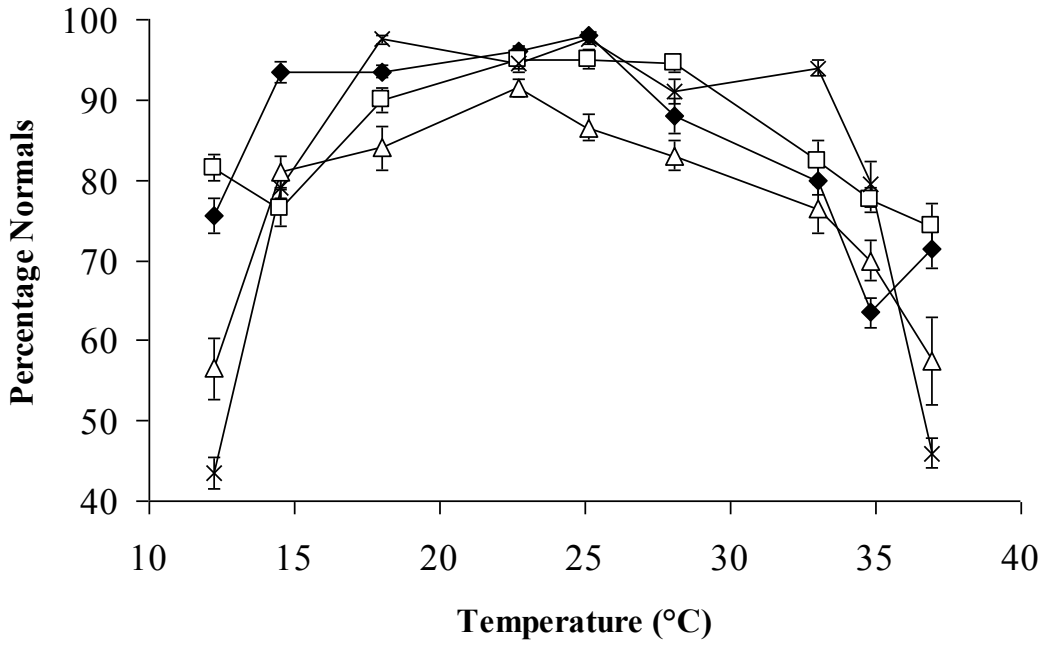
Experiment 2

Seed-lot ($F_{3,108}$ = 9.2; P<0.001) and temperature ($F_{8,108}$ = 35.4; P<0.001) had highly significant effects on the percentage of normal seedlings developed. There was also a highly significant seed-lot by temperature interaction ($F_{24,108}$ = 3.8; P<0.001). By decreasing the lowest temperature to 12.2°C, compared to the lowest temperature of 15.0°C tested in Experiment 1, there was a marked reduction in the percentage of normal seedlings attained in three of the four seed lots (Figure 2a). Averaged across temperatures, seed-lot Glob 228 produced the highest percentage of normal seedlings and seed-lot Glob 244 had the lowest (Figure 2a). The differential response of the seed-lots to temperature was mainly due to seed-lots KI 133 and Glob 244 exhibiting greater inhibition of seedling development at the extreme low and high temperatures. Germination and normal seedlings attained in seed-lot KI 133 dropped the most sharply from 34.8°C to 36.9°C. Seed-lot KI 133 showed a broader optimum for percentage normal seedlings attained. It had the highest percentage of normal seedlings attained at 33.0°C, but dropped to the lowest level of all seed-lots at 36.9°C. The development of normal seedlings in seed-lots Glob 244 and KI 133 were also markedly impaired at the low temperature of 12.2°C.

The seed-lot ($F_{3,108}$ = 14.1; P<0.001) and temperature ($F_{8,108}$ = 109.6; P<0.001) effects were also highly significant for the time taken to reach 50% (T50) of the maximum normal seedlings obtained, however there was no seed-lot by temperature interaction. The rate of normal seedling development was high (T50 was low) for all seed-lots across the temperature range 22.6°C to 29.0°C and declined sharply (T50 increased) above and below this range (Figure 2b). For all seed-lots the rate of normal seedling development was slowest (T50 highest) at the highest and lowest temperatures tested (Figure 2b). Averaged across temperatures, the rate of normal seedling development was fastest (T50 was lowest) for seed-lot Glob 185 and slowest (T50 was highest) for Glob 244. At the lowest temperature tested, the percentage of normal seedlings attained varied by almost 40% between seed-lots (Figure 2a), yet the rate of normal seedling development was very similar for the four seed-lots (Figure 2b). Across all temperatures and seed-lots, the rate of normal seedling development was correlated with the mean percentage of normal seedlings

attained (Pearson's r=-0.77; P<0.001). Nevertheless, while there was variation between seed-lots in their overall development of normal seedlings, both above and below the optimum germination temperature, the absence of an interaction effect indicated that seed-lots did not respond differentially to temperature in their rate of normal seedling development.

a)



b)

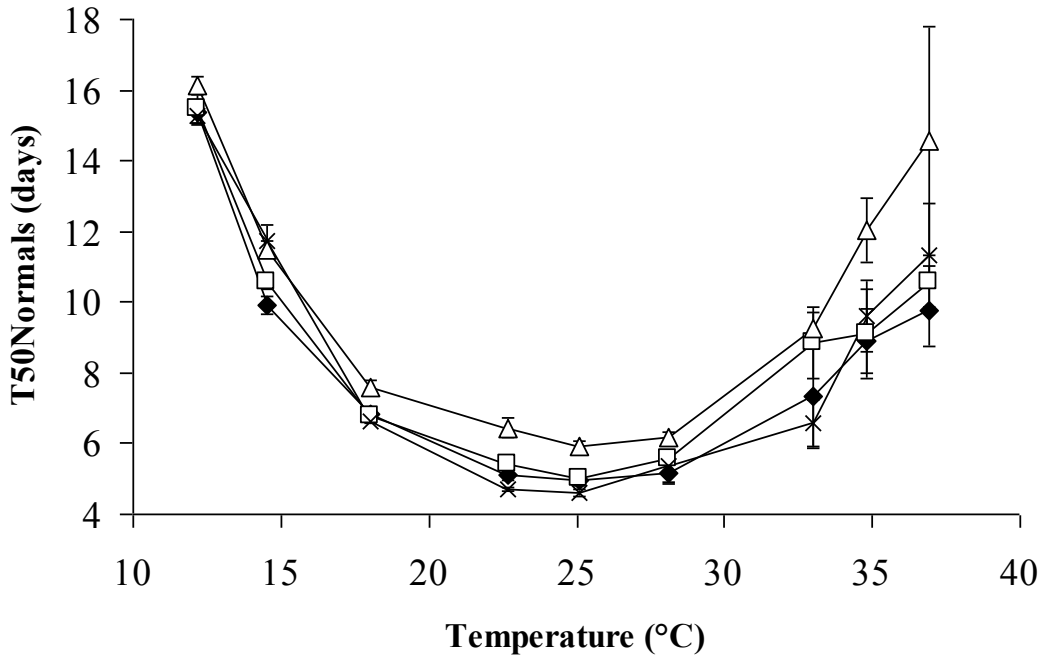


Figure 2. Mean (±s.e.) **a)** percentage of seeds that produced normal seedlings and **b)** rate (T50) of normal seedling development (days) for seed-lots Glob 185 (♦), Glob 228 (□), Glob 244 (Δ) and KI 133 (x) after 27 days from sowing. Seeds were germinated at nine different temperatures and each point is the mean of four replicates of 50 seeds. Where s.e. bars are not visible they are smaller than the plot symbol.

The lower and maximum temperature limits for the rate of normal seedling development of the four seed-lots studied were similar and in the range 9.1°C to 10.5°C and 40.3°C to 41.4°C, respectively. The optimum temperature for rate of normal seedling development was between 24.8°C and 25.5°C (Table 1) and between 21.2°C and 24. 8°C for percentage normal seedlings attained (Table 1). Seed-lot KI 133 showed the highest optimum for the percentage of normal seedlings attained and seed-lot Glob 185 showed the lowest. For all seed-lots the optimum temperature for attaining normal seedlings was lower than the optimum temperature for rate of normal seedling development (Table 1).

Table 1. Estimated optimum temperatures for four seed-lots of *Eucalyptus globulus* based on percentage normal seedling development (Normals/Sown) and rate (days) of normal seedling development (T50Normals). The column titled „Range' represents the range of temperatures over which the means obtained were not significantly different from the temperature tested closest to the optimum and the minimum and maximum indicated. Seed-lot Glob 185 used in Experiments 1 and 2 was the same seed-lot

Seed-lot:	**Normals/Sown**		**T50Normals (days)**	
	Optimum °C:	Range °C:	Optimum °C:	Range °C:
Experiment 1				
Glob 185	21.2	15.0 - 25.5	25.5	22.6 - 28.1
Experiment 2				
Glob 185	22.3	14.4 - 25.1	25.4	18.0 - 33.0
Glob 244	24.0	14.4 - 36.9	24.8	18.0 - 28.1
Glob 228	23.9	12.2 - 28.1	25.1	18.0 - 28.1
KI 133	24.8	18.0 - 33.0	25.2	18.0 - 33.0

When expressed as straight proportions of seeds sown (Figure 3), seed-lot and temperature had a significant effect on the proportions of fresh-ungerminated seeds, dead seeds and abnormal seedlings (Figure 3). However, there was a differential response of the seed-lots to temperature for the proportions of fresh-ungerminated seeds ($F_{24,108} = 2.8$; P=0.001), and abnormal seedlings ($F_{24,108} = 2.2$; P=0.004). When data were expressed on a proportion of total seeds that did not give normal seedlings basis (Figure 4), temperature had a significant effect on the proportion which were classified as fresh-ungerminated seeds ($F_{8,108} = 21.4$; P<0.001); dead seeds ($F_{8,108} = 21.4$; P<0.001); and abnormal seedlings ($F_{8,108} = 3.1$; P=0.004), however there were no significant seed-lot effects or seed-lot by temperature interactions. At low temperatures most ungerminated seeds were fresh whereas at high temperatures, particularly 34.8°C and 37.0°C, there were a high proportion of dead seeds (Figures 3 and 4). Averaged across temperatures, seed-lot Glob 185 had the highest proportion of dead seeds and seed-lot KI 133 had the lowest. At high temperature the majority of abnormalities were accounted for by stunted radicles.

Compared to Experiment 1, the results from Experiment 2 show that when the low temperature is reduced by another 3°C to 12.2°C, there is a reduction in not only the commencement of normal seedling development but also in the maximum level obtained. This suggests that when the temperature is too low, the physiological processes governing seedling development are slowed down to the point that the maximum level of normal seedlings developed is reduced. It is possible that a reduction in the rate of imbibition at 12.2°C contributed to a reduction in radicle protrusion and hence normal seedling development. The proportion of seeds which did not germinate and therefore did not develop into normal seedlings at 12.2°C were largely accounted for by fresh-ungerminated seeds and abnormal seedlings.

High temperature exposure resulted in a higher fraction of dead seeds and abnormal seedlings (Figures 3 and 4). This suggests that low temperature imposed conditional dormancy on seed-lots. High temperature caused irreversible effects on cellular functioning whereas low temperature prevented normal processes associated with germination without being lethal. Seed-lot KI 133 was particularly sensitive to the lowest temperature and in response expressed the highest level of conditional dormancy. This seed-lot was also the most suppressed in maximum development of

normal seedlings at the highest temperature.

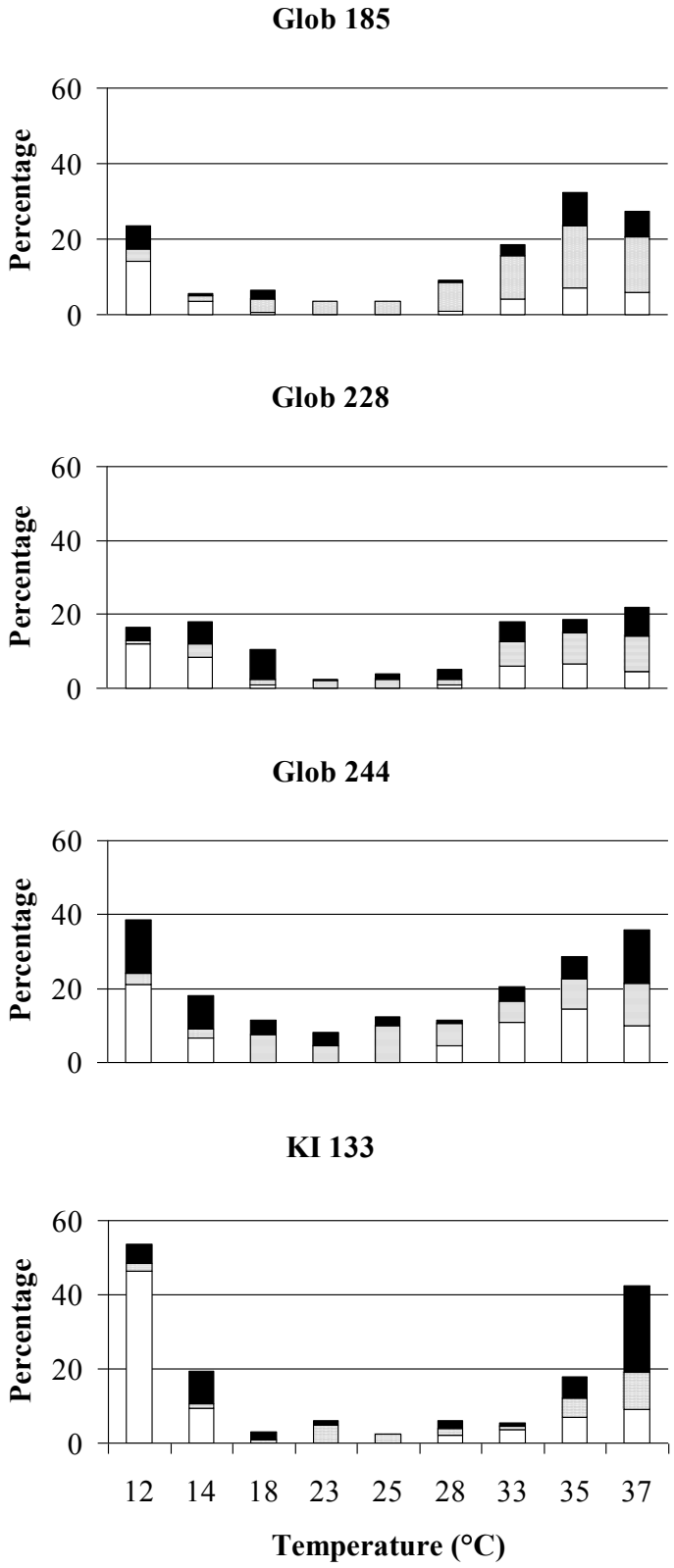


Figure 3. Percentage fresh-ungerminated seeds (□), dead seeds (⚅) and abnormal seedlings (■), after twenty seven days from sowing for four commercial *E. globulus* seed-lots across nine temperatures ranging from 12°C to 37°C. Each value is the mean of 4 replicates of 50 seeds at each temperature. Data represents outright proportions of seeds sown.

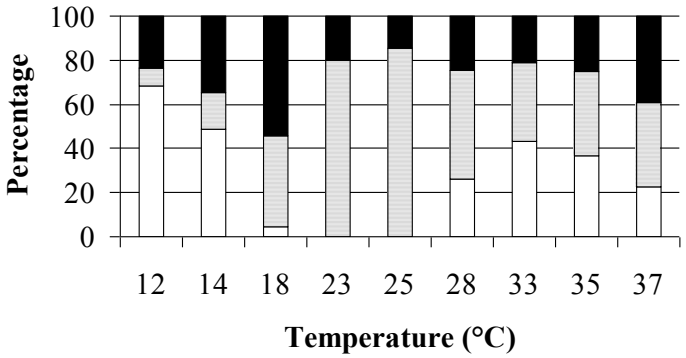


Figure 4. Percentage fresh-ungerminated seeds (□), dead seeds (▨) and abnormal seedlings (■), expressed as a proportion of total seeds that did not give normal seedlings, after 27 days from sowing across nine temperatures ranging from 12°C to 37°C. Data have been averaged across the four commercial seed-lots. Each value is the mean of 4 seed-lots with 4 replicates of 50 seeds from each seed-lot at each temperature.

Discussion

This study showed that the optimum temperature for germination rate was 24.4°C (Experiment 1) and for normal seedling development rate was 24.8°C to 25.4°C (Experiment 2), consistent with the ISTA (1999) guidelines. The optimum for germination rate was lower than the 28°C reported by Lopez *et al.* (2000). Although the optimum temperature for germination rate in seed-lot Glob 185 was found to be 24.4°C in the present study, the rate at the temperatures from 19.9°C to 28.8°C did not differ significantly from the temperature tested closest to this optimum. Similarly, the optimum temperature for normal seedling development rate in seed-lot Glob 185 was found to be 25.5°C and the rate at the temperatures from 22.6°C to 28.8°C did not differ significantly from the temperature tested closest to this optimum. The calculated optimum for normal seedling development rate in seed-lot Glob 185 was 25.4°C in Experiment 1 and 25.5°C in Experiment 2. Given that these two experiments were conducted over a different range of temperatures, this gives confidence in the prediction of the optimum temperature for normal seedling development rate. The seed-lot used in the study by Lopez *et al*. (2000) was from the Furneaux provenance, whereas the seed-lots used in the present study were from a broad genetic background (except for KI 133). This difference in genetic composition between seed-lots may account for the difference in the temperature optimum for germination rate.

This study showed that temperatures above 30°C generally delayed germination, reduced total germination percentage, caused a high proportion of seed death and suppressed the development of normal seedlings. This study confirmed the results found by Lopez *et al.* (2000) for *E. globulus*, showing that germination rate and total percentage germination of *E. globulus* seeds declines at temperatures above 28°C. The germination response of *E. nitens* seeds has also been shown to be significantly affected by variation in temperature, with germination rate increasing with temperature to an optimum of 28°C or 18°C (depending on provenance seeds were obtained from) and then declining (Humara *et al.* 2000). The rate and percentage of normal seedling development was also found to decline at temperatures above 29°C in both experiments (except for seed-lot KI 133 which retained a high percentage at

33°C). By exposing seeds to a greater range of temperatures above and below optimum than the study by Lopez *et al*. (2000) the present study determined the cardinal temperatures for rate of normal seedling development and the optimum temperatures for maximum attainment of normal seedlings for the four seed-lots studied.

Temperatures below 19°C were found to slow the onset of germination and development of normal seedlings in seed-lot Glob 185, however the maximum levels of these parameters attained over the 21 day period were similar to those at the optimum temperature. Of the low temperatures tested in Experiment 1 with seed-lot Glob 185, the lowest (15.2°C) was found to reduce the onset of germination and normal seedling development most significantly. The delay of commencement of germination at low temperatures can most likely be explained by thermodynamics, with the physiological processes occurring in seeds related to the commencement of metabolism being slowed down by the reduced temperature (Kader and Jutzi 2002). In *E. globulus* (Lopez *et al.* 2000) and *E. nitens* (Humara *et al.* 2000) it has been shown that reduced germination at lower temperatures was associated with lower rates of seed imbibition. Water uptake by seeds under optimal conditions of supply is triphasic, with a rapid initial uptake phase followed by a lag phase leading to phase 3, which is concurrent with radicle elongation (Bewley and Black 1994). The durations of both the lag phase and the second rapid uptake of water phase are dependent on imbibition temperature, with lower temperatures lengthening the lag phase and therefore delaying radicle protrusion (Kader and Jutzi 2002).

The delayed commencement of germination at low temperatures did not affect percentage germination or percentage normal seedlings developed in Experiment 1. However, high temperature exposure negatively affected percentage germination and percentage normal seedlings developed and this could be explained by the fact that the lower temperatures did not impair the physiological processes but merely reduced their rate of action. Conversely, high temperatures have been shown to cause damage to metabolic functions such as protein denaturation, reduced enzyme function and membrane damage (Bewley and Black 1994; Corbineau *et al.* 2002). It is this impairment at the cellular level that most likely led to not only the reduced

germination rate but also the reduction in percentage germination and normal seedlings developed.

The mechanisms preventing germination under high temperature stress are known to vary in different species. If thermoinhibition is involved then ungerminated seeds should germinate immediately upon transfer to a temperature suitable for germination (Horowitz and Taylorson (1983). The control of thermoinhibition, has been classified into two broad categories by Hills and van Staden (2003). The first is where germination at high temperatures can be prevented through the action of the embryo coverings, which either place a mechanical restraint on embryo extension, form a permeability barrier interfering with water uptake and gaseous exchange or possess endogenous germination inhibitors. The second category includes embryo factors, where the presence of endogenous chemicals, mainly hormonal, prevent germination at high temperatures. There is often a complex interplay between both sets of factors leading to thermoinhibition (Hills and van Staden 2003).

While the exact mechanism preventing the germination of *E. globulus* seeds at high temperatures is unresolved, it is clear that thermoinhibition as defined can not be the sole factor as exposure to high temperatures resulted in a high proportion of seed death and is thus not reversible. The increased level of abnormal seedlings observed at high temperatures suggests that there is a negative effect on seedling development as well as on seed germination. Seedlings were generally found to be stunted at high temperature, relative to those germinated under optimal conditions, suggesting an impairment of the seedling development process.

The responses observed in these experiments at high temperatures could be due partly to protein denaturation. Protein denaturation is related to temperature such that as the temperature rises, changes in protein conformation occur which actually promote the germination process (Bewley and Black 1994). As temperatures become too high, further conformational changes occur which are deleterious. Analysis of protein expression during the onset and release of thermoinhibition in a study by Hills *et al*. (2001) found that a number of proteins are specifically expressed in response to thermoinhibition. These proteins imposed a block on germination which was presumed to actively prevent radicle elongation. Changes in membrane states

could also have contributed to the responses to high temperature observed. Corbineau *et al.* (2002) found that deterioration of sunflower seeds during incubation at a high temperature (45°C) was mainly related to membrane damage and alteration of energy metabolism. This study also revealed that electrolyte and potassium leakage was higher when seeds were germinated at high temperatures compared to the optimum temperature. A study by Taylor *et al*. (2007) found that endogenous embryo factors, which act mainly in the radicle, prevent germination of *Tagetes minuta* at high temperatures.

Endogenous embryo factors could have been involved in the high temperature response in the present study. In the study by Taylor *et al*. (2007) the stage of development at which seeds were most sensitive to exposure to high temperatures was also shown to be prior to radicle emergence, as once this had occurred at 25°C, subsequent exposure to 36°C did not inhibit further emergence and development of those seeds. It has been suggested by Taylor *et al*. (2007) that DNA synthesis can be inhibited by high temperatures in *Tagetes minuta* achenes and this inhibits subsequent germination under optimal conditions. The optimum temperature for net photosynthesis of *E. globulus* was found to be in the range 17 to 23°C by Battaglia *et al*. (1996), depending on the mean daily temperature. Although photosynthesis is not the main process influencing development from germination to normal seedling development, the temperature optimum for photosynthesis is similar to that found in this study for normal seedling development (21.2 – 24.8°C) but slightly lower than the optimum for normal seedling development rate (24.8 – 25.5°C).

The variation in response of the commercial seed-lots studied to low and high temperature exposure during germination could be due to numerous factors including genetics, storage time of seeds and seed orchard environment. Seed-lot KI 133 was very sensitive to low temperatures. This seed-lot was harvested 2-3 years earlier than the other seed-lots and this extra time in storage may have affected its performance. However, the possibility of a genetic difference can not be dismissed as it was the least genetically diverse, containing only selections from King Island, whereas the other three seed-lots were from a broad genetic background. Indeed, it is possible that the response observed could reflect adaptation to less extreme temperatures in the maritime environment of King Island.

The fact that seed-lots responded differentially to temperature in maximum percentage normal seedling development suggests that through suitable pre-screening there is the opportunity to avoid the use of seed-lots which are sensitive to high temperatures during either germination or seedling development. This strategy would be expected to result in a more uniform crop of seedlings in the nursery. Commercial germination testing is conducted at 25°C and as these results show this generally results in a high level of germination for most seed-lots. This temperature is not reflective however of those that the seeds could be exposed to in the nursery and this test does therefore not give very useful information about susceptibility to high temperature stress. It would be more useful if seed-lots were screened at temperatures higher than 33°C prior to dispatch, as these results show that this would allow identification of sensitive seed-lots. It has been observed that seed-lots showing poor germination in nurseries had displayed high viability, uniformity and vigour in laboratory based germination tests carried out under ISTA guidelines for *Eucalyptus globulus* (ISTA 1999). The results show that germination testing at 25°C can pick up rank differences in percentage germination but the commercial test does not assay germination rates nor allow non-optimal responses to be detected.

In conclusion, germination and normal seedling development rate were more sensitive to temperatures outside the optimum range than percentage germination or normal seedling development. Percentage germination and normal seedling development were relatively stable over a broad temperature range, whereas rate was more specific. Commercial seed-lots varied in percentage normal seedling development at low and high temperatures. There was a differential response of seed-lots to temperature in percentage normal seedling development, however this was not evident for rate of normal seedling development. While the seed-lots tested differ in their response to low and high temperatures, the basis of this variation is unclear as the seed-lots were different in numerous factors including their genetic composition, storage time and seed orchard environment.

Chapter 3

The genetic regulation of *Eucalyptus globulus* seed germination

Introduction

The maternal parent may influence the phenotype of offspring through direct contributions to the nuclear genotype of the embryo and through specific maternal effects. Maternal effects may be either genetically or environmentally based and are signalled when the maternal effect on offspring performance exceeds the 50% expectation from Mendelian inheritance (Galloway *et al.* 2009; Lacey 1998). Maternal effects have been widely documented in plants and can affect offspring vigour and fitness (Byers *et al.* 1997; Donohue 2009; Galloway *et al.* 2009; Lipow and Wyatt 1999; Roach and Wulff 1987). Most studies of maternal effects on plants have focused on characters expressed early in the life cycle, such as seed size or seed mass (Bischoff and Muller-Scharer 2010; Mazer and Wolfe 1998), and dormancy and germination (Galloway 2001b). Although maternal effects are most common in the early stages of the life cycle (Roach and Wulff 1987), they can persist into adulthood (Galloway *et al.* 2009; Helenurm and Schaal 1996) and even flow across generations (Byers *et al.* 1997). These effects may result from direct or indirect causes. Maternal effects on traits such as seed size and germination, for example, are common and because these traits can greatly affect early seedling growth (Galloway *et al.* 2009; Roach and Wulff 1987), the maternal effects may persist into the later stages of the life cycle.

Maternal effects have been partitioned by Roach and Wulff (1987) into: cytoplasmic genetic, endosperm nuclear, and maternal phenotypic effects. Maternal phenotypic effects result from the environment or genotype of the maternal parent and are probably the least well understood class of maternal effects. These effects include epigenetic as well as biotic and abiotic environmental effects (Agrawal 2001; Lacey 1998; Mazer and Gorchov 1996; Roach and Wulff 1987; Schmid and Dolt 1994). Studies of direct and indirect maternal effects in plants have focused on short-lived annuals (Byers *et al.* 1997; Helenurm and Schaal 1996), and there are relatively few

studies of long-lived forest trees. Studies of forest tree systems have demonstrated that the environment experienced by parents in a seed orchard may affect the expression of phenotypic variation in the subsequent generation used for deployment (termed "seed aftereffects") (Andersson 1994; Stoehr *et al.* 1998).

Maternal environment effects are considered by Roach and Wulff (1987) as "transient influences that endure one generation or, with diminishing effect, into the second generation." These influences may have a structural or physiological basis. The tissues immediately surrounding the embryo and endosperm, for example, are all maternal (Bischoff and Muller-Scharer 2010; Roach and Wulff 1987). These tissues eventually form the seed coat, fruit and accessory seed structures, which become important determinants of seed dormancy, dispersal and germination traits (Monty *et al.* 2009; Roach and Wulff 1987) and can influence the phenotype of an individual at maturity (Donohue 2009; Galloway 2001a; Mazer and Gorchov 1996). The availability of resources to a maternal plant may influence progeny phenotype due to the quantity or quality of nutrients available to offspring (Donohue 2009; Galloway 2001a; Mazer and Gorchov 1996; Pico *et al.* 2004). An example of this is angiosperms growing in resource-rich environments often producing larger or higher quality seeds than those growing in resource-poor environments (Mazer and Wolfe 1992; Miao *et al.* 1991; Stratton 1989; Valencia-Diaz and Montana 2005). Phenotypic maternal effects can be caused by a number of different environmental factors. Seed size, for example, has shown a sensitivity to maternal temperature, water availability, resource availability, and hormone level (Roach and Wulff 1987). Maternal photoperiod has been shown to influence the percentage and rate of seed germination in *Arabidopsis thaliana* (Munir *et al.* 2001).

Maternal effects can also influence the sensitivity of seeds to environmental conditions (Roach and Wulff 1987). A study by Sung *et al*. (1998) showed that the sensitivity of lettuce seeds to high temperature stress during germination was at least partly related to maternal environment. The study showed that the upper temperature limit for germination of lettuce seeds could be modified by manipulating the temperature of the maternal environment during seed production, with seeds developing under higher temperature conditions having increased tolerance to high

temperature stress. There is no published information on the effect of maternal environment on *E. globulus* seed germination or response to high temperature stress.

Cytoplasmic genetic maternal effects are derived from the fact that organelles such as plastids and mitochondria can be directly transferred, independent of nuclear genes, from the maternal plant to the offspring during ovule formation and development (Donohue 2009; Roach and Wulff 1987). Molecular and quantitative genetic studies have shown that cytoplasmic factors contribute to heritable variation in both qualitative and quantitative traits in plants (Galloway *et al.* 2009; Rasanen and Kruuk 2007; Roach and Wulff 1987). Maternal nuclear effects result from the nuclear genotype of the mother plant directly or indirectly affecting offspring phenotype. Variance at nuclear loci among gametes produced by different parents might be expected to affect the survival and reproduction of offspring (Helenurm and Schaal 1996). Variation in nuclear genes tends to affect developmental characters such as the number of leaflets on the second leaf, the timing of stem production relative to leaf production, size at two months and flowering time of *Lupinus texensis* (Helenurm and Schaal 1996). Maternal nuclear effects have also been suggested to influence seedling growth rate, leaf area and tiller number (Roach and Wulff 1987).

A second class of maternal genetic effects originate from the endosperm. Endosperm nuclear effects arise because during angiosperm development, multiple fertilisation usually results in 3N endosperm with two nuclei from the maternal and only one from the paternal parent (Donohue 2009; Galloway 2001a; Roach and Wulff 1987). The endosperm therefore always contains more doses of maternal than paternal genes (Donohue 2009). The endosperm contains enzymes important for germination (Harvey and Oaks 1974) and is also the source of nutrients for the developing embryo. The female parent may have a more important role in determining the characteristics of this nutrient source due to the differential dosage of male and female genes (Donohue 2009; Roach and Wulff 1987).

There are few studies that allow separation of maternal embryonic nuclear and maternal effects (Bischoff and Muller-Scharer 2010; Monty *et al.* 2009; Rasanen and Kruuk 2007), particularly in the case of forest trees (Besnard *et al.* 2008). This is partly because many studies confound genetic effects with maternal environment, by

not having replicated maternal genotypes growing in common environment trials (Monty *et al.* 2009; Rasanen and Kruuk 2007). The present study focuses on the role maternal genetics plays in seed germination responses in the Australian forest tree *Eucalyptus globulus*.

Eucalyptus globulus is one of the few eucalypt species in which maternal and seed after-effects have been studied. A study by Lopez *et al*. (2003) showed that seed mass exhibited a significant maternal effect, increasing seed germinative capacity but not germination rate. This study could not, however, differentiate maternal genetic from maternal environment effects on germination, because seeds were harvested from trees in native stand localities and therefore maternal genetic effects were confounded with maternal environment effects. The present study allows separation of maternal genetics from maternal environment effects, by including randomised replicates of maternal genotypes at a single site.

The present study aimed to determine: (i) whether maternal genetic variation at the race and genotype level affects the germination of *E. globulus* seeds, (ii) whether these effects are differentially expressed in stressful germination environments, and (iii) the stability of maternal influences on germination across seasons and sites.

Materials and Methods

Study system

E. globulus is grown in pulpwood plantations throughout the temperate regions of the world (Eldridge *et al.* 1993; Potts 2004) and is mainly deployed through seed production systems rather than cloning (Potts *et al.* 2008). *E. globulus* is native to south-eastern Australia where it intergrades with three closely related taxa (Jordan *et al.* 1993). It is mainly pollinated by birds and insects, and has a mixed mating system with average outcrossing rates in undisturbed native forests higher than 85% (Mimura *et al.* 2009). The genetic diversity across the natural range of *E. globulus* and intergrade populations has been summarised using a hierarchy of races and subraces (Dutkowski and Potts 1999).

E. globulus trees used in this study were located in a seed orchard in Cambridge, south-eastern Tasmania (42°48’27.23"S 147°25’58.48"E) containing selected

germplasm from the Australian National *E. globulus* breeding program run by the Southern Tree Breeding Association (STBA) and mainly included genotypes from three of the *E. globulus* races identified by Dutkowski and Potts (1999) as the Furneaux Group, Strzelecki Ranges and Western Otway races. Cambridge has an altitude of 40 m with a long term average annual rainfall of 507 mm and average annual maximum and minimum temperatures of 17.4°C and 8°C, respectively. Trials were carried out over three consecutive seasons 2007/08, 2008/09 and 2009/10. All trees were treated in accordance with standard commercial practices.

Seeds were also harvested from *E. globulus* trees in a seed orchard in Manjimup, Western Australia (34°15'3"S 116°8'42"E). Manjimup has an altitude of 290 m with a long term average annual rainfall of 1007 mm and average annual maximum and minimum temperatures of 20.3°C and 9.6°C, respectively. Seeds were harvested from this orchard on the 13th January 2009.

Genotype selection

Genotypes were selected from each of the three races, hereafter referred to as Furneaux, Otway and Strzelecki. From the Cambridge seed orchard, four genotypes were selected from the Furneaux race, nine from the Otway race and four from the Strzelecki race. From the Manjimup seed orchard, three genotypes were selected from the Furneaux race, six from the Otway race and four from the Strzelecki race. There were generally four replicates (ramets) from each genotype, however in some cases this was not possible and less were harvested. Ramets of different genotypes had been planted in line plots which were randomly distributed through the seed orchards. Ramets of each genotype were selected from different line plots, where possible.

In the 2007/08 season seeds from all trees were harvested at 13 months elapsed time from when flowering records for the orchard indicated the genotypes first flowered. In the 2008/09 season seeds from Cambridge were harvested at varying times elapsed from peak flowering, ranging from 10-13 months. It was unknown how much time had elapsed from peak flowering when the trees from Manjimup were harvested, however all samples were picked on the same day. In the 2007/08 season

at Cambridge, seeds derived from two trees out of 50 were from mass-supplementary pollination (MSP) and the remainder were derived from open-pollination (OP). In the 2008/09 season at Cambridge, seeds derived from 16 trees out of 49 were from MSP and the remainder were derived from OP. In the 2008/09 season at Manjimup, all seeds were derived from OP. For harvesting, trees were divided into four sections, lower and upper sections of the canopy on the north and south sides of the tree. An equal proportion of capsules was harvested from each section in order to provide a sample that was as representative of the whole tree as possible. After capsules were harvested, seeds were extracted by placing capsules in an oven at 40°C for 24 hours. Chaff and impurities were removed from samples using a vertical air column and by hand-picking to 100% seed purity. Seed-lots were cool-stored in a 5°C room in darkness until the commencement of germination testing. The number of seeds per capsule and weight of 100 seeds were recorded for the sample from each ramet.

Seed germination

Seeds were germinated at three separate temperatures, in three separate incubators set at 25°C (optimal), 32°C and 37°C (high temperatures). For the 2007/08 season at Cambridge, seed germination tests were undertaken in two runs (runs 1 and 2). Half of the replicates were germinated in the first run and the other half in the second run. This was done because there was insufficient space in the germination cabinets to complete the experiment in a single run. For the 2008/09 season, seeds from Cambridge and Manjimup were germinated in single, separate runs (runs 3 and 4 respectively). In all cases, seeds were germinated under continuous fluorescent light. Although it has been reported that uniformity and germination rate of *E. globulus* seeds can be improved with constant darkness (Nair *et al.* 2009; Nair *et al.* 2007) the present study used constant light because the experiment included normal seedling development which requires light. Seeds from each tree (seed-lot) were represented once at each temperature. Seed-lots were completely randomised within perspex cabinets at each temperature. Three to six replicates of the control "reference" seed-lot Glob 185 (used in Chapter 2) were also germinated at each temperature.

Seeds were germinated on sections of Advantec number 1 type filter paper placed in the perspex germination cabinets as described in Chapter 2. One length of filter paper

was placed over each section in the perspex cabinet. 75 mL of distilled water was added to each well in each cabinet and water levels were checked daily, with distilled water added where necessary. The incubator set at 25°C was a Linder and May LMRIL – Series 320-1-SD refrigerated incubator (Queensland, Australia). The incubator set at 32°C was a Contherm Digital Series cooled incubator (New Zealand). The incubator set at 37°C was an Axyos Technologies Micro Digital Control refrigerated incubator control system (Queensland, Australia). For each replicate, 50 seeds were placed on each section of filter paper, giving 400 seeds per perspex cabinet. The commercial seed-lot Glob 185, described in Chapter 2, was used as a reference in all runs. For the reference seed-lot Glob 185, three to six replicates of 50 seeds were germinated at each temperature during each run. All seeds were counted using a Contador electronic seed counter (Baumann Saatzuchtbedarf D-74638, Waldenburg). Cabinets were placed in a random order within incubators and their location within the incubators was changed each time the trial was scored.

Trial commencement

All seeds were germinated within 8 to 17 months from harvest. Run 1 commenced on the 5th of August, 2008 (day 0) and concluded on the 29th of August 2008. Run 2 commenced on the 15th of May 2009 (day 0) and concluded on the 5th of June 2009. Run 3 commenced on the 7th August 2009 (day 0) and concluded on the 28th August 2009. Run 4 commenced on the 4th September 2009 (day 0) and concluded on the 25th of September 2009. Tiny Tag Data Loggers (Gemini Data Loggers, United Kingdom) were placed in each of the incubators and logged temperature for the duration of the trials.

Trial assessment

Run 1 was assessed daily (with the exception of day 23) for germination and normal seedlings, as outlined above. Runs 2, 3 and 4 were assessed on days 3, 4, 5, 6, 7, 10, 14, 18 and 21. For run 2, only seeds at 25°C were scored on day 6 and only seeds at 37°C were scored on day 18. For runs 3 and 4, only seeds at 25°C were scored on days 4 and 6 and only seeds at 37°C were scored on day 18. For all runs, a seed was recorded as germinated when there was visible protrusion of the radicle through the

seed coat. A seedling was classified as normal, in accordance with ISTA guidelines (Bekendam and Grob 1993; ISTA 1999) and removed for all runs when it had a radicle at least one third the length of the seedling, a hypocotyl and two expanded cotyledons which were not covered by the seed coat. The day at which a seed germinated or seedling was exhibiting normal development was recorded. On the final day of scoring for all runs, remaining seedlings were classified as normal or abnormal. Abnormalities included stunted radicles, failure of radicles to emerge but emergence of cotyledons, and failure of seedlings to "push-off" seed coats, in accordance with ISTA guidelines for seedling evaluation (Bekendam and Grob 1993). The final day of scoring for all runs was done as outlined in Chapter 2.

Statistical analysis

To estimate the response rate of each experimental unit of 50 seeds, the time taken for 50% of the maximum germination or normal seedling development (T50) was calculated using the logistic function which was fitted to the cumulative curves for germination or seedling development (Shafii and Price 2001). This was done using the Marquardt's iterative non-linear procedure PROC NLIN of SAS version 9.1 (SAS Institute Inc 2003). The procedure for calculation was detailed in Chapter 2. The data were summarised into six response traits, three measuring proportions and three measuring rates. The proportional data included the proportion of seeds sown that germinated (germ/sown); the proportion of seeds that germinated which developed into normal seedlings (normals/germ); and the proportion of seeds sown that developed into normal seedlings (normals/sown). The rate data included the time taken to reach 50% (T50) of the maximum germination attained (T50germ); the time taken to reach 50% (T50) of the maximum normal seedlings attained (T50normals) and the difference between the T50 for germination and T50 for normal seedling development (T50normals-T50germ).

The germination tests were carried out in multiple runs and in each run three to six replicates of 50 seeds of commercial seed-lot Glob 185 were germinated at each temperature. The amount each of the runs deviated from the grand mean of the control seed-lot was calculated for each trait at each temperature. The data for each run and each temperature were then additively adjusted to account for the deviation

of the control seed-lot from the grand mean. The data were analysed in several stages by fitting linear mixed models (as detailed in results section) PROC MIXED of SAS Version 9.1 (SAS Institute Inc 2003). Prior to analysis the proportional data were arcsine transformed and rate data were subjected to log transformation to optimise normality and homogeneity of residuals.

Initially a fixed genotype model was fitted to the data for each site/season separately, where genotype effects were tested ignoring race. This test treated run, temperature, genotype and the two way interaction as fixed effects (genotype model). Tree within genotype was treated as a random effect and used to test the fixed genotype effect. The temperature and interaction terms were tested against the residual error. To determine whether seed weight influenced germination, the same model was also fitted to the data but including seed weight as a covariate.

A race model was fitted, where run, temperature, race and the two way interaction were treated as fixed effects and genotype within race and its two way interaction with temperature and tree within genotype were treated as random effects. Genotype within race was used to test the race effect. Genotype within race by temperature was used to test the temperature and race by temperature fixed effects. The other fixed effects were tested against the residual error term. The effect of race and site on seed weight and number of seeds per capsule was also specifically tested. In this case, race, site, temperature and their two and three way interactions were treated as fixed effects and genotype within race and site by genotype within race were treated as random effects and used to test the other fixed effects. To test whether seed weight (measured as the weight of 100 seeds) or pollination type (OP or MSP) affected the germination response, seed weight (covariate) and pollination type (factor) were included separately in the previously described genotype model and race model.

Analysis of season effects was carried out on the data from Cambridge, seasons 2007/08 and 2008/09. The data were restricted to only include genotypes where two or more ramets matched across the seasons. For the six germination traits, season, run within season, genotype, temperature and their two and three way interactions were treated as fixed effects. Tree within genotype was treated as a random effect and was used to test the fixed genotype effect. Other fixed effects were tested using

the residual error. Higher order interactions including tree within genotype were rarely significant and were submerged with the residuals. Using the same individuals, the fixed effect of genotype, season and their interaction on the number of seeds obtained per capsule and seed weight were tested using a linear model and the residual as the error term. The race effects on the number of seeds obtained per capsule and seed weight were also tested using a linear mixed model with race, season and their interaction as fixed effects. The genotype within race and its interaction with season were fitted as random effects and used to test terms involving race.

Analysis of site and temperature effects on germination traits was carried out on a subset of genotypes which matched at both sites in season 2008/09 and that had more than one ramet represented at each site. For the six germination traits, site, temperature, genotype and their two and three way interactions were treated as fixed effects. Tree within genotype was treated as a random effect and used to test the fixed genotype effect. All other fixed effects were tested with the residual term. For seed set and weight, the fixed effect of genotype, site and their interaction were tested using a linear model and the residual as the error term.

The correlation of germination traits within individual temperatures, proportion germination and germination rate, proportion normal seedlings and normal seedling development rate and rate traits at 25°C and 37°C were performed using the PROC CORR procedure in SAS version 9.1 (SAS Institute Inc 2003). Pearson's correlations were calculated (i) using genotype least square means averaged across three temperatures (25°C, 32°C and 37°C) from 17 genotypes from Cambridge 2007/08 between proportion germination and germination rate and between proportion normal seedlings and normal seedling development rate, (ii) between germination rate at 25°C and 37°C and normal seedling development rate at 25°C and 37°C using least square means for 17 genotypes from Cambridge 2007/08 and (iii) for combined data which contained the genotype means for germination traits at 25°C and 37°C for Cambridge 2007/08, 2008/09 and Manjimup 2008/09.

Results

Effect of genotype on seed set and seed size

At Cambridge, there was a significant effect of both genotype and season on the number of seeds per capsule and seed weight (Table 2). These effects of genotype could not be attributable to racial differences. The seasonal effect was due to fewer seeds per capsule and heavier seeds being obtained in 2007/08 (seeds per capsule was 7.54 ± 1.38 and seed weight was 0.23 ± 0.01) than in 2008/09 (seeds per capsule was 10.63 ± 1.38 and seed weight was 0.20 ± 0.01), although this effect varied slightly with genotype for both traits.

When testing across sites in the same season, genotype effects were only detected for seed weight but there was a significant site effect for both seed weight and the number of seeds per capsule (Table 3). This was due to the Cambridge site producing more and heavier seeds per capsule than the Manjimup site (seeds per capsule for Cambridge was 12.51 ± 1.44 and seed weight was 0.17 ± 0.01; seeds per capsule for Manjimup was 7.49 ± 1.64 and seed weight was 0.14 ± 0.01).

Within site and season effects of genotype on seed germination

Genotype and temperature had a significant effect on virtually all seed germination traits examined, however the interaction between genotype and temperature was only significant in 3 of the 18 tests (Table 4). Only at the Cambridge site for the rate of normal seedling development for both seasons was the variation between genotypes explicable by a difference between the race of origin of the genotype (Table 5). This was due to seeds from Strzelecki developing normal seedlings faster than those from Otway and Furneaux (Figure 5). The same trend was evident for the rate of seed germination (Table 5; $P<0.1$; data not plotted). Inclusion of seed weight as a covariate in the genotype analysis showed that seed weight only had an effect (negative) on rate of normal seedling development (Cambridge 2008/09 and Manjimup 2009). However, its inclusion in the model did not influence the significance of the effect of genotype or its interaction for any trait (as reported in Table 4). The race differences observed for the rate of seed germination and normal seedling development were accentuated when seed weight was included as a

covariate in the model. The number of seeds per capsule as a covariate had no effect on any of the germination traits in any of these analyses.

Table 2. Effect of genotype, race and season on seed weight and the number of seeds per capsule
The data were derived from seeds collected in 2007/08 and 2008/09 from 14 genotypes growing in the Cambridge seed orchard, Tasmania. The analyses are presented for separate genotype and race level analyses

			Seed Weight		Seeds per Capsule	
Fixed Effects	Num d.f.	Den d.f.	F	P	F	P
Genotype	13	52	22.4	<0.001	3.3	0.001
Season	1	52	32.8	<0.001	6.2	0.016
Season*Genotype	13	52	2.2	0.024	2.2	0.022
Race	2	11	2.6	0.120	1.0	0.406
Season	1	11	12.6	0.005	3.2	0.101
Race*Season	2	11	0.2	0.803	1.1	0.366

Table 3. Effect of genotype and site on seed weight and the number of seeds per capsule
The data were derived from seeds collected in 2007/08 and 2008/09 from 14 genotypes growing in the Cambridge seed orchard, Tasmania

			Seed Weight		Seeds per Capsule	
Fixed Effects	Num d.f.	Den d.f.	F	P	F	P
Genotype	4	18	4.8	0.008	2.7	0.067
Site	1	18	7.8	0.012	5.3	0.034
Site*Genotype	4	18	0.3	0.853	0.8	0.522

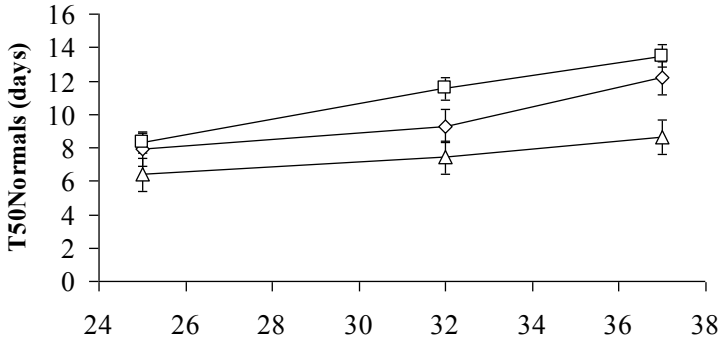
Table 4. **Effect of genotype and temperature on six germination traits**
Analyses were undertaken on seeds collected from Cambridge 2007/08, Cambridge 2008/09 and Manjimup 2008/09. The effects were tested on the proportion of seeds germinated (Germ/Sown), the proportion of germinated seeds which developed into normal seedlings (Normals/Germ), the proportion of normal seedlings developed (Normals/Sown), germination rate (T50Germ), rate of development from germination to normal seedlings (T50Normals-T50Germ), and normal seedling development rate (T50Normals). F is the F value and P is the probability. Num d.f. is the numerator degrees of freedom and Den d.f. is the denominator degrees of freedom

			Proportion						Rate (days)					
			Germ/Sown		Normals/Germ		Normals/Sown		T50Germ		T50Normals-T50Germ		T50Normals	
Fixed Effects	Num d.f.	Den d.f.	F	P	F	P	F	P	F	P	F	P	F	P
Cambridge 2007/08														
Genotype	16	35	4.4	0.000	1.6	0.122	2.9	0.004	7.7	<0.001	1.1	0.398	6.5	<0.001
Temperature	2	70-71	54.8	<0.001	137.6	<0.001	180.7	<0.001	84.5	<0.001	1.3	0.271	107.3	<0.001
Genotype*Temperature	32	70-71	1.7	0.042	1.4	0.112	1.5	0.086	1.3	0.165	1.9	0.013	1.6	0.061
Cambridge 2008/09														
Genotype	15	29	6.3	<0.001	3.8	0.001	6.2	<0.001	19.4	<0.001	0.7	0.724	13.7	<0.001
Temperature	2	56-57	39.9	<0.001	162.6	<0.001	141.9	<0.001	107.3	<0.001	1.6	0.216	116.2	<0.001
Genotype*Temperature	29-30	56-57	0.9	0.623	1.3	0.227	0.7	0.814	1.3	0.174	0.5	0.963	0.7	0.866
Manjimup 2008/09														
Genotype	12	13	2.7	0.047	2.4	0.069	3.1	0.029	2.1	0.107	1.6	0.202	4.5	0.006
Temperature	2	26	34.0	<0.001	74.6	<0.001	68.6	<0.001	17.6	<0.001	1.3	0.300	31.0	<0.001
Genotype*Temperature	23-24	26	2.1	0.038	1.5	0.178	1.9	0.055	0.8	0.734	0.9	0.641	1.5	0.176

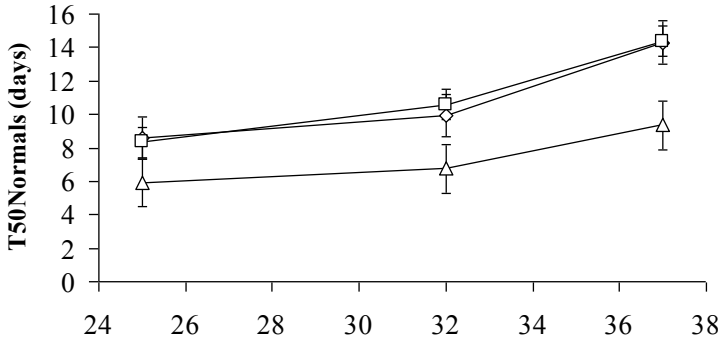
Table 5. Effect of race and temperature on six germination traits
Analyses were undertaken on seeds collected from Cambridge 2007/08, Cambridge 2008/09 and Manjimup 2008/09. For details on the germination traits, refer to Table 4

			Proportion						Rate (days)					
			Germ/Sown		Normals/Germ		Normals/Sown		T50Germ		T50Normals-T50Germ		T50Normals	
Fixed Effects	Num d.f.	Den d.f.	F	P	F	P	F	P	F	P	F	P	F	P
Cambridge 2007/08														
Race	2	14	1.6	0.233	2.3	0.135	2.4	0.130	3.6	0.056	0.6	0.581	5.1	0.022
Temperature	2	28	32.8	<0.001	101.3	<0.001	137.6	<0.001	59.8	<0.001	0.3	0.721	68.8	<0.001
Race*Temperature	4	28	2.2	0.097	0.8	0.537	2.1	0.110	1.9	0.142	0.9	0.507	2.2	0.093
Cambridge 2008/09														
Race	2	13	1.6	0.233	1.9	0.184	1.7	0.215	3.5	0.059	0.8	0.485	3.8	0.050
Temperature	2	25-26	46.2	<0.001	122.2	<0.001	157.0	<0.001	89.7	<0.001	1.5	0.254	129.5	<0.001
Race*Temperature	4	25-26	1.7	0.183	0.6	0.645	0.2	0.940	1.8	0.165	0.2	0.948	1.7	0.183
Manjimup 2008/09														
Race	2	10	0.5	0.646	1.9	0.194	0.8	0.483	0.5	0.622	0.3	0.720	0.9	0.422
Temperature	2	19-20	16.5	<0.001	82.8	<0.001	46.3	<0.001	24.4	<0.001	1.2	0.319	23.6	<0.001
Race*Temperature	4	19-20	0.9	0.464	4.1	0.014	2.1	0.114	0.8	0.514	0.5	0.749	0.5	0.708

a) Cambridge 2007/08



b) Cambridge 2008/09



c) Manjimup 2008/09

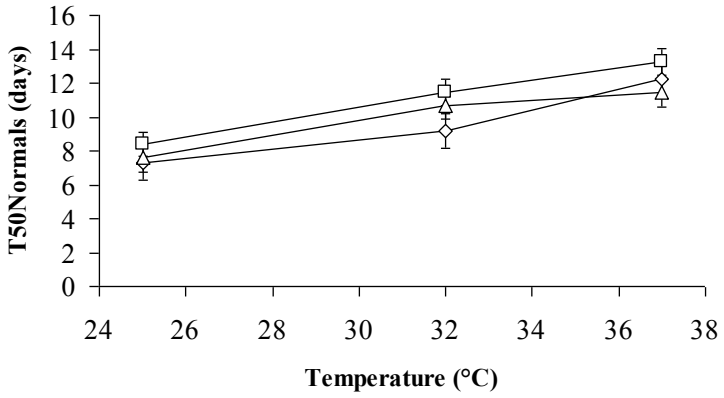


Figure 5. Least square means (±s.e.) for three races (◊ Furneaux; □ Otway; Δ Strzelecki) for rate (T50) of normal seedling development (days) from **a)** Cambridge 2007/08 **b)** Cambridge 2008/09 and **c)** Manjimup 2008/09 across three temperatures (25°C, 32°C and 37°C). The race effect was significant for Cambridge 2007/08 and Cambridge 2008/09 but there were no significant race by temperature interactions for rate of normal seedling development. Where s.e. bars are not visible they are smaller than the plot symbol.

Variation between seasons at Cambridge

To test differences between seasons of sampling at the Cambridge site, the effect of season was included in the model using data from the 14 genotypes which had samples taken from the same two ramets in both seasons (Table 6). It should be noted that the season term includes differences in actual season, seed development time and pollination type. However, when pollination type (OP or MSP) was tested in the analysis of the data from Cambridge, for the 2008/09 season, no significant effect was detected on any trait.

Neither season nor its interaction terms affected any of the rate traits, however proportional traits were affected (Table 6) and this effect varied with both temperature and genotype (Figures 6 and 9). Averaged across genotypes, the proportion of germinated seeds which developed into normal seedlings was more sensitive to increasing temperature for the 2008/09 season, compared to the 2007/08 season (Figure 6b).

When data were averaged across seasons there was a significant genotype effect for all but one trait, and one of these traits, proportion of seeds germinated, exhibited a significant genotype by temperature interaction (Table 6). This interaction was mainly due to the atypical high temperature sensitivity of genotype 6071, although other genotypes become more differentiated in their response at 37°C (Figure 7). The poor germination of 6071 at high temperatures was evident in individual season analyses, even in the 2007/08 season when its germination at the optimal temperature was well above 90% (Figure 8). The overall proportion germination and normal seedling development also changed with season depending upon genotype (Table 6). This genotype by season interaction could not be explained by a race by season interaction ($F_{2, 11} = 0.4$; $P>0.05$). The interaction was due to marked changes in ranks of several genotypes (e.g. 6071 and 5741), one markedly increasing the proportion germination and normal seedling development in 2008/09 (5741), the other exhibiting marked decreases (6071) (Figure 9). The germination of 6071 was notable as the genotype exhibited sensitivity to high temperature and season.

Both the number of seeds per capsule and seed weight were influenced by season (Table 2). When included as a covariate in the mixed model analysis, the number of seeds per capsule had no significant effect on any germination trait whereas seed weight affected proportion germination, and rates of germination and normal seedling development. The inclusions of the covariates did not affect the significance of season or other model terms in Table 6, with the exception of the seasonal effect on the proportion of normal seedlings which became non-significant.

Table 6. Effect of season and genotype on six germination traits
Analyses were undertaken on seeds collected from 14 genotypes from Cambridge seasons 2007/08 and 2008/09 that had two or more ramets matching across the seasons. For details of the germination traits, refer to Table 4

			Proportion						Rate (days)					
			Germ/Sown		Normals/Germ		Normals/Sown		T50Germ		T50Normals-T50Germ		T50Normals	
Fixed Effects	Num d.f.	Den d.f.	F	P	F	P	F	P	F	P	F	P	F	P
Genotype	13	26	6.5	<0.001	2.6	0.018	5.0	0.000	19.0	<0.001	1.0	0.543	16.9	<0.001
Season	1	120-122	0.1	0.761	19.9	<0.001	6.0	0.016	0.1	0.819	0.5	0.483	1.0	0.326
Temperature	2	120-122	93.2	<0.001	247.7	<0.001	266.7	<0.001	134.0	<0.001	0.6	0.533	139.0	<0.001
Season*Temperature	2	120-122	0.2	0.860	15.6	<0.001	5.1	0.008	1.2	0.307	0.8	0.456	2.1	0.133
Genotype*Temperature	26	120-122	2.0	0.006	1.6	0.054	1.4	0.096	1.3	0.146	0.6	0.959	1.3	0.184
Season*Genotype	13	120-122	2.8	0.002	2.8	0.002	3.5	0.000	1.4	0.152	1.3	0.216	1.5	0.112
Season*Genotype*Temperature	25-26	120-122	0.9	0.618	1.5	0.073	0.9	0.584	1.1	0.366	0.9	0.640	0.5	0.961

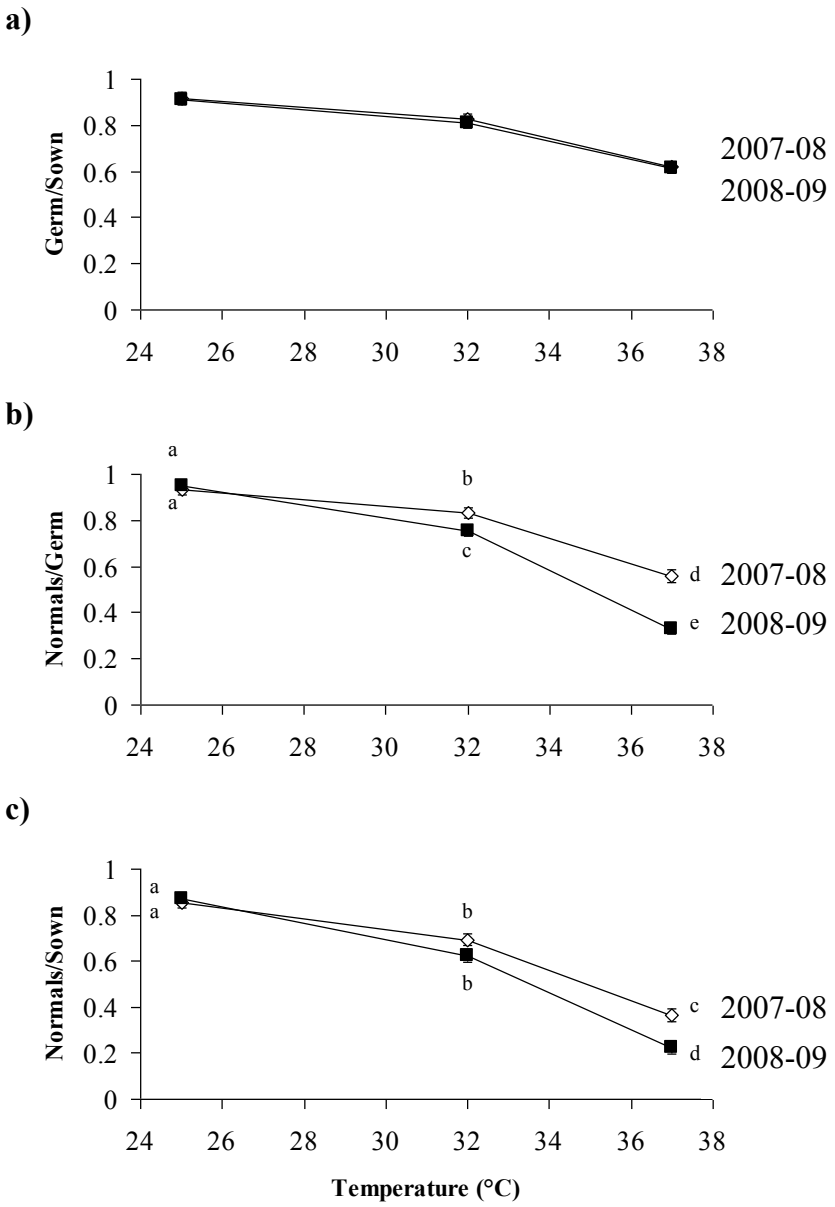


Figure 6. Least square means (±s.e.) from season by temperature interactions for **a)** proportion of germinated seeds **b)** proportion of germinated seeds which developed into normal seedlings and **c)** proportion of normal seedlings developed. Data are averaged across 14 genotypes which had 2 or more ramets matching across the two seasons at Cambridge. When interaction effects were significant common letters across both seasons and temperatures represent least square means across seasons and temperatures which were not significantly (P>0.05) different using the Tukey-Kramer multiple range test. Where s.e. bars are not visible they are smaller than the plot symbol.

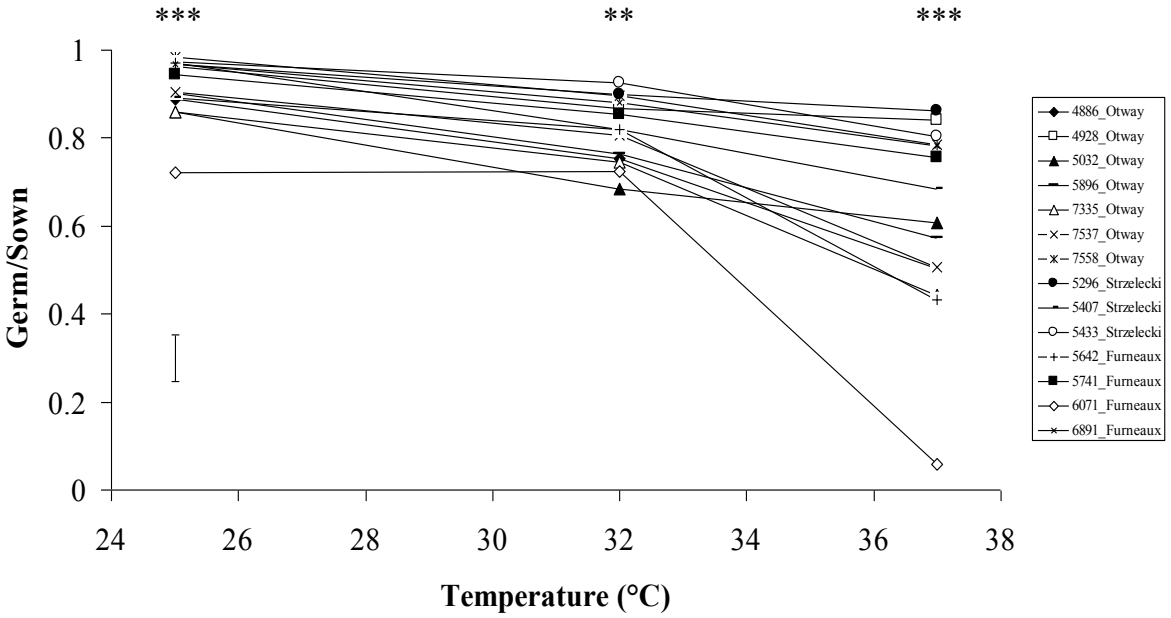


Figure 7. Least square means from genotype by temperature interactions for proportion of germinated seeds. Data are averaged across 14 genotypes which had two or more ramets matching across the two seasons at Cambridge. The bar indicates the pooled standard of n = 4. Significance of the fixed genotype effect tested separately at each temperature is indicated (ns: not significant * P<0.05 ** P<0.01 *** P<0.001).

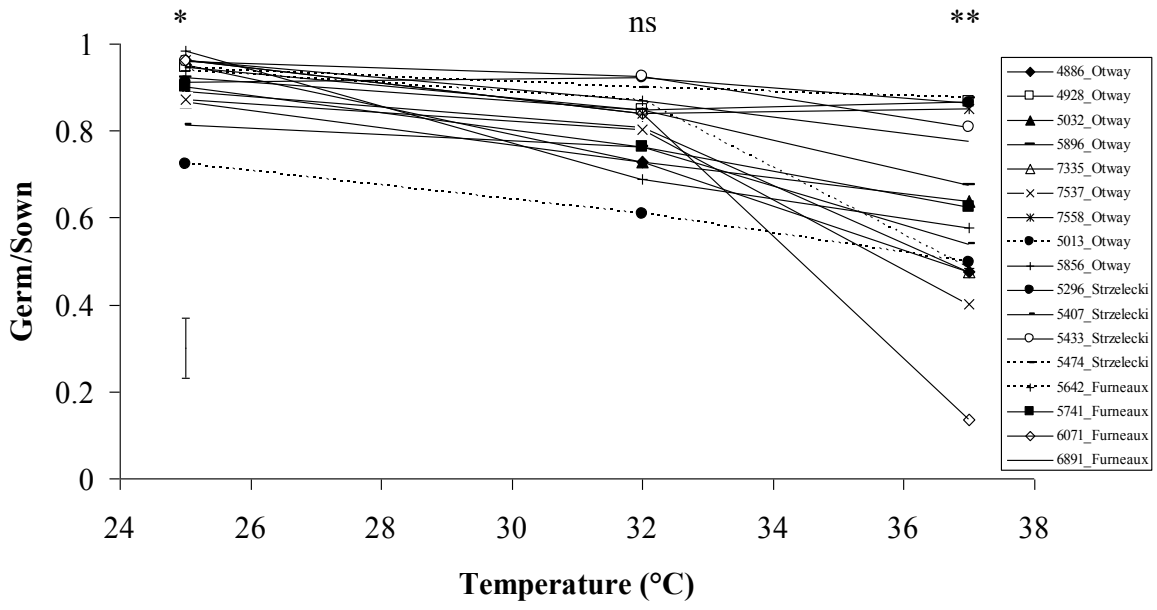


Figure 8. Least square means for proportion of germinated seeds for Cambridge 2007/08 (17 genotypes). The bar indicates the pooled standard of n = 4. Significance of the fixed genotype effect tested separately at each temperature is indicated (ns: not significant * P<0.05 ** P<0.01 *** P<0.001).

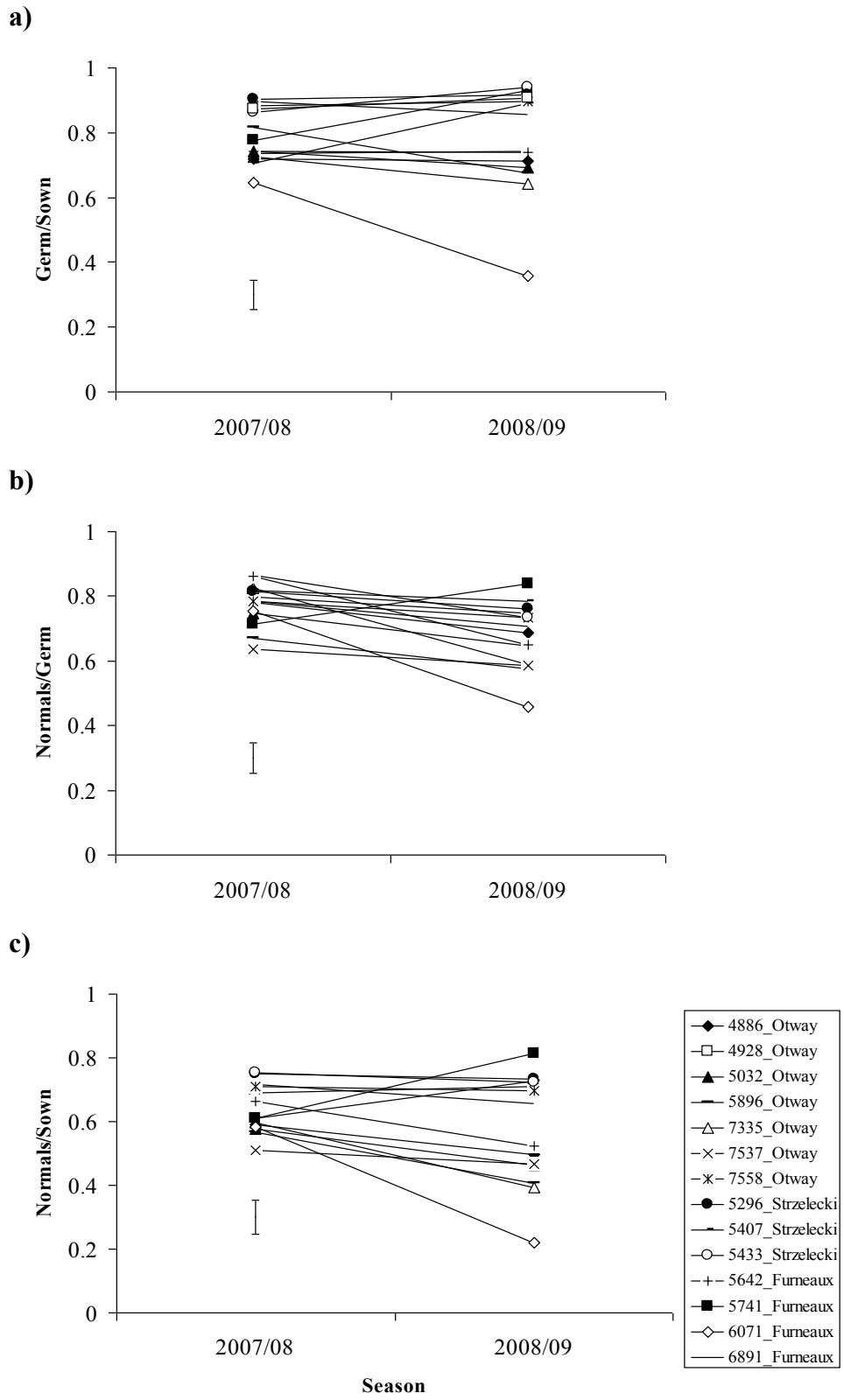


Figure 9. Least square means from season by genotype interactions for **a)** proportion of germinated seeds **b)** proportion of germinated seeds which developed into normal seedlings and **c)** proportion of normal seedlings developed for 14 genotypes with two or more ramets matching across seasons 2007/08 and 2008/09 at Cambridge. The bar on each graph indicates the pooled standard error of n = 4.

Site effects

To test whether maternal site of origin impacted on germination traits, a similar model was fitted including site as a fixed effect and data for five genotypes which matched at both sites and had more than one ramet represented at each site. These genotypes were harvested in the 2008/09 season from both Cambridge and Manjimup.

Site had a large effect on the proportion traits (Table 7), with seeds collected from the Cambridge site having a greater proportion seed germination and normal seedling development than that collected from Manjimup (Figure 10). However this effect was temperature dependent for the proportion of normal seedlings, as the difference between sites was only significant at the optimal temperature (Figure 10b, c). Similarly, the differences between genotypes was site dependent for five of the six traits assessed (Table 7). There was little consistency apparent in these genotype by site interactions, as in for some genotypes site mainly affected rate (e.g. 5642 and 5032), whereas in others both proportions and rates (5741, 5407) were adversely affected at the Manjimup site (Figure 11). While the overall site effects were small for rate traits, this was due to some genotypes exhibiting faster germination and seedling development in seeds from Manjimup, while the reverse was so for other genotypes.

Seed weight was significantly greater at Cambridge compared with Manjimup, and its inclusion as a covariate in the mixed model showed that increased seed weight was associated with faster germination ($F_{1,36}$ = 6.1; P=0.019) and faster normal seedling development ($F_{1,36}$ = 10.3; P=0.003). When seed weight was accounted for, there was no significant difference between sites for germination rate ($F_{1,36}$ = 0.6; P=0.430), but there was still highly significant effects of site on the proportion traits.

Table 7. Effect of site of sampling on six germination traits
Analyses were undertaken on seeds from five genotypes which matched at Cambridge and Manjimup and had more than one ramet represented at each site. For details of the germination traits, refer to Table 4

			Proportion						Rate (days)					
			Germ/Sown		Normals/Germ		Normals/Sown		T50Germ		T50Normals-T50Germ		T50Normals	
Fixed Effects	Num d.f.	Den d.f.	F	P	F	P	F	P	F	P	F	P	F	P
Genotype	4	19	2.6	0.070	3.0	0.046	3.3	0.034	16.8	<0.001	0.2	0.957	9.9	0.000
Site	1	38	24.5	<0.001	8.4	0.006	18.7	0.000	5.2	0.028	1.3	0.260	3.6	0.067
Temperature	2	38	32.7	<0.001	90.6	<0.001	98.4	<0.001	53.1	<0.001	1.7	0.196	58.6	<0.001
Site*Temperature	2	38	0.4	0.700	6.9	0.003	5.0	0.012	1.1	0.341	0.1	0.953	2.3	0.119
Genotype*Temperature	8	38	1.2	0.314	1.4	0.221	1.8	0.104	3.6	0.004	0.4	0.928	2.3	0.038
Site*Genotype	4	38	4.0	0.008	3.8	0.011	4.6	0.004	10.7	<0.001	0.4	0.825	6.3	0.001
Site*Genotype*Temperature	8	38	0.5	0.815	1.0	0.466	0.8	0.634	0.9	0.511	0.5	0.849	0.3	0.959

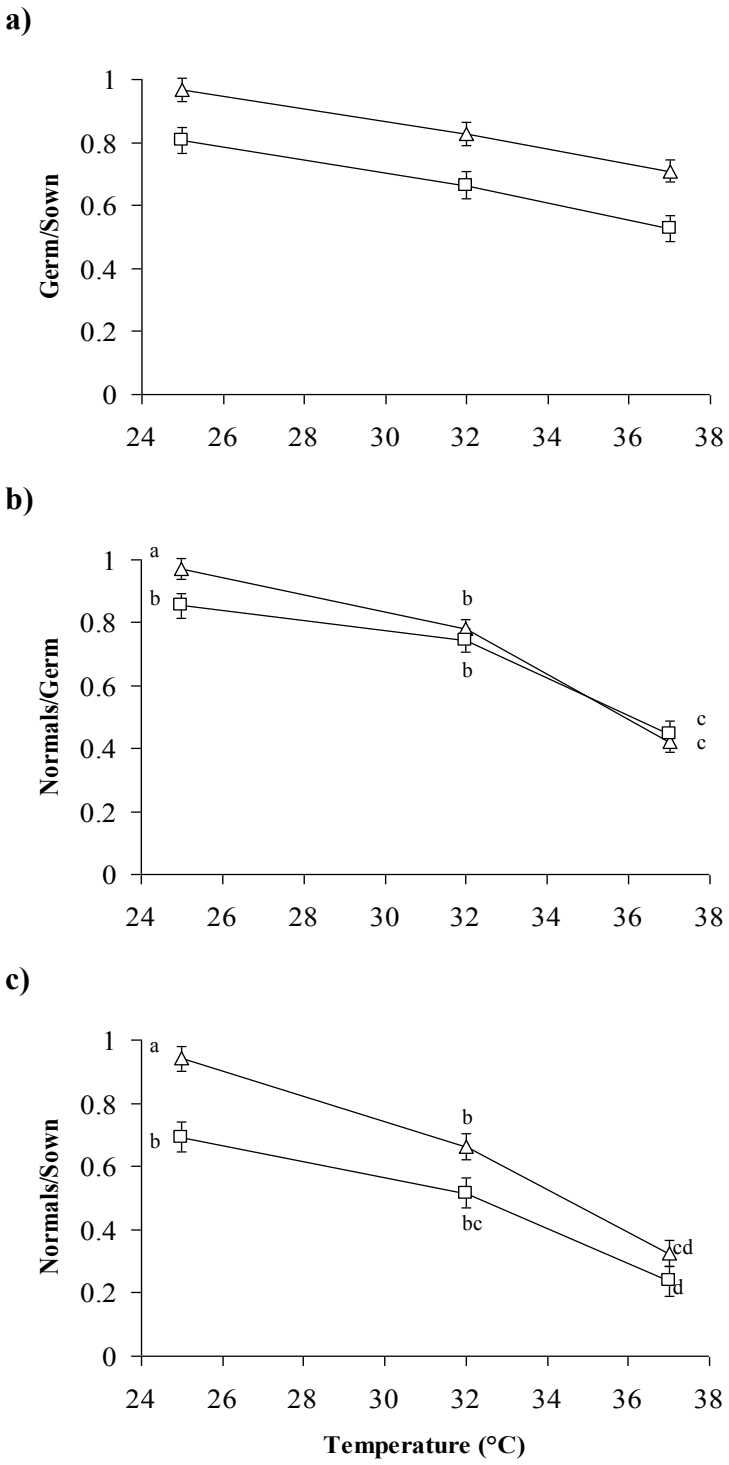


Figure 10. Least square means (±s.e.) from site by temperature interactions for (Δ Cambridge and □ Manjimup) for **a)** proportion of germinated seeds **b)** proportion of germinated seeds which developed into normal seedlings and **c)** proportion of normal seedlings developed. When interaction effects were significant common letters across sites and temperatures were not significantly ($P>0.05$) different using the Tukey-Kramer multiple range test. Sites were significantly different ($P<0.05$) at 25°C and 37°C.

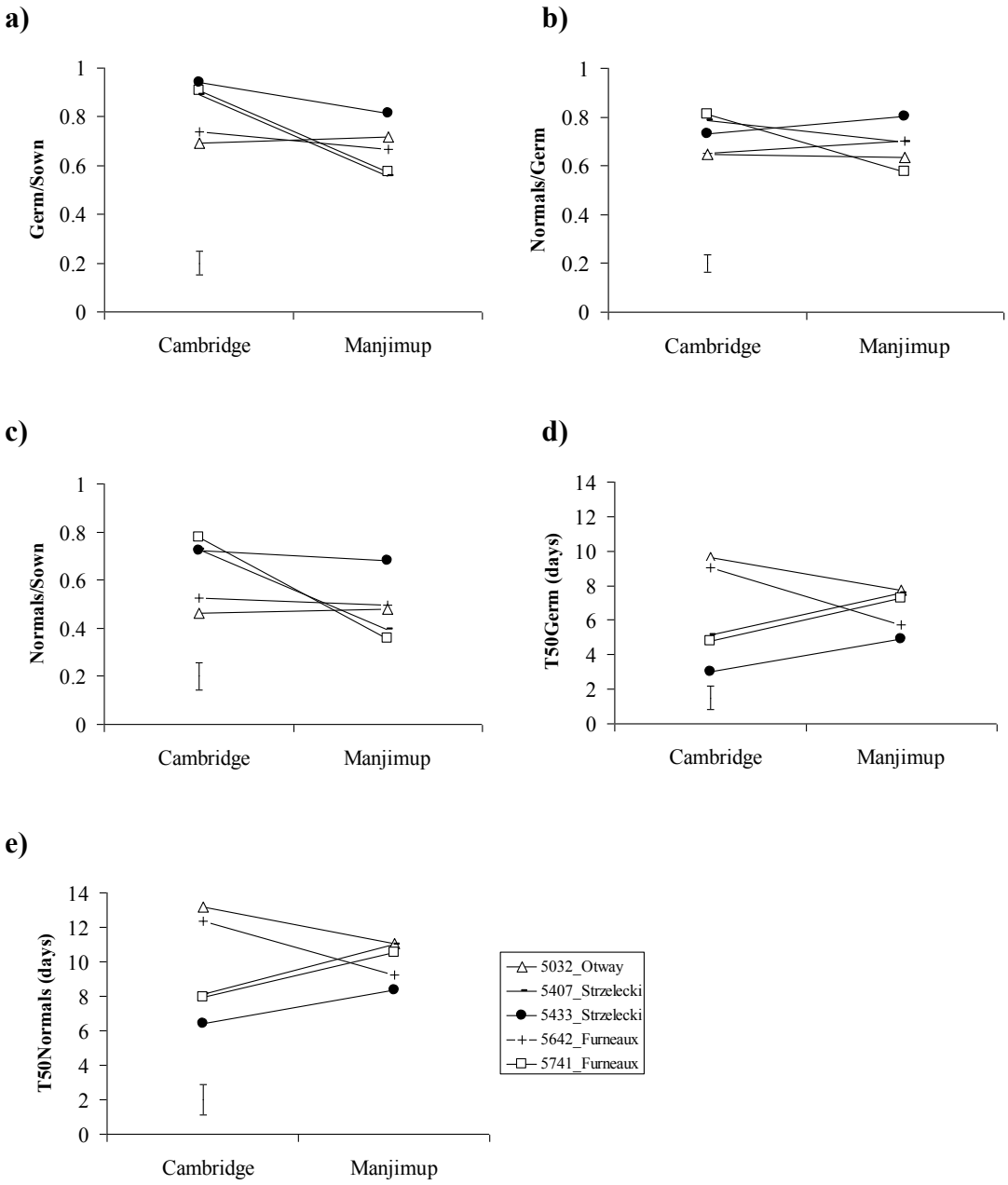
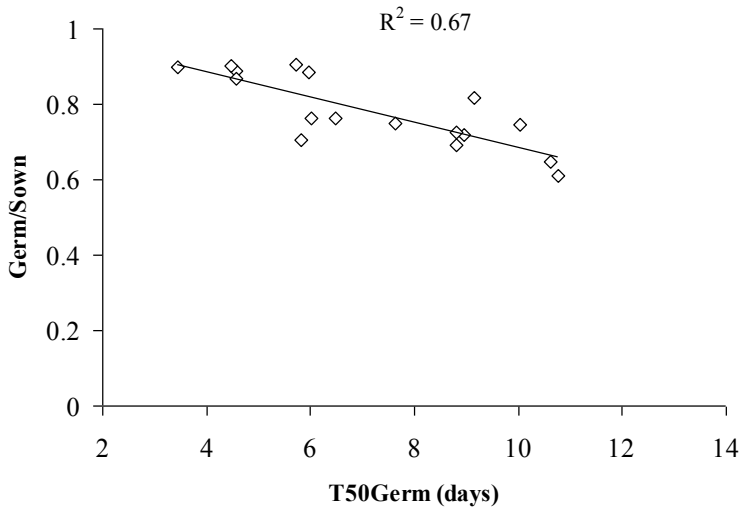


Figure 11. Least square means from site by genotype interactions for **a)** proportion of germinated seeds **b)** proportion of germinated seeds which developed into normal seedlings **c)** proportion of normal seedlings developed **d)** rate (T50) of germination (days) and **e)** rate (T50) of normal seedling development (days) for five genotypes which matched at Cambridge and Manjimup and had more than one ramet represented at each site. The bar on each graph indicates the pooled standard error of n = 4.

Relationship between germination traits

The germination traits studied were generally inter-correlated both within and between temperatures at the genotype level. This is clearly seen in the genotype level correlations from Cambridge 2007/08 (Table 8). Correlations were generally positive amongst proportion and amongst rate traits, but negative between rate and proportion traits. Of the rate traits, germination rate was generally the most significantly correlated with proportion traits. The proportion of germinated seed increased as the germination rate increased (i.e. T50 decreased) ($r = -0.82$, $n=17$, $P<0.001$, Figure 12a). Similarly the proportion of normal seedlings increased as the rate of normal seedling development increased (i.e. T50 decreased) ($r = -0.85$, $n=17$, $P<0.001$, Figure 12b). When comparing across temperatures, rate traits were generally highly positively correlated between the optimal temperate at which testing is normally undertaken (25°C) and the highest temperature tested (37°C) (Table 9; Figure 13). Proportion traits however were less well correlated across these temperatures, and while all correlations were positive only six of the ten calculated were significant (Table 9). The proportion of germination at 25°C was not correlated with rate traits at 37°C (germination rate, $r=-0.35$; $P = 0.172$; rate of normal seedling development $r=-0.24$; $P = 0.344$).

a)



b)

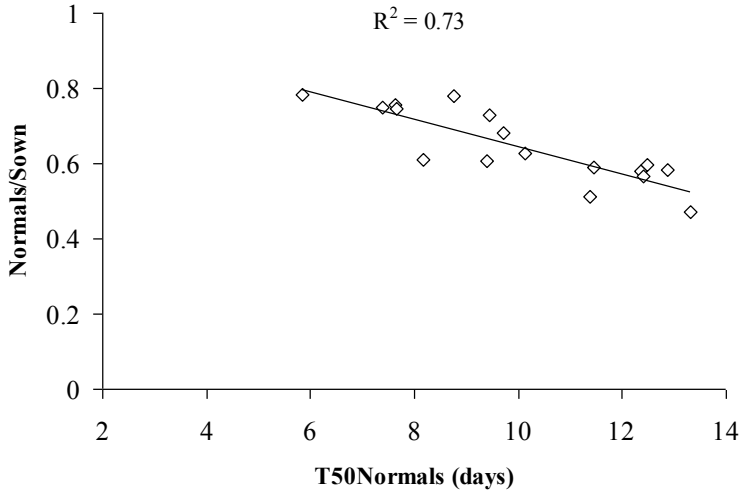


Figure 12. Genotype least square means from Cambridge 2007/08 for **a)** rate (T50) of germination (days) and proportion of germinated seeds and **b)** rate (T50) of normal seedling development (days) and proportion of normal seedlings developed averaged across three temperatures (25°C, 32 and 37°C). Each point represents one of 17 genotypes from the Cambridge site. R^2 values are shown.

Table 8. Genotype mean correlations amongst germination traits within temperatures

Pearson's correlation coefficients are shown based on least square means from 17 genotypes from Cambridge season 2007/08

25°C	Proportion		Rate (days)		
	Normals/Germ	Normals/Sown	T50Germ	T50Normals-T50Germ	T50Normals
Germ/Sown	-0.11 ns	0.72 **	-0.40 ns	-0.06 ns	-0.40 ns
Normals/Germ		0.61 **	-0.46 ns	0.08 ns	-0.41 ns
Normals/Sown			-0.64 **	0.03 ns	-0.61 **
T50Germ				0.04 ns	0.96 ***
T50Normals-T50Germ					0.30 ns

32°C	Proportion		Rate (days)		
	Normals/Germ	Normals/Sown	T50Germ	T50Normals-T50Germ	T50Normals
Germ/Sown	0.48 *	0.89 ***	-0.59 *	-0.08 ns	-0.61 **
Normals/Germ		0.83 ***	-0.49 *	-0.32 ns	-0.57 *
Normals/Sown			-0.64 **	-0.22 ns	-0.69 **
T50Germ				-0.14 ns	0.97 ***
T50Normals-T50Germ					0.10 ns

37°C	Proportion		Rate (days)		
	Normals/Germ	Normals/Sown	T50Germ	T50Normals-T50Germ	T50Normals
Germ/Sown	0.37 ns	0.93 ***	-0.67 **	0.50 *	-0.57 *
Normals/Germ		0.64 **	-0.55 *	0.47 ns	-0.45 ns
Normals/Sown			-0.77 ***	0.56 *	-0.67 **
T50Germ				-0.50 *	0.95 ***
T50Normals-T50Germ					-0.19 ns

Table 9. Correlations between genotype least square means for five germination traits assessed at 25°C and 37°C
Pearson's correlations were performed at each of the sites separately and also on the combined dataset. Within each column the correlation for each germination trait at each site between 25°C and 37°C is shown. P values denote the significance of the correlations. Blank cells denote where genotype did not have a significant effect on the trait (Table 1). The combined analysis included genotype means from all three tests

		Proportion						Rate (days)			
		Germ/Sown		Normals/Germ		Normals/Sown		T50Germ		T50Normals	
Site	n	r	P	r	P	r	P	r	P	r	P
Cambridge 2007/08	17	0.24	0.362			0.30	0.241	0.73	0.001	0.84	<0.001
Cambridge 2008/09	16	0.88	<0.001	0.16	0.562	0.76	0.001	0.91	<0.001	0.88	<0.001
WA 2008/09	13	0.36	0.222			0.37	0.213			0.49	0.087
Combined	46	0.58	<0.001	0.34	0.145	0.53	<0.001	0.82	<0.001	0.77	<0.001

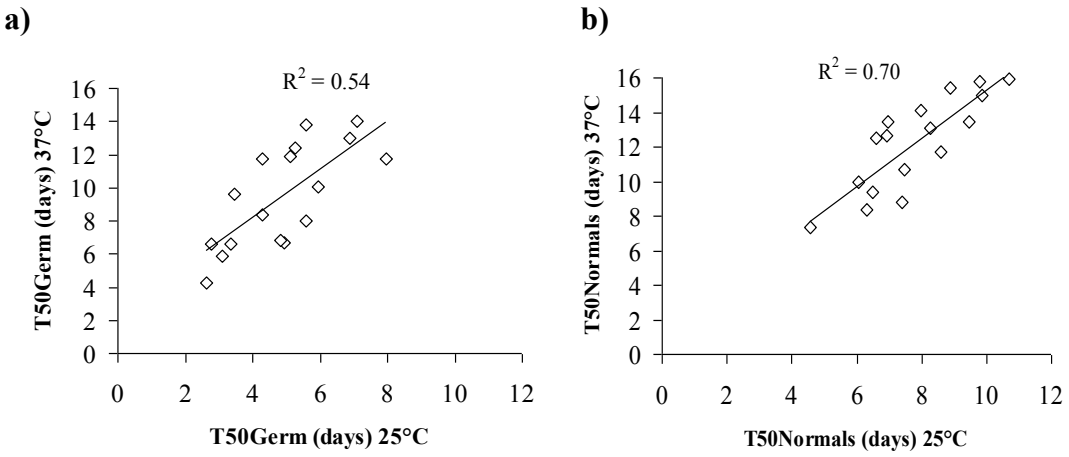


Figure 13. Genotype least square means for rate (T50) (days) of **a)** germination and **b)** normal seedling development at 25°C and 37°C. Analysis is based on data from Cambridge 2007/08. R^2 values are shown.

Discussion

This is the first study to show a genetic basis to intra-specific variation in seed germination traits in *Eucalyptus*. Several previous studies have reported differences in germination characteristics of seeds sourced from different geographic origins (Battaglia 1993; Humara *et al.* 2000; López *et al.* 2003). However, many of these studies confound differences in maternal environment and genetics. In the present study for seeds collected from Cambridge, a component of the genetic variation in one trait, rate of normal seedling development could be attributed to the race of origin. In this case one race, Strzelecki had significantly faster seedling development than either the Otway and Furneaux races. While Strzelecki has been reported to have smaller capsules and smaller seeds than these two races (McGowen *et al.* 2004a), no significant race difference in seed weight was detected between the races in the present study. Lopez *et al*. (2003) found that *E. globulus* seed mass exhibited a significant maternal effect, increasing seed germinative capacity but not germination rate. In other *E. globulus* studies, the rate of germination increases with increasing seed size (Lopez *et al.* 2000; Watson *et al.* 2001), consistent with the trends observed for seed weight in the present study. When seed weight was included in analyses of the germination traits, strong differences between genotypes remained, including the observed differences between races. This argues that the genetic differences in seed germination traits observed are not due to genetic differences in seed weight, and thus have a more complex underlying mechanism. Indeed, the complexity of the genetic effects on germination is exemplified by the differential response of seed-lots from different genotypes to temperature in the proportion of seeds germinated.

While significant genetic differences in many traits were observed within a season or site, the genotype effect was actually relatively unstable. The genotype by season and site interaction effects were highly significant for many traits, even when seed weight was fitted as a covariate. For season, the interaction was caused by two genotypes which changed rankings markedly for proportion traits but not rate traits, arguing that this effect is not due to differences in seed harvest maturity as Chapter 5 suggests that it is the rate rather than the proportion traits which are more sensitive to harvest time. This was similarly the case for the site by genotype interaction, but in this case two genotypes were interactive for proportion traits but a different two genotypes

were interactive for the rate traits, indicating that the site effect impacts on genotypes in different ways.

The influence of the maternal genotype on germination traits could have been due to the maternal contribution to the nucleus of the embryo (one-half maternal), or true maternal effects arising from the maternally derived seed coat, maternal plastid inheritance (McKinnon *et al.* 2001), or maternal provisioning of the seeds (Bischoff and Muller-Scharer 2010; Donohue 2009; Roach and Wulff 1987; Schmuths *et al.* 2006). Additional maternal provisioning may occur via an endosperm dosage effect, due to the endosperm containing two-thirds of its genotype from maternal origin (Bischoff and Muller-Scharer 2010) and containing a number of enzymes important for germination (Donohue 2009). In the present study this can be ruled out as eucalypts produce seeds with no endosperm, rather the newly emerged seedling is sustained by the photosynthetic cotyledons (Boland *et al.* 1980). It is still possible for different maternal genotypes to differentially provision seeds through resource allocation to the ovules or developing seeds (Bischoff and Muller-Scharer 2010; Donohue 2009). However, in the present study, the crossing design does not allow the cause of the maternal genetic influence on seed germination traits to be determined.

While there are clearly genetic effects on seed germination traits, the present study also argues that maternal environment can also influence these traits. This is evident by significant site and season effects on many of the traits examined, as well as the response of several of these traits to temperature (e.g. proportion of seeds or germinated seeds which developed into normal seedlings). Variation in seed weight did not explain the highly significant effects of site and season on the proportional traits, nor the interactions with temperature observed. Self pollination does reduce the proportion of seeds or germinated seeds which developed into normal seedlings (Chapter 4). Thus variation in the proportion of self pollination or survival of selfed embryos across seasons or sites (McGowen *et al.* 2010) could account for the variation in proportion traits observed in the present study. The other possibility is that the site or season effects reflect differences in maturity of the seeds at harvest. It is possible that the seasonal effect at Cambridge is due to differences in elapsed time between flowering and seed harvest. In the 2007/08 season all samples were

harvested when 13 months had elapsed from peak flowering. In the 2008/09 season, this time period was not set and the development time varied between approximately 10 and 13 months from peak flowering. However, the harvest time study in Chapter 5 suggests that it is the rate rather than the proportion traits which are more sensitive to harvest time.

There are other mechanisms which could explain the site and season effects on germination traits, including the interaction with temperature. Seed germination and dormancy is often strongly affected by the testa, and there may be environmental effects on its chemical and mechanical properties (Donohue 2009). Another option is that differences in resource availability to developing seeds (Mazer and Gorchov 1996) differs between sites and this could directly or indirectly affect germination traits. Seeds from Manjimup were significantly smaller than seeds from Cambridge, which could be due to the seeds from Manjimup receiving fewer resources than those from Cambridge. Seed size can be influenced by maternal environment (Cavers and Steel 1984; Krannitz *et al.* 1999; Lee 1988; Sultan 1996; Van Hinsberg 1998; Wulff *et al.* 1999), and it affects the amount of food reserves that will be available to the embryo (Valencia-Diaz and Montana 2005). While seed size differences could explain differences between sites in germination rate, it did not explain the site differences on the proportion of normal seedlings developed.

There was evidence for an environment by temperate interaction in the proportion of seeds or germinated seeds which developed into normal seedlings. This was due to the site differences being only significantly expressed at the optimal temperature, whereas the season effect was most significantly expressed at the highest temperature. This different response would suggest different mechanisms are involved in temperature effects of season and site. The high temperature sensitivity of the Cambridge 2008/09 seed sample was quite specific to the seedling development as opposed to the proportion germination or rates traits, suggesting the higher temperatures increase the proportion of abnormal seedlings. This effect is unlikely to be due to differences in harvest time between these seasons as Chapter 5 showed that while the harvest time by temperature interaction was significant for rate traits it was not significant for proportion traits. Despite a report of seasonal stability in outcrossing rates in *E. globulus* (McGowen *et al.* 2004), the possibility that this

interaction is due to differences in outcrossing rates between seasons can not be dismissed. There is a trend in Chapter 4 for selfed seeds to exhibit greater sensitivity to high temperatures for the proportion of germinated seeds which developed into normal seedlings. The other option is that the interaction effect for this trait is a true effect of differences in the maternal environment between seasons. For example, Sung *et al*. (1998) suggested that lettuce seeds which developed under higher temperature conditions had increased tolerance to high temperature stress.

In summary, this study has shown that both maternal genetics and maternal environment can affect the germination response of *E. globulus* seeds. There is also evidence for differential expression of the genotype effects across years and sites. In addition, when it occurs, a differential response of various germination traits to high temperature stress appears to be due to both genetic and environmental effects. However, the differential genetic effect with temperature appears to be mainly affecting proportion germination whereas the environmental effect appears to be mainly affecting the proportion of germinated seeds which developed into normal seedlings.

From an applied perspective it may be possible to improve laboratory germination testing procedures to better predict deleterious effects in the nursery. Commercial germination tests only measure percentage germination. The present study shows that at each temperature this measurement does predict the proportion of normal seedlings developed and the rates of germination and normal seedling development. While variation between genotypes in the rate traits measured at 25°C does correlate well with rate variation at 37°C, this across temperature correlation is less for the proportion traits. The proportion germination at 25°C does not correlate with the rate traits at 37°C. Thus the trait traditionally assessed in commercial germination tests poorly predicts the germination response at 37°C. Germination tests at high temperatures would be required to quantify variation between genotypes in their proportion germination at high temperatures. By including these additional measurements of germination and normal seedling development in commercial testing prior to seed dispatch, a better indication of genotype performance would be provided to nursery operators. However, as there are both site and seasonal effects on these parameters it is important that tests are applied to

different seed collections even from the same genotype.

Chapter 4

Paternal and maternal genetic influences on *Eucalyptus globulus* seed germination

Introduction

In plants, the genotype of the maternal parent may influence early life cycle traits through both Mendelian contributions to the embryo nuclear genotype as well as through additional „maternal genetic effects' (Donohue 2009; Roach and Wulff 1987). A „maternal genetic effect' arises when there is an additional genetic contribution of the maternal genotype beyond the chromosomal contribution expected (Roach and Wulff 1987). In angiosperms, maternal genetic effects may arise from (i) the maternal genetic effects through maternal inheritance of plastids, (ii) the effects of endosperm, which is triploid and two-thirds of its genotype is of maternal origin, (iii) the effects of the seed coat and other maternal tissue immediately surrounding the embryo, (iv) the effects of maternal provisioning during seed development, with hormones, proteins, transcripts and nutrient resources all provisioned to seeds by the maternal parent, and (v) the maternal determination of the progeny environment via dispersal (Donohue 2009). These influences mean that in most angiosperms, the maternal plants have a greater effect on offspring phenotype and fitness than paternal plants because they provide nourishment of the seeds, two-thirds of the endosperm genetic material and the extra nuclear DNA in plastids and mitochondria of the embryo (Donohue 2009; Galloway 2001a; Roach and Wulff 1987; Schmid and Dolt 1994). Indeed, many studies suggest that the primary control of seed dormancy and germination is through the maternal tissues surrounding the embryo (Baskin and Baskin 1998; Bischoff and Muller-Scharer 2010).

Maternal effects on seed mass have been previously reported in the forest tree *Eucalyptus globulus* (López *et al.* 2003), which have carry-over effects on germinative capacity and early seedling growth (Lopez *et al.* 2000; López *et al.* 2003; Martins-Corder *et al.* 1998). While no direct effects on seed germination traits were

detected in the study of Lopez *et al*. (2003), Chapter 3 showed a maternal influence on seed germination and early seedling development, including tolerance to high temperature stress. Through sampling replicated grafts of the maternal genotype it was also shown that there was a maternal genetic component to this influence. However, it was not possible to determine whether this was simply due to the maternal contribution to the nuclear genotype of the embryo, through for example an additive genetic effect (general combining ability), or whether there was an additional maternal genetic effect. The experiment in Chapter 3 did not allow examination of the regulation by the paternal (pollen-donating) parent, or comparison of the maternal versus the paternal influence on seed germination traits. Variation between males is the best way to estimate the general combining ability based on nuclear genes, as variation between females includes the general combining ability and maternal genetic effects (Lynch and Walsh 1998). In eucalypts a direct maternal genetic effect may occur through DNA in cytoplasmic organelles, as both the chloroplast (McKinnon *et al.* 2001) and mitochondrial (Vaillancourt *et al.* 2004) genomes have been shown to be maternally inherited.

The results from Chapter 3 revealed variability in the sensitivity of genotypes to optimum and high temperature stress. This high temperature sensitivity was evident as reduced germination and seedling development rate, reduced percentage germination and reduced development of seedlings under high temperature conditions (ISTA 1999). Maternal effects can influence the sensitivity of seeds to environmental conditions (Roach and Wulff 1987). A study by Sung *et al*. (1998) showed that the sensitivity of lettuce seeds to high temperature stress during germination was at least partly related to maternal environment. The study showed that the upper temperature limit for germination of lettuce seeds could be modified by manipulating the temperature of the maternal environment during seed production, with seeds developing under higher temperature conditions having increased tolerance to high temperature stress. Another factor that may influence the response of seeds to high temperature stress is inbreeding. It is frequently reported that inbreeding depression is greater in more stressful environments (Frankham *et al.* 2002; Roff 1997). In a review by Armbruster and Reed (2005), based on data from 34 studies, inbreeding depression was found to increase under relatively stressful conditions in 76% of the cases examined, although this increase was only statistically

significant in 48% of cases. It appears that the expression of deleterious recessive alleles underlying inbreeding depression may often depend on specific environmental conditions (Bijlsma *et al.* 1999; Dahlgaard and Hoffman 2000; Haag *et al.* 2003; Heywood 1993; Reed and Bryant 2001; Reed *et al.* 2003). Inbreeding in *E. globulus* results in marked inbreeding depression for performance traits (Costa e Silva *et al.* 2010a; Hardner and Potts 1995; Hardner *et al.* 1998; Potts *et al.* 2008). In the study of Hardner and Potts (1995), selfing was shown to severely depress seed set and field growth relative to out-crossing, but no significant effect was found on germination percentage or germination rate, or survival of seedlings to 4 months in the glasshouse. However, these germination tests were undertaken under a temperature (22ºC) which was near optimal for the species (see Chapter 2) and it is unknown if self-pollinated seeds are more sensitive to high temperature stress during germination, compared to open-pollinated or out-crossed seeds.

The present study aimed to determine the maternal and paternal genotype influence on seed germination traits in *E. globulus*, particularly the response to high temperature stress. There is no published information on the paternal influences on seed germination in eucalypts and the relative importance of the embryonic nuclear genes compared with maternal genetic effects is unknown. The present study specifically aimed to determine if there is (i) an effect of the embryonic nuclear genotype, (ii) a maternal genetic effect, and (iii) an effect of inbreeding.

Materials and Methods

Study system

Eucalyptus globulus Labill. (Tasmanian blue gum) is a hardwood forest tree, native to south-eastern Australia (Dutkowski and Potts 1999; Jordan *et al.* 1993). *E. globulus* is the hardwood species most widely planted for pulpwood in temperate regions of the world (Eldridge *et al.* 1993; Potts 2004). It is mainly pollinated by birds and insects, and has a mixed mating system with average outcrossing rates in undisturbed native forests higher than 85% (Mimura *et al.* 2009). Self-pollination results in significantly less viable seeds per flower and per capsule, compared to outcrossed seeds (Hardner and Potts 1995; McGowen *et al.* 2010). Progeny derived from self-fertilisation exhibit severe inbreeding depression for survival and growth

(Costa e Silva *et al.* 2010a; Hardner and Potts 1995; Hardner *et al.* 1998), and there is also evidence for inbreeding depression following biparental breeding in the wild (Hardner *et al.* 1998). Unless derived from a 100% self-incompatible tree, seeds obtained from open-pollination can contain self-pollinated individuals, and thus low outcrossing rates in seed orchards may result in seeds which are sub-optimal for deployment purposes (Patterson *et al.* 2004b).

Eucalyptus globulus trees used in this study were located in a seed orchard in Cambridge, south-eastern Tasmania (42°48’27.23"S 147°25’58.48"E), encompassing genotypes from different races as classified by Dutkowski and Potts (1999). Cambridge has an altitude of 40 m with a long-term average annual rainfall of 507 mm and average annual maximum and minimum temperatures of 17.4°C and 8°C, respectively. Trials were carried out over two consecutive seasons 2008/09 and 2009/10. All trees were treated in accordance with standard commercial practices. The genotypes in the orchard were selections from the Southern Tree Breeding Association (STBA) National *E. globulus* Breeding Program.

Maternal versus paternal influence

To estimate the paternal and maternal genetic influence on seed germination traits, genotypes were used as both a male and female parent in a diallel crossing trial. It was investigated whether the sensitivity to high temperature stress was attributable to maternal or paternal genetic influence, or both. Four genotypes from the Western Otway race (hereafter referred to as Otway) exhibiting a range of germination responses in season 2007/08 at the Cambridge site and showing consistency in relative rankings in season 2008/09 were selected as parents (Figures 7 and 8, Chapter 3). Genotypes were selected from a single race to aid pollen extraction and crossing due to the more synchronous flowering between genotypes within the same race, compared to working with genotypes from different races. The four genotypes were 7558, 7335, 4886 and 7537. There were two ramets each from different plots in the seed orchard selected from each genotype, giving a total of eight trees in the trial. A reciprocal crossing trial was designed with pollen from each genotype being applied to all other genotypes, with the exception of self-pollination. This design resulted in a total of 24 crosses being performed (4 genotypes x 2 ramets per

genotype x 3 crosses per ramet). For each cross, fifteen flowers were pollinated from around the canopy of each tree. 360 flowers were control-pollinated in total.

Flowers were collected from multiple ramets of each genotype, except for 7558 where pollen was collected from a single ramet, for pollen extraction and then combined to give a single pollen for each genotype. The ramets used for flower collection were from different plots in the seed orchard for two genotypes (7335 and 4886) but not for the other two genotypes (7558 and 7537). Flowers were selected at the stage when the operculum was just lifting away from the receptacle, to rule out any contamination from other pollen sources. The flowers were placed on trays wrapped with aluminium foil and placed in an oven set at 30°C for 48 hours. The flowers were then rubbed against a sieve to extract the pollen, with a smaller aperture sieve placed underneath to separate other material. The pollen fell through both sieves and was collected on a sheet of foil placed underneath.

The pollen was then placed in glass tubes and stored at -18°C in darkness. For testing viability, pollen were streaked across the surface of an agar medium (into quadrants) containing 30% sucrose and 150ppm of boric acid (Potts and Marsden-Smedley 1989) in 8 x 8 cell petridishes, then incubated at 25ºC for 24 h with continuous fluorescent light, with at least three replicates in each petridish for each pollen source. Percentage germination was scored with a light microscope at 100X magnification. The average pollen germination for genotypes 7335, 7558, 4886 and 7537 was 55%, 15.6%, 17.6% and 14%, respectively.

Controlled pollinations were undertaken using the single visit pollination procedure outlined by Williams *et al*. (1999). 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. Capsules were harvested from the trees 12 months after the pollinations were performed, on the 5th of January 2010. They were placed in an oven set at 40°C in darkness for 24 hours and extracted seeds were then stored at 5°C in darkness until germination testing commenced. Chaff was removed from samples using a vertical air column and by hand picking to 100% purity. The number of seeds per capsule and weight of 100 seeds were recorded from each cross on each ramet.

Effect of self-pollination

Most trees in the seed orchard have some level of self compatibility and there will be some level of self-pollination when trees are open-pollinated or mass-supplementary pollinated (MSP) since flowers are not emasculated (Patterson *et al.* 2004a). A trial was designed to test the effect of self-pollination, compared to open-pollination (OP) and MSP on the sensitivity of derived seeds to exposure to high temperature stress during germination.

To increase the number of viable seeds obtained, it was important that a genotype was selected which was relatively self compatible. The genotype selected was 7970, from the Western Otway race. This genotype has a self-incompatibility (SI) index of 57, which was the lowest known in the seed orchard. This means that self pollination of a tree from this genotype results in a 57% reduction in the number of seeds obtained per flower, compared to when the tree is outcrossed. Five ramets of genotype 7970 were selected for this trial.

Flowers were collected from a single ramet of this genotype for pollen extraction. Flower selection, pollen extraction, pollen viability testing and storage were undertaken as outlined above. The average germination of pollen from genotype 7970 was 68.9%. Fifty flowers on each of the five ramets of genotype 7970 were self-pollinated, giving 250 flowers in total. The fifty flowers self-pollinated on each tree were selected from around the canopy, both high and low, to be as representative of the canopy as possible. Controlled self-pollinations and flower isolation were undertaken as outlined above.

Fifteen flowers on each of the five trees had mass-supplementary pollinations (MSP) performed on them (Patterson *et al.* 2004a). The pollen used for MSP was from genotype 4862. Flowers were selected from around the canopy of each tree and all branches were labelled. Any flowers on the same branch which had not had MSP done on them were removed. Fifty flowers were also selected on each tree for open-pollination (OP). These flowers were selected as having not shed the operculum and were from around the canopy of each tree. The branches which had pollinated flowers on them were labelled.

Capsules were harvested from the trees 12 months after the pollinations were performed, on the 27th of October 2009. Seeds were extracted, cleaned and stored as outlined above. The number of seeds per capsule and weight of 100 seeds were recorded from each cross on each ramet.

Seed germination

Seeds were germinated on sections of Advantec number 1 type filter paper placed in the perspex germination cabinets as described in Chapter 2. Fifty seeds from each cross from each tree (seed-lot) were germinated at each of three temperatures (25°C, 32°C and 37°C). Each lot of 50 seeds represented an experimental unit and was placed on separate lengths of filter paper which were placed in separate sections of the perspex germination cabinets and spaced evenly. One replicate from each cross on each ramet was germinated at each temperature. Allocation of seed-lots to spaces within and between perspex germination cabinets was random. 75 mL of distilled water was added to each section in the germination cabinets and the water wicked up to the seeds, maintaining a consistent level of moisture for the duration of the experiment. Water levels within sections were checked daily and distilled water was added where necessary. Four replicates of 50 seeds each of the commercial seed-lot Glob 185, described in Chapter 2, were also germinated at each temperature.

Incubators

Seeds were germinated at three constant temperatures, in three separate incubators set at 25°C (optimal), 32°C and 37°C (high temperatures) with continuous fluorescent light, as outlined in Chapter 3. Temperature was logged for the duration of the experiments inside the incubators using Tiny Tag Data Loggers (Tiny Tag, United Kingdom).

Trial commencement

Seeds from the inbreeding experiment and the diallel crossing were tested in separate runs. The trial comparing selfing with open-pollination (OP) and MSP commenced on the 13th of November 2009 (day zero) and concluded on the 4th of December 2009. The diallel trial commenced on the 22nd of January 2010 (day zero) and concluded on the 12th of February 2010. Seeds from both experiments were germinated 17 days after harvest.

Trial assessment

Seeds germinated at 25°C were assessed on days 3, 4, 5, 6, 7, 10, 14, and 21. Seeds germinated at 32°C were assessed on days 3, 5, 7, 10, 14, and 21. Seeds germinated at 37°C were assessed on days 3, 5, 7, 10, 14, 18, and 21. At each assessment a seed was recorded as germinated when there was visible protrusion of the radicle through the seed coat. Seedlings with a radicle at least one third the length of the seedling, a hypocotyl and two expanded cotyledons were classified as normal, in accordance with ISTA guidelines (Bekendam and Grob 1993; ISTA 1999) and removed. For both experiments, the day at which a seed germinated or a seedling was exhibiting normal development was recorded. On the final day of scoring for both experiments, remaining seedlings were scored as normal or abnormal. Abnormalities included stunted radicles, failure of radicles to emerge but emergence of cotyledons and failure of seedlings to "push-off" seed coats, in accordance with ISTA guidelines for seedling evaluation (Bekendam and Grob 1993). Final scoring for both experiments was undertaken on day 21 and was done as outlined in Chapter 2.

Statistical analysis

To estimate the response rate of each experimental unit of 50 seeds, the time taken for 50% of the maximum germination or normal seedling development (T50) was calculated using the logistic function which was fitted to the cumulative curves for germination or seedling development (Shafii and Price 2001). This was done using the Marquardt's iterative non-linear procedure (PROC NLIN) of SAS version 9.1 (SAS Institute Inc 2003). The procedure for calculation was detailed in Chapter 2.

The data were summarised into six response traits, three measuring proportions and three measuring rates. The proportional data included: the proportion of seeds sown which germinated (germ/sown); the proportion of seeds which germinated that developed into normal seedlings (normals/germ); and the proportion of seeds sown which developed into normal seedlings (normals/sown). The rate data included: the time taken to reach 50% (T50) of the maximum germination attained (T50germ); the T50 for maximum normal seedlings developed (T50normals); and the difference between the T50 for germination and T50 to develop a normal seedling (T50normals-T50germ).

For analysis of seed characteristics including seed weight and the number of seeds per capsule from the diallel experiment, female genotype, male genotype and female genotype by male genotype were treated as fixed effects and tree within female genotype was treated as a random effect and used as the error term to test the female fixed effect. All other fixed effects were tested with the residual term. One-way analysis of variance was used to test the difference between genotype means at individual temperatures.

For analysis of germination traits from the diallel experiment, a linear mixed model was fitted to the data with male genotype, female genotype, male genotype by female genotype, temperature, male genotype by temperature, female genotype by temperature and female genotype by male genotype by temperature terms treated as fixed effects. Tree within female genotype was the random term used to test the female genotype effect. Male genotype by tree within female genotype was the random term used to test the male genotype effect and male genotype by female genotype interaction. All other fixed effects were tested with the residual error.

For analysis of seed characteristics including seed weight and the number of seeds per capsule for the experiment comparing self-pollination, OP and MSP treatments pollination type was treated as a fixed effect and tree was treated as a random term and used to test the fixed pollination type effect. To test whether seed weight (measured as the weight of 100 seeds), the number of seeds per capsule and the number of seeds per flower affected the germination response, seed weight or

number of seeds per capsule or flower were included separately as covariates in this model.

For analysis of germination traits for the experiment comparing self-pollination, OP and MSP treatments a linear mixed model was fitted to the data which included pollination type, temperature and pollination type by temperature as fixed effects and tree and tree by pollination as random effects. The tree by pollination type term was used as the error to test the pollination type main effect. The temperature and temperature by pollination type interaction were tested with the residual term. All analyses were undertaken by fitting linear mixed models PROC MIXED, using SAS version 9.1 (SAS Institute Inc 2003). Prior to analysis the proportional data were arcsine transformed and rate data were subjected to log transformation to optimise normality of residuals.

Results

Maternal versus paternal influence

Neither female nor male genotype had a significant effect on seed weight, the number of seeds per capsule or the number of seeds per flower (Table 10). Female genotype significantly affected three out of the six germination traits, whereas male genotype significantly affected five out of the six (Table 11). No significant male by female genotype interactions were detected. Genotype performance as male and female was very similar (Figure 14). Genotype 7558 generally showed the best performance for all traits as a male and female parent. Genotype 7537 generally showed the poorest performance for all traits as a male and female parent.

As the paternal parent, genotypes did not exhibit a differential response to temperature for any traits, but as the maternal parent a differential response to temperature was evident for all germination traits except rate of normal seedling development (Table 9, Figure 15f). When included as a covariate in the linear mixed model analyses, the only significant negative effect detected for seed weight was on the rate of normal seedling development ($F_{1,21} = 5.5$; $P = 0.029$), however this did not change the significance of any of the fixed effects for this trait. Inclusion of the number of seeds per capsule or flower as covariates had no significant effect on any

of the germination response traits (data not shown).

Table 10. Effects of female and male genotypes on seed weight, the number of seeds per capsule and the number of seeds per flower

			Seed Weight		Seeds per Capsule		Seeds per Flower	
Fixed Effects	Num d.f.	Den d.f.	F	P	F	P	F	P
Female_Genotype	3	4	3.9	0.112	4.9	0.079	0.9	0.514
Male_Genotype	3	8	1.9	0.202	2.0	0.190	0.5	0.674
Female_Genotype*Male_Genotype	5	8	0.6	0.705	1.2	0.391	2.2	0.157

Table 11. Effects of female and male genotypes on six germination traits
For details of the germination traits, refer to Table 4

			Proportion						Rate (days)					
			Germ/Sown		Normals/Germ		Normals/Sown		T50Germ		T50Normals-T50Germ		T50Normals	
Fixed Effects	Num d.f.	Den d.f.	F	P	F	P	F	P	F	P	F	P	F	P
Female_Genotype	3	4	0.5	0.732	19.6	0.007	8.4	0.034	13.7	0.014	4.2	0.101	3.6	0.126
Male_Genotype	3	8	2.5	0.131	9.7	0.005	9.1	0.006	19.8	0.001	4.1	0.049	9.0	0.006
Female_Genotype*Male_Genotype	5	8	1.5	0.299	1.7	0.251	2.3	0.138	1.2	0.372	0.9	0.506	0.3	0.894
Temperature	2	23	29.4	<0.001	58.4	<0.001	97.6	<0.001	70.5	<0.001	3.6	0.043	68.6	<0.001
Male_Genotype*Temperature	6	23	0.6	0.711	1.2	0.353	1.3	0.296	2.0	0.102	1.4	0.256	0.8	0.572
Female_Genotype*Temperature	6	23	2.7	0.042	3.3	0.018	6.7	0.000	2.9	0.028	3.4	0.014	1.1	0.374
Female_Genotype*Male_Genotype²	10	23	1.4	0.252	1.6	0.164	1.3	0.305	1.6	0.180	2.5	0.036	0.8	0.667

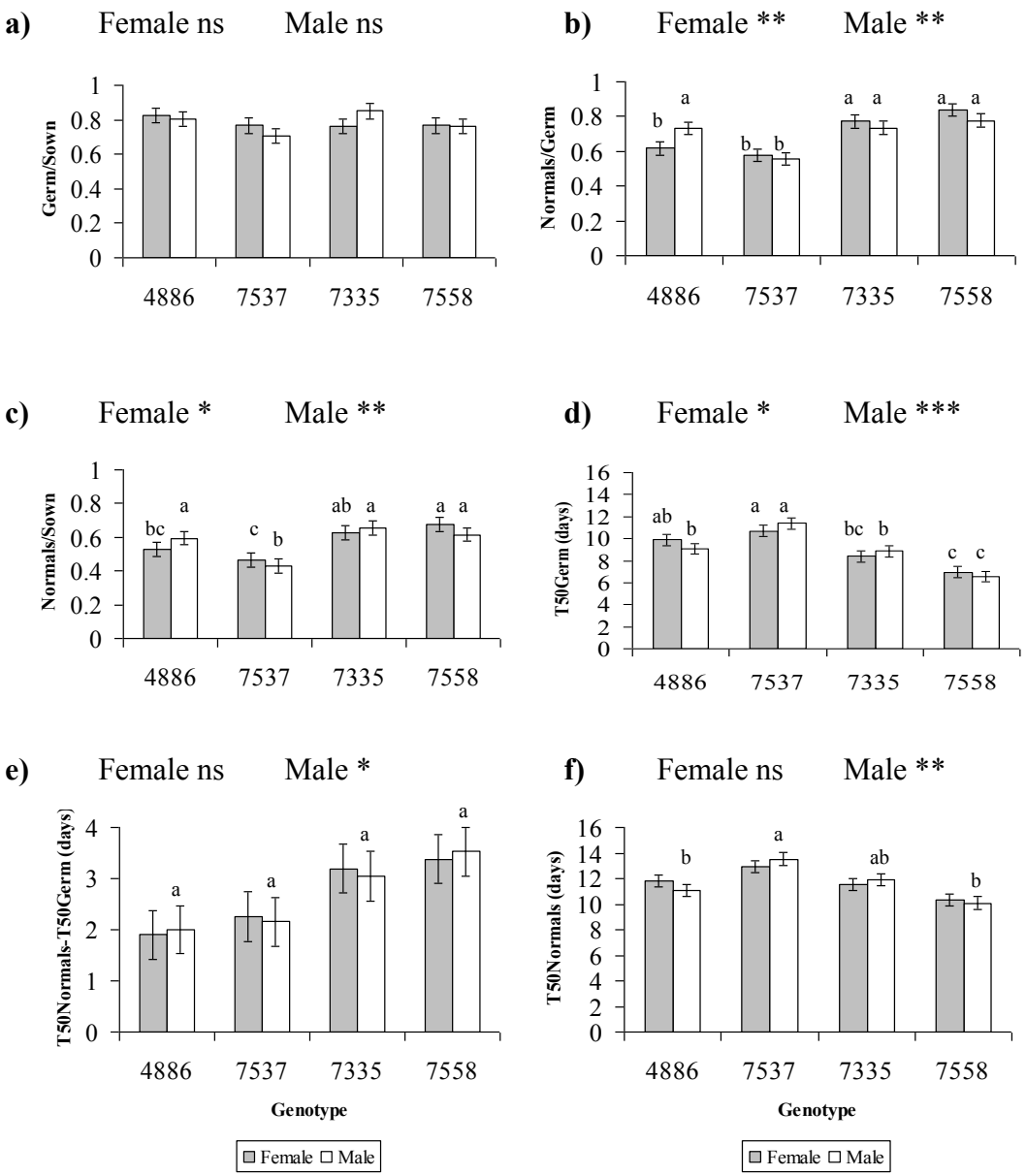


Figure 14. Least square means (±s.e.) for female (solid) and male (open) effects on **a)** proportion of germinated seeds **b)** proportion germinated seeds which developed into normal seedlings **c)** proportion of normal seedlings developed **d)** rate (T50) of germination (days) **e)** rate (T50) of development from germination into normal seedlings (days) and **f)** rate (T50) of normal seedling development (days). Results are averaged across the three temperatures (25°C, 32°C, and 37°C) tested. Where the female or male effect was significant (Table 9), common letters represent least squares means within a male or female series which were not significantly (P>0.05) different using the Tukey-Kramer multiple range test.

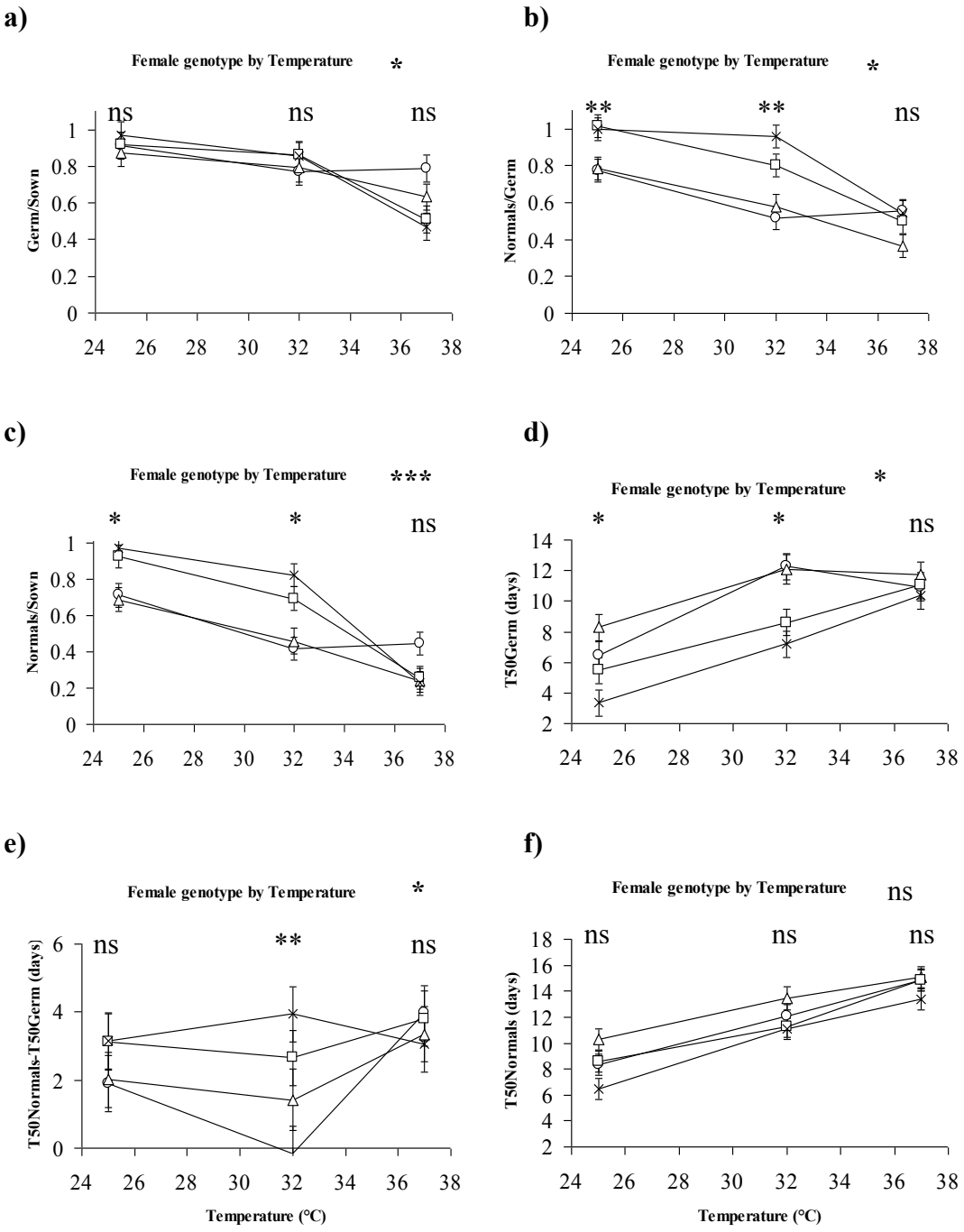


Figure 15. Least square means (±s.e.) for four genotypes (○ 4886; Δ 7537; □ 7335; x 7558) as females for **a)** proportion of germinated seeds **b)** proportion of germinated seeds which developed into normal seedlings **c)** proportion of normal seedlings developed **d)** rate (T50) of germination (days) **e)** rate (T50) of development from germination into normal seedlings (days) and **f)** rate (T50) of normal seedling development (days). Results are across the three temperatures tested (25°C, 32°C and 37°C). Significance of the genotype by temperature interaction for each trait is indicated next to each figure heading (n.s. not significant, * P<0.05 ** P<0.01 *** P<0.001). Significance of the fixed female genotype effect tested separately at each temperature is indicated (n.s. not significant, * P<0.05 ** P<0.01 *** P<0.001). The negative mean value in e) is due to later germinating seeds not developing into normal seedlings.

The overall ranking of genotypes matched relatively well with that determined in Chapter 3 (Figures 7 and 8). As a female, the genotype 4886 was more negatively affected in the transition from 25°C to 32°C for germination rate than the other genotypes (Figure 15d) and genotype 7558 was generally least affected for several traits (Figure 15b, c, d, e). Maternal genotype 7558 was relatively tolerant to high temperature (32°C) for rate of development from germination to normal seedlings (Figure 15e). However, there was a significant ($F_{10, 3} = 2.5$; $P = 0.036$) three-way female genotype by male genotype by temperature interaction for rate of development from germination to normal seedlings. This three-way interaction was no longer significant when male genotypes 4886 or 7537 were excluded from the analysis.

Effect of self-pollination

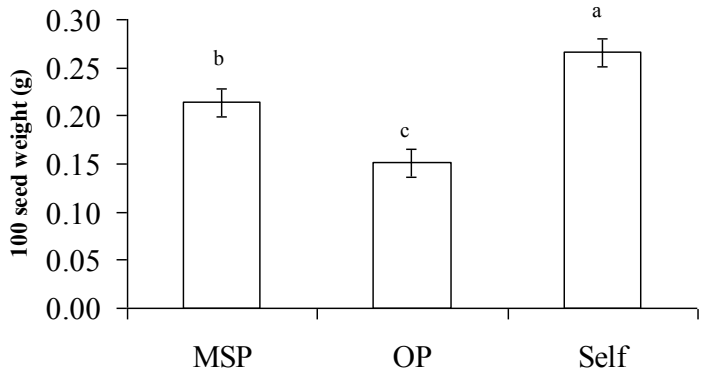
Pollination type had a significant effect on the number of seeds per capsule ($F_{2,8} = 15.7$; $P = 0.002$). There were significantly fewer seeds per capsule derived from self-pollination (8.97), compared to MSP (23.75) and OP (35.14) (Figure 16b). Pollination type also had a significant effect on the number of seeds per flower ($F_{2,8} = 15.1$; $P = 0.002$). There were significantly fewer seeds per flower derived from self-pollination (6.78), compared to MSP (21.87) and OP (31.40) (Figure 16b). For both the number of seeds per capsule and seeds per flower, the highest levels were attained from OP, while MSP was again intermediate (Figure 16b). Pollination type had a significant ($F_{2,8} = 21.5$; $P = 0.001$) effect on seed weight. Seeds derived from self-pollination were significantly heavier than seeds derived from OP and MSP (Figure 16a). Seeds derived from OP were significantly lighter than seeds derived from MSP and selfing (Figure 16a).

Pollination type had a significant effect on the proportion of germinated seeds that developed into normal seedlings ($F_{2,8} = 10.5$; $P = 0.006$) and the proportion of normal seedlings developed ($F_{2,8} = 7.4$; $P = 0.015$), but not on the proportion of seeds germinated ($F_{2,8} = 1.4$; $P = 0.307$), germination rate ($F_{2,8} = 0.4$; $P = 0.690$), rate of development from germination to normal seedlings ($F_{2,8} = 1.0$; $P = 0.406$), or normal seedling development rate ($F_{2,8} = 0.7$; $P = 0.537$). Self-pollination reduced the proportion of normal seedlings developed from germinated seeds and seeds sown,

compared to OP and MSP (Figure 17). This could not be explained by smaller seed size as selfing resulted in heavier seeds (Figure 16a).

While the pollination type by temperature interaction for the six germination traits was not statistically significant at the P=0.05 level (data not shown), there was a trend for seeds derived from self-pollination to be more sensitive to high temperature stress for the proportion of germinated seeds which developed into normal seedlings ($F_{4,23} = 1.9$; P = 0.142) and proportion of normal seedlings developed ($F_{4,23} = 2.2$; P = 0.101) (Figures 18a and 18b).

a)



b)

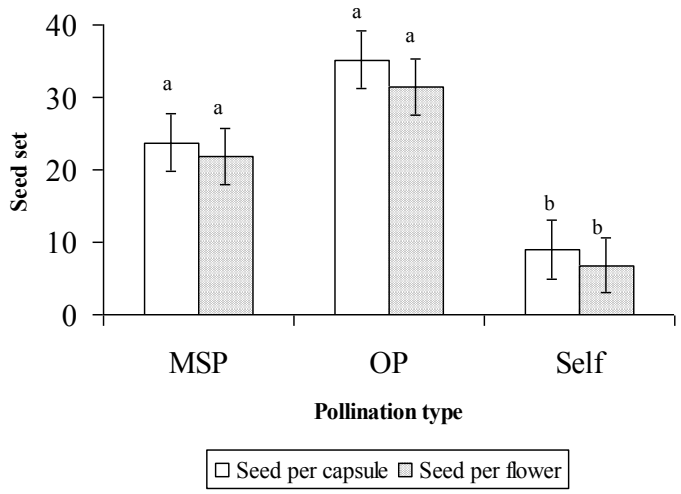


Figure 16. Least square means (±s.e.) for **a)** seed weight (x100 in grams) and **b)** seed set (number of seeds per capsule and number of seeds per flower) for three different pollination types (MSP, OP and self-pollination) using the Otway genotype (7970). Common letters represent least squares means within a seed weight, seeds per capsule or seeds per flower series which were not significantly (P>0.05) different using the Tukey-Kramer multiple range test.

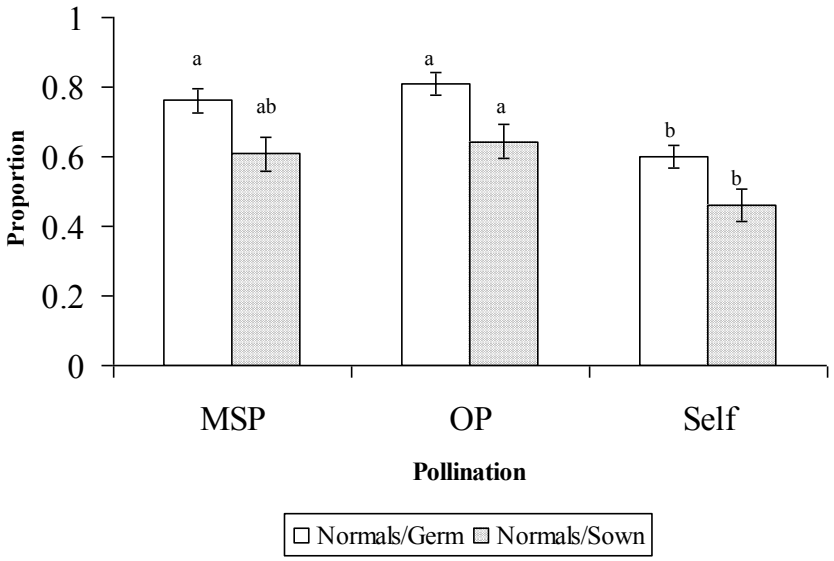


Figure 17. Least square means (±s.e.) for proportion of germinated seeds which developed into normal seedlings and proportion of normal seedlings developed for three different pollination types (MSP, OP and self-pollination), averaged across three temperatures (25°C, 32°C and 37°C). Common letters represent least squares means within a Normals/Germ or Normals/Sown series which were not significantly (P>0.05) different using the Tukey-Kramer multiple range test.

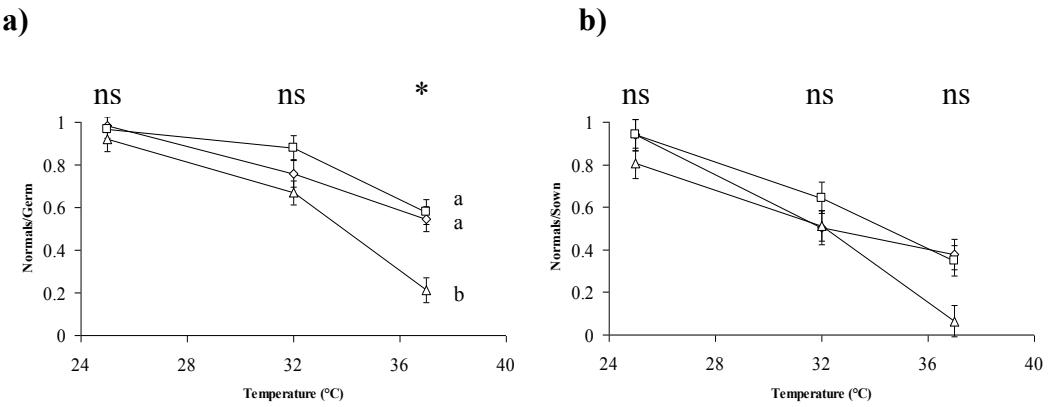


Figure 18. Least square means (±s.e.) for (◊ MSP; □ OP; Δ Selfed) for **a)** proportion of germinated seeds which developed into normal seedlings and **b)** proportion of normal seedlings developed for three pollination types across three temperatures (25°C, 32°C and 37°C). Each point is the mean of five replicates at each temperature. Common letters represent least squares means which were not significantly (P>0.05) different using the Tukey-Kramer multiple range test. Comparisons of means were made at individual temperatures. Significance of pollination type at each temperature is indicated next to each figure heading (n.s. not significant; * P<0.05; ** P<0.01; *** P<0.001.

Discussion

Consistent with previous studies of *E. globulus* (Hardner and Potts 1995; Hardner *et al.* 1998; McGowen *et al.* 2010; Pound *et al.* 2002), a significant reduction in seed set following self-pollination when compared with outcross and open-pollination was found. While the genotype tested had low self-incompatibility, the effect of selfing still caused deleterious effects on seed set. Self-pollination in *E. globulus* appears to result in both fewer fertilised ovules (total seeds per capsule) and an increase in the rate of abortion of fertilised ovules (ratio of viable seeds to total seeds) (Hardner and Potts 1995; Pound *et al.* 2002a). The effect on seed size was consistent with findings that showed a negative correlation between the number of seeds per capsule and mean seed weight in *E. globulus*, possibly due to seeds in lower yielding capsules receiving relatively more resources than seeds in higher yielding capsules (Suitor *et al.* 2010). The high seed set obtained from open-pollination and the fact that seed set from open-pollination did not differ significantly from MSP suggests that there was a high level of out-crossing for the trees tested and little self-pollination occurred in the open-pollinated flowers. However, the possibility that this effect is expressed as abortion of capsules which have less seeds following selfing cannot be dismissed (McGowen *et al.* 2010; Suitor *et al.* 2010).

There are direct reports of depressed growth of inbred compared to outcrossed progeny in *E. globulus* (Costa e Silva *et al.* 2010a; Hardner and Potts 1995; López *et al.* 2000a) and increased expression of chlorophyll mutants following selfing (McGowen *et al.* 2004; Patterson *et al.* 2000). The study by Hardner and Potts (1995) showed that inbreeding depression for height in *E. globulus* occurred between germination and eight months after planting and there was a trend for inbreeding depression to increase with age. The present study showed early expression of inbreeding depression for the development of germinated seeds into normal seedlings and the overall development of normal seedlings but not proportion germination or rate. This result suggests that post-germination development processes are more sensitive to inbreeding depression than germination.

Inbreeding depression is thought to be caused by two main non-mutually exclusive genetic mechanisms: directional dominance, where homozygotes produced by

inbreeding unmask recessive and/or partially recessive deleterious alleles; and overdominance, where the heterozygote itself is advantageous (Charlesworth and Charlesworth 1987; Charlesworth and Willis 2009; Lynch and Walsh 1998). It has been argued that rare and partially recessive deleterious alleles as opposed to overdominance drives inbreeding depression in *E. globulus* (Costa e Silva *et al.* 2010a; Costa e Silva *et al.* 2010b).

There was evidence for inbreeding depression on the proportion of normal seedlings developed. The present study thus suggests that partially recessive deleterious alleles are expressed post-germination and can cause inbreeding depression very early in the life cycle consistent with their potential role in post-zygotic abortion of developing seeds (Hardner and Potts 1995; Pound *et al.* 2002a). The absence of an effect of selfing on germination or germination rate was supported by Hardner and Potts (1995). They found that although there is a severe impact of inbreeding at seed set, the effect of genetic load on germination and early survival appears to be masked and only becomes evident again after field planting. A review of literature by Husband and Schemske (1996) similarly found that inbreeding depression was usually greatest at seed production or at growth/reproduction and was less expressed during seed germination. However this review did not include data from long-lived woody angiosperms, such as *E. globulus*. The present study shows that inbreeding depression can be manifest in the 21 day window of time that the germination tests were conducted over.

Although not significant in the full statistical analysis, there was a trend for self-pollinated seeds to be more sensitive to high temperature stress in the present study, which warrants further investigation. As with any genetic effect, the magnitude of inbreeding depression can also be influenced by environmental factors, with fitness traits often exhibiting inbreeding-by environment interaction (Armbruster and Reed 2005; Lynch and Walsh 1998). The findings from the present study fit with the frequently claimed finding that inbreeding depression is greater in more stressful environments (Frankham *et al.* 2002; Roff 1997). This could be due to the expression of deleterious recessive alleles underlying inbreeding depression being dependent on specific environmental conditions (Bijlsma *et al.* 1999; Dahlgaard and Hoffman 2000; Haag *et al.* 2003; Heywood 1993; Reed and Bryant 2001; Reed *et al.* 2003). A study

by Silva *et al*. (2010c) showed that expression of inbreeding depression in *E. globulus* was related to inter-tree competition for resources, such that on a more productive site there was higher suppression of inbred progeny by outcrossed progeny due to greater inter-tree competition for resources at the more productive site.

By employing a diallel crossing design the present study provides a direct comparison of maternal and paternal genetic contributions to germination in *E. globulus* because the same genotypes are compared as males and females and there is randomisation of female and male genotypes within a single site. As plants were located at a common site non-nuclear maternal effects are likely to result from asymmetries in genetic contributions via plastid DNA or to differences in provisioning by the parental genotype, rather than from differences in environment among parents (Burgess and Husband 2004; Husband and Gurney 1998). The female parent affected three out of the six germination traits. However the male parent affected five out of the six germination traits studied indicating that the nuclear genes transmitted by the pollen of distinct paternal genotypes caused phenotypic differences among the progeny (Mazer and Gorchov 1996). These paternal effects were consistent with trends in the female effects, suggesting that embryonic nuclear genetic effects dominate these germination traits and that maternal genetic effects are small.

Extranuclear paternal genetic or environmental effects on progeny phenotype are generally considered to be of less importance than extranuclear maternal genetic effects because of the absence of plastids in the generative or sperm cells of many taxa (Corriveau and Coleman 1988), lower volume of pollen cytoplasm relative to egg cells, and the relatively low dose of paternally derived genes present in endosperm cells (Mazer and Gorchov 1996). Several studies have reported significant effects of the paternal environment on offspring traits but they are smaller than maternal environmental effects (Galloway 2001a; Roach and Wulff 1987; Schmid and Dolt 1994). The present study provides strong evidence that embryo nuclear genes are a major determinant of the overall germination response in *E. globulus* as paternal genetic influence was large and correlated with female influence. This finding contrasts with previous reviews which argue that characters measured

during early growth are strongly influenced by maternal effects that overshadow any nuclear contribution (Helenurm and Schaal 1996).

In contrast to the overall germination response in *E. globulus*, there is evidence to suggest that the differential response to high temperature may be more under maternal genetic than the nuclear control of the embryo as there were significant interaction effects with temperature for the female but not the male parent. The differential maternal effect on the response to temperature of germinating *E. globulus* seeds may be caused by maternal environment or maternal genetic effects. The maternal environment can affect the temperature response (Chapter 3), but is unlikely to be a contributing factor in the present study, as trees were located at a common site, seeds developed in a common environment, and female genotypes were replicated in the study. A maternal genetic effect could arise through many causes including contributions to the endosperm, influence of the maternal genotype on offspring/seed provisioning, maternally inherited cytoplasm or plastid DNA (chloroplast and mitochondrial DNA) cytonuclear interactions, or through effects of the maternally derived seed coat (Burgess and Husband 2004; Roach and Wulff 1987). Maternal effects mediated through the endosperm is unlikely in the case of eucalypts as the endosperm degenerates early in seed development and the newly emerged seedling is sustained by the photosynthetic cotyledons (Boland *et al.* 1980).

One of the primary controls of germination and dormancy is through the maternal tissues surrounding the embryo (Mayer and Poljakoff-Mayber 1982). It is possible that the maternal genetic effects observed are due at least in part to differences in the maternal tissue. Nutrient resources, proteins, transcripts and hormones are all capable of being provisioned to seeds by the maternal parent (Donohue 2009). The seed coat is perhaps the most direct manner whereby the maternal genotype exerts control over germination (Donohue 2009) and it is possible that it regulates the response to temperature in *E. globulus*. The seed coat imposes mechanical constraints on germination and acts as an environmental filter, regulating the permeability to both oxygen and water and thereby regulating imbibition dynamics and oxidative processes (Donohue 2009). The seed coat also alters the intensity of particular light wavelengths that reach the embryo (Finch-Savage and Leubner-Metzger 2006; Leubner-Metzger 2005). Alternatively, the maternal genetic effect dominating the

differential response to temperature could be due to maternally transmitted plastids and their genomes (Helenurm and Schaal 1996). Previous studies have shown that plastid inheritance is mostly maternal in various *Epilobium* species (Schmitz and Kowallik 1986) and it has been argued that this can cause maternal effects which influence germination and seed set (Husband and Gurney 1998). In the case of *Eucalyptus globulus*, both the chloroplast (McKinnon *et al.* 2001) and mitochondrial DNA (Vaillancourt *et al.* 2004) are maternally inherited. These plastid genomes contain genes which are directly involved in photosynthesis and respiration (Steane 2005; Vaillancourt *et al.* 2004). The observed female by temperature effects could have been due to differences between females in the sensitivity of plastid gene translation or metabolic processes in the plastid to high temperature stress. Another possibility is that nuclear genes from the paternal parent which may affect response to temperature are „silenced' during germination (Andersson *et al.* 2008). This would rely on the existence of genomic imprinting which is the differential expression of genes depending on whether they were inherited from the maternal or paternal parent (Barlow 1993).

In conclusion, there is strong evidence that embryo nuclear genes are major determinants of overall germination response as paternal genetic influence was large and correlated with female influence. However, the female genotype was the main cause of the differential response to high temperature during the germination process, arguing that differential temperature responses were driven by maternal genetic effects. While the specific mechanisms leading to parental effects could not be determined from the crosses conducted in this study, it is clear that the maternal and paternal nuclear genes both have significant effects on several germination traits in *E. globulus*. Also clear is the fact that maternal genetic effects have more influence on the response of germinating *E. globulus* seeds to high temperature stress. This suggests that the choice of female genotype in MSP programs will have influence on the sensitivity of seeds to high temperatures within the nursery. Although the pollen source will influence several germination traits, the evidence suggests it will not influence the response to high temperature. Characters measured during early growth are reported to be strongly influenced by maternal effects that overshadow any nuclear contribution. The present study shows that this is not necessarily the case for germination and early seedling development in *E. globulus*, however it may be true

for the response to high temperature stress.

Chapter 5

The effect of time of harvest, irrigation treatments and kilning temperature on *Eucalyptus globulus* seed germination

Introduction

Eucalyptus globulus is the premier eucalypt grown in industrial plantations in the temperate regions of the world (Cotterill *et al.* 1999; Eldridge *et al.* 1993; Potts *et al.* 2004). Breeding programs have been established in Argentina, Australia, Chile, China, Ethiopia, Portugal, Spain and Uruguay (Hunde *et al.* 2007; Potts *et al.* 2004) and many of these are two or more generations removed from the native populations. The majority of plantations are grown for pulpwood production, however their use for veneer and solid wood products is increasing (Greaves *et al.* 2004; Hamilton *et al.* 2010; Raymond 2000).

Some clonal plantations of *E. globulus* have been established, however deployment has been limited as plant costs are high (Dutkowski and Whittock 2004) and there is low average rooting success for the majority of genotypes (Cañas *et al.* 2004; Potts 2004). Deployment from seeds is the more common method (Griffin 2001) and the majority of plantations in Australia have been established using seedlings. Most seedlings are now obtained from improved germplasm from open-pollinated seedling (Griffin 2001; McGowen *et al.* 2004a; Tibbits *et al.* 1997) or grafted (Patterson *et al.* 2004b) seed orchards, or through large-scale manual pollination systems (Callister and Collins 2007; Patterson *et al.* 2004a).

The establishment of plantations of *E. globulus* from seedlings relies on successful seed germination in nurseries. Seed germination has been variable and uneven in many nurseries in Australia and in some cases the time taken for complete germination of seed-lots has been extended from the expected 14 day period to six weeks, by which point complete germination has still not occurred. Nursery output of specification seedlings can be reduced by up to 25% through low or asynchronous germination (Potts *et al.* 2008) and in nurseries which may sow about 10 million

seeds annually, this amounts to considerable loss. In some cases this situation has been linked to periods of high temperature (C. Spurr unpubl. data). Results from Chapter 2 showed that high temperature exposure (above 29°C) during germination of commercial seed-lots had a negative effect on the percentage and rate of seed germination and normal seedling development. The location of several major seed nurseries in Australia places them at significant risk of high temperature exposure during the summer months.

It is unknown whether agronomic practices influence the performance of seeds at optimum and high temperatures. Seeds are generally harvested commercially after 12 months have elapsed from flowering. Each year seed producers are under pressure to supply seeds within tight timelines, which can lead to earlier harvesting of some genotypes. It is unknown whether harvesting earlier or later than the standard 12 months from flowering would influence seed germination and particularly its sensitivity to high temperature stress. In addition, capsules harvested commercially are normally kilned at 40°C for 24 hours, to extract the seeds. It is unknown whether kilning at a higher or lower temperature would influence the sensitivity of seeds to high temperature stress.

The impact of irrigation management of the maternal trees on seed sensitivity to high temperature stress is also unresolved. Work by Suitor *et al*. (2010) showed that reducing irrigation inputs resulted in greater capsule retention, compared to normally irrigated control trees, however the capsules retained under normal irrigation had higher seed yield than those from reduced irrigation treatments. The work by Suitor *et al*. (2010) did not include studying the germination of the seeds collected from reduced and normally irrigated trees.

The present study aims to address agronomic factors affecting seed and germination traits, response to high temperature stress and specifically (i) the effect of time of seed harvest; (ii) the effect of kilning temperatures above and below the commercial standard used for seed extraction; and (iii) the effect of irrigation treatments applied to maternal trees.

Materials and Methods

Experimental design

Eucalyptus globulus trees used in this study were located in a seed orchard in Cambridge, south-eastern Tasmania (42°48’27.23"S 147°25’58.48"E) containing selected germplasm from the Australian National *E. globulus* Breeding Program run by the Southern Tree Breeding Association (STBA). The orchard mainly included genotypes from three of the *E. globulus* races identified by Dutkowski and Potts (1999) as the Furneaux Group, Strzelecki Ranges and Western Otway. Cambridge has an altitude of 40 m with a long-term average annual rainfall of 507 mm and average annual maximum and minimum temperatures of 17.4°C and 8°C respectively. Trials were carried out over two consecutive seasons 2007/08 and 2008/09. All trees were treated in accordance with standard commercial practices.

Tree selection

A single genotype from each of the three races was selected for the time of harvest trial. The races were the Furneaux Group (early flowering), Strzelecki Ranges (intermediate flowering), and Western Otway race (late flowering), hereafter referred to as Furneaux, Strzelecki and Otway, respectively. These races commence flowering at different times, staggered by approximately 1 month intervals.

Genotype 6891 was selected from Furneaux, genotype 5407 was selected from Strzelecki and genotype 7335 was selected from Otway. Six ramets from each of the Furneaux and Strzelecki genotypes and 12 ramets from the Otway genotype were selected. The extra six ramets from the Otway genotype were used as controls, with no capsules harvested from them until 15 months after commencement of flowering. The remaining six ramets from the Otway genotype and those from the other genotypes were harvested at three separate dates. In all cases, the time of seed harvest was based on the time elapsed from commencement of flowering. Ramets of different genotypes had been planted in line plots which were randomly distributed through the seed orchard. Ramets of each genotype were selected from different line plots where possible.

Harvest dates

The harvest dates were 11 months from when flowering records for the orchard indicated the genotypes first flowered (early harvest), 13 months (commercial harvest) and 15 months (late harvest). The seeds were derived from open-pollination for all trees except one tree from the Otway genotype, which was mass-supplementary pollinated. The early harvest dates for the 2007/08 season were 22nd August, 19th September, and 16th October 2007 for the Furneaux, Strzelecki, and Otway genotypes, respectively. The commercial harvest dates were 24th October, 22nd November, and 18th December 2007 and the late harvest dates were 19th December 2007, 17th January, and 12th February 2008, for the Furneaux, Strzelecki, and Otway genotypes, respectively.

It was possible that early harvesting of fruit may have an effect on the development of fruit remaining on the tree, as early harvesting would result in less competing reproductive structures on the tree (Suitor *et al.* 2010). The change in resource allocation by harvesting early could therefore affect development of remaining fruit and introduce an effect on seeds, other than time elapsed from commencement of flowering. To account for this a control was included consisting of six trees which were only harvested at 15 months from commencement of flowering. These six trees were from genotype 7335 from the Otway race.

Kilning temperature

A kilning experiment was also incorporated into the time of harvest trial. The kilning experiment involved kilning capsules at 30°C, 40°C (standard temperature used by some seed producers) and 50°C. One replicate of 50 seeds from each tree and each kilning temperature (experimental unit) were germinated at each temperature. Samples for the kilning experiment were harvested from the same six ramets from genotype 7335 (Otway race) which were used in the time of harvest trial. Samples were taken at the early (11 months elapsed from commencement of flowering) and commercial harvest (13 months elapsed) dates only.

Irrigation treatments

Seeds from the irrigation trial undertaken by Suitor (2008) were harvested. Suitor *et al.* (2010) selected seven genotype groups (i.e. blocks) of three ramets each. Five of the groups were five different genotypes (5411, 7335, 5741, 5592, and 6029) and the other two groups were the same genotype (7537). Within each group, the three ramets were located at close proximity within the seed orchard. Within each group, one of the ramets received conventional irrigation, one received half conventional irrigation to one side of the tree, with sides swapped every two weeks, to induce partial root zone drying (PRD), and one ramet received no irrigation.

All trees were exposed to rainfall during the trial. Conventional irrigation (CI) was delivered using micro-irrigators; trees received 14 litres per hour, for one hour intervals, three times a week so that each tree received 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 of its root system. The treatments were applied from December 2006 to March 2007. Treatments therefore commenced at the time of flowering, which for the selected genotypes occurred in December but the date varied slightly due to genotypic variation (Potts and Gore 1995), and proceeded until the end of the capsule set period.

Total rainfall for the duration of the trial from December 2006 to March 2007 was 193.4 mm. Soil moisture was recorded using Hansen data logger (AM400). One sensor was placed at 60 cm 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 PRD or no irrigation and the PRD treated trees had more water available for the majority of the season than those that received no irrigation, consistent with the amount of irrigation water applied in each treatment (Suitor 2008).

Capsules from the trees exposed to the irrigation treatments were harvested during the 2007/08 season. The capsules harvested therefore represented those which were developing during the period over which irrigation treatments were applied the

previous season. Representative 100 capsule samples were harvested from all trees at 13 months elapsed time from commencement of flowering. One tree was not harvested as it had been commercially harvested.

Harvest method

For all experiments, the trees were divided into four sectors for harvesting purposes. These were low in the tree (north and south sides) and high in the tree (north and south sides). Where possible, an equal quantity of capsules was harvested from each sector. This was done to avoid confounding the results with variation in the rate of out-crossing which can change with heights in the tree canopy (Patterson *et al.* 2001). After picking, the capsules were placed in paper bags. The capsules were then placed in an oven set at 40°C for 24 hours, for seed extraction, with a temperature and humidity logger. If insufficient seeds were extracted initially, extra capsules were taken from the tree within 1 to 7 days of the initial harvest.

Seed extraction and cleaning

The seeds were then separated from the capsules and sieved to separate the seeds from the ovulodes and other trash. A vertical Dakota air column was used to remove any remaining trash or hollow seeds. All samples were then hand-picked to 100% purity. The number of seeds per capsule and weight of 100 seeds were recorded for the sample from each ramet. Seeds were counted into lots of 50 with a Contador electronic seed counter (Baumann Saatzuchtbedarf D-74638, Waldenburg).

Seed germination

Seeds from all experiments were germinated at three constant temperatures, in three separate incubators, as described in Chapter 3, set at 25°C (optimal), 32°C and 37°C (high temperatures). All seeds were germinated under continuous white fluorescent light. Temperature was logged for the duration of the experiments inside the incubators using Tiny Tag Data Loggers (Tiny Tag, United Kingdom). Seeds were germinated on sections of Advantec number 1 type filter paper placed in the perspex germination cabinets as described in Chapter 2. One length of filter paper was placed over each section in the perspex germination cabinets. 75 mL of distilled water was

added to each well in each perspex germination cabinet and the water wicked up to the seeds, maintaining a consistent level of moisture for the duration of the experiment. Water levels within sections were assessed daily and distilled water was added where necessary. An experiment commenced when seeds were placed on the moist filter paper and this point in time was considered day zero of the test.

For all experiments the replication was at the tree (ramet) level. A sub-lot of 50 seeds from each of the ramets (seed-lots) was germinated at each temperature. Each lot of 50 seeds represented an experimental unit. Allocation of experimental units of 50 seeds to sections within and between perspex germination cabinets was random. Three to four replicates of 50 seeds each of the commercial seed-lot Glob 185 (described in Chapter 2) were germinated at each temperature during each run. Perspex germination cabinets were placed in a random order within the incubators and their location within the incubators was changed each time the trial was scored.

Trial design

For the time of harvest experiment, germination testing was undertaken in three runs. Seeds from half of the ramets were germinated in the first run (run 1) and seeds from the other half of the ramets in the second run (run 2). Germination testing at 37°C was undertaken in a third run (run 3). Seeds from the kilning temperature experiment were germinated in the same three runs and randomised with the time of harvest treatment samples. Seeds from the irrigation experiment were germinated in a single, separate run (run 4) and all seed-lots were randomised across genotypes and irrigation treatments. Run 1 commenced on the 9th of September 2008 (day zero) and concluded on the 30th of September 2008. Run 2 commenced on the 20th of February 2009 and concluded the 13th of March 2009. Run 3 commenced on the 2nd of October 2009 and concluded on the 23rd of October 2009. Run 4 commenced on the 4th of September 2009 and concluded on the 25th of September 2009. A control seed-lot (Glob 185) showed no difference between runs and therefore no run level adjustment was done.

Trial assessment

Run 1 was scored daily from day 2 to day 21 from sowing. For run 2, seeds germinated at 25°C were scored on days 2, 3, 4, 5, 6, 7, 10, 14, and 21 from sowing. Seeds germinated at 32°C were scored on days 2, 3, 5, 7, 10, 14, and 21 from sowing. Seeds germinated at 37°C were scored on days 3, 5, 7, 10, 14, 18, and 21 from sowing. For run 3, seeds were scored on days 3, 5, 7, 10, 14, 19, and 21 from sowing. For run 4, seeds germinated at 25°C were scored on days 3, 4, 5, 6, 7, 10, 14 and 21 from sowing. Seeds germinated at 32°C were scored on days 3, 5, 7, 10, 14 and 21 from sowing. Seeds germinated at 37°C were scored on days 3, 5, 7, 10, 14, 18 and 21 from sowing. At each assessment a seed was recorded as germinated when there was visible protrusion of the radicle through the seed coat. A seedling was classified as normal, in accordance with ISTA guidelines (Bekendam and Grob 1993; ISTA 1999) and removed for all runs when it had a radicle at least one third the length of the seedling, a hypocotyl and two expanded cotyledons which were not covered by the seed coat. The day at which a seed germinated or a seedling was exhibiting normal development was recorded. On the final day of scoring for all runs, remaining seedlings were scored as normal or abnormal. Abnormalities included stunted radicles, failure of radicles to emerge but emergence of cotyledons and failure of seedlings to "push-off" seed coats, in accordance with ISTA guidelines for seedling evaluation (Bekendam and Grob 1993). The final day of scoring for all runs was done as outlined in Chapter 2.

Statistical analysis

To estimate the response rate of each experimental unit of 50 seeds, the time taken for 50% of the maximum germination or normal seedling development (T50) was calculated by fitting the logistic function to the cumulative curves for germination or normal seedling development (Shafii and Price 2001). This was done using the Marquardt's iterative non-linear procedure PROC NLIN of SAS version 9.1 (SAS Institute Inc 2003). The procedure for calculation was detailed in Chapter 2.

The data were summarised into six response traits, three measuring proportions and three measuring rates. The proportional data included: the proportion of seeds sown

which germinated (germ/sown); the proportion of seeds which germinated that developed into normal seedlings (normals/germ); and the proportion of seeds sown which developed into normal seedlings (normals/sown). The rate data included: the time taken to reach 50% (T50) of the maximum germination attained (T50germ); the T50 for maximum normal seedling development (T50normals); and the difference between the T50 for germination and T50 for normal seedling development (T50normals-T50germ).

Data from the time of harvest trial were analysed using a linear mixed model which treated genotype, harvest date, temperature, temperature by genotype, genotype by harvest date, temperature by harvest date and temperature by genotype by harvest date as fixed effects. Tree within genotype was treated as random effect and used to test the genotype fixed effect. Harvest date by tree within genotype was treated as a random term and used to test the harvest date and genotype by harvest date fixed effects respectively. Other fixed effects were tested using the residual error. The effect of early harvesting (preharvesting) of seeds on the seeds remaining on the trees was tested using a subset of data with the trees from the Otway genotype which were harvested at the three dates (early, commercial and late) and those which were not harvested until the latest date. This analysis was done using a mixed model which treated temperature, preharvest and preharvest by temperature as fixed effects. The variation between trees was treated as a random term and used to test the fixed preharvest effect. The residual error was used to test the preharvest by temperature interaction. The effect of preharvesting on seed weight and the number of seeds per capsule was tested using a mixed model with preharvest as a fixed effect. The residual error was used to test this fixed effect.

Data from the kilning temperature experiment were analysed using a linear mixed model which treated harvest date, kilning temperature, harvest date by kilning temperature, temperature, temperature by harvest date, temperature by kilning temperature and temperature by kilning temperature by harvest date as fixed effects. Tree by harvest date by kilning temperature was treated as a random term and used to test the kilning temperature and kilning temperature by harvest date fixed effects. Tree by harvest date was treated as a random term and used to test the harvest date

fixed effect. Tree was also treated as a random term. Other fixed effects were tested using the residual error.

Data from the irrigation experiment were analysed using a linear mixed model which treated irrigation, temperature and irrigation by temperature as fixed effects. Block by irrigation was treated as a random effect and used to test the irrigation fixed effect. Block was also treated as a random effect and the other fixed effects were tested with the residual error.

The data were analysed by fitting linear mixed models using PROC MIXED of SAS version 9.1 (SAS Institute Inc 2003). Prior to analysis the proportional data were arcsine transformed and rate data were subjected to log transformation to optimise normality of residuals.

Results

Time of harvest

Time of harvest had a significant effect on seed weight ($F_{2,27} = 4.6$; $P = 0.020$) and seed set (number of seeds per capsule) ($F_{2,29} = 8.0$; $P = 0.002$), with larger (Figure 19a) and fewer seeds per capsule (Figure 19b) being obtained from early harvesting, compared to the commercial and late harvests. Neither seed weight nor seed set had a significant effect on any of the six germination traits when included in the linear mixed models as covariates.

Genotype had a significant effect on all traits except rate of development from germination to normal seedlings in the time of harvest study (Table 12). The Otway genotype (7335) was slower to germinate and produced fewer germinated seeds and normal seedlings than the Strzelecki and Furneaux genotypes (5407 and 6891) (Figure 20). Genotypes responded differentially to temperature for all traits (Table 12). The Otway genotype was more negatively affected by increasing temperature than the Strzelecki and Furneaux genotypes for all traits except rate of development from germination to normal seedlings (data not shown), consistent with the findings from Chapter 3.

Time of harvest had a significant effect on rates of germination and normal seedling development (Table 12), but in these and several other cases the response was genotype specific. For four traits the response to harvest date was dependent on genotype (Table 12). Genotypes responded differentially to harvest date for all traits except the proportion of germinated seeds which developed into normal seedlings and rate of development from germination to normal seedlings. The proportion of seed germination and proportion of normal seedlings developed were negatively affected by early harvesting in the Furneaux genotype only (Figure 20a and Figure 20c). Germination rate was relatively stable across harvest dates for the Strzelecki and Furneaux genotypes, however germination rate was faster for early harvested seeds from the Otway genotype, compared to commercially and late harvested seeds (Figure 20d). A similar trend was observed for normal seedling development rate.

There was a significant harvest time by temperature interaction for the rate of germination (Table 12). There was little delay in germination rate at 32°C in early harvested seeds compared with that experienced by the commercially and late harvested seeds (Figure 21), suggesting that it has a higher temperature threshold. No significant differences were detected between trees which were harvested at the three dates (early, commercial and late) and the control trees which were only harvested at the latest date for any germination trait or seed weight or the number of seeds per capsule (data not shown).

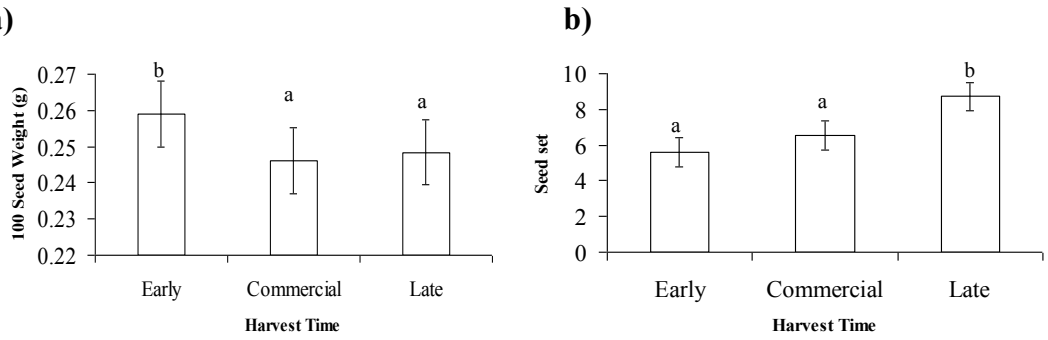


Figure 19. Least square means (±s.e.) for **a)** seed weight (x100 in grams) and **b)** seed set (seeds per capsule) for seeds harvested at three different times from commencement of flowering. Common letters represent least squares means which were not significantly (P>0.05) different using the Tukey-Kramer multiple range test.

Table 12. Effect of harvest date and genotype on six germination traits
For details of the germination traits, refer to Table 4

			Proportion						Rate (days)					
			Germ/Sown		Normals/Germ		Normals/Sown		T50Germ		T50Normals-T50Germ		T50Normals	
Fixed Effects	Num d.f.	Den d.f.	F	P	F	P	F	P	F	P	F	P	F	P
Genotype	2	15	14.7	0.000	45.3	<0.001	33.1	<0.001	37.7	<0.001	0.1	0.869	45.2	<0.001
Harvest_Date	2	29-30	1.2	0.321	2.7	0.081	1.4	0.268	5.9	0.007	1.0	0.392	4.0	0.029
Genotype*Harvest_Date	4	29-30	6.8	0.001	1.1	0.361	5.4	0.002	5.6	0.002	0.9	0.470	5.2	0.003
Temperature	2	84-85	29.5	<0.001	184.7	<0.001	155.9	<0.001	170.1	<0.001	5.9	0.004	168.6	<0.001
Genotype*Temperature	4	84-85	4.5	0.003	6.4	0.000	7.4	<0.001	15.3	<0.001	4.3	0.003	6.2	0.000
Harvest_Date*Temperature	4	84-85	0.6	0.643	2.2	0.073	1.2	0.320	2.7	0.038	3.1	0.020	1.3	0.296
Genotype*Harvest_Date*Temperature	8	84-85	0.3	0.954	1.0	0.445	0.6	0.810	2.0	0.054	3.3	0.003	1.5	0.161

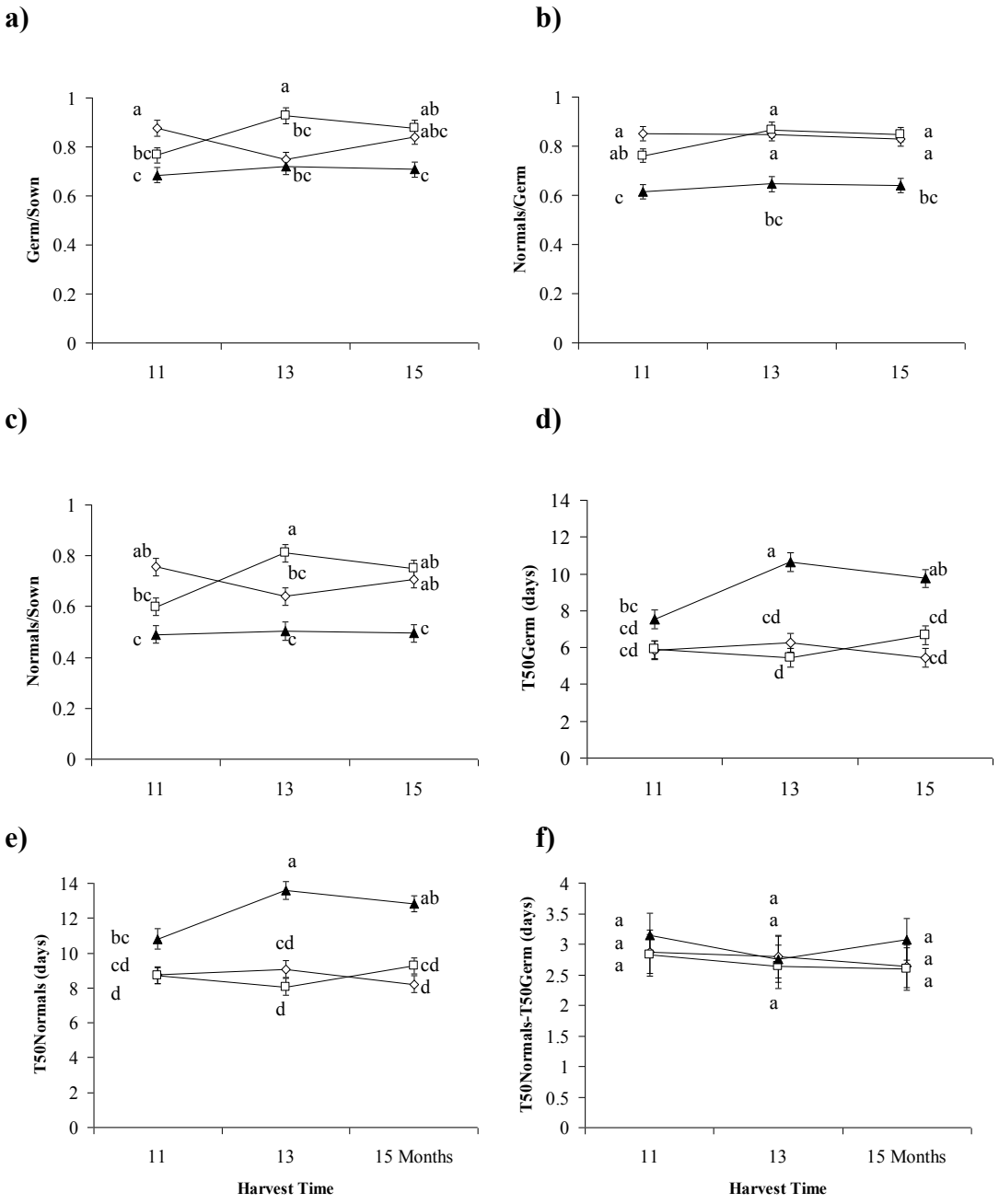


Figure 20. Least square means (±s.e.) for three genotypes (□ 6891 Furneaux; ◊ 5407 Strzelecki; and ▲ 7335 Otway) harvested at 11 months (early), 13 months (commercial) and 15 months (late 1) after commencement of flowering for **a)** proportion of germinated seeds **b)** proportion of germinated seeds which developed into normal seedlings **c)** proportion of normal seedlings **d)** rate (T50) of germination (days) **e)** rate (T50) of normal seedling development (days) and **f)** rate (T50) of development from germination into normal seedlings (days). When interaction effects were significant common letters across genotypes and harvest dates were not significantly (P>0.05) different using the Tukey-Kramer multiple range test. Where s.e. bars are not visible they are smaller than the plot symbol.

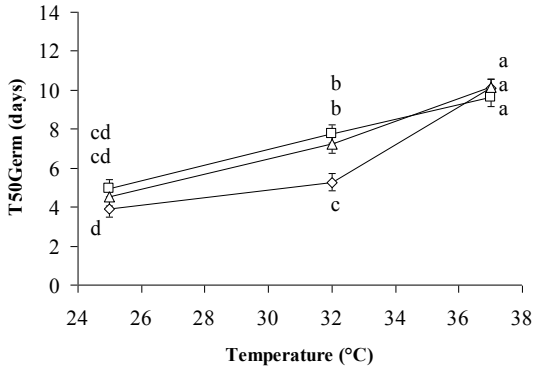


Figure 21. Least square means (±s.e.) for seeds harvested at (◊ 11; □ 13; and Δ 15 months after commencement of flowering) for rate (T50) of germination (days), averaged across three genotypes at 25°C, 32°C and 37°C. Common letters across harvest dates and temperatures were not significantly (P>0.05) different using the Tukey-Kramer multiple range test. Where s.e. bars are not visible they are smaller than the plot symbol.

Effect of kilning temperature

Kilning temperature had no effect on the six germination traits studied as a main effect or its interaction with harvest time and temperature (Table 13). Genotype effects and genotype by temperature interactions were consistent with the previous analysis. The germination rate of the Otway genotype declined less as temperature increased for seeds harvested at 11 months compared with 13 months from commencement of flowering (Figure 22a). This result is consistent with the previous analysis showing earlier harvested seeds had a higher temperature threshold for delayed germination rate (Figure 21a).

Table 13. Effect of harvest date and kilning temperature on six germination traits
For details of the germination traits, refer to Table 4

			Proportion						Rate (days)					
	Num	Den	Germ/Sown		Normals/Germ		Normals/Sown		T50Germ		T50Normals-T50Germ		T50Normals	
Fixed Effects	d.f.	d.f	F	P	F	P	F	P	F	P	F	P	F	P
Harvest_Date	1	4 to 5	0.7	0.434	0.4	0.542	1.0	0.357	39.4	0.002	3.7	0.129	27.2	0.007
Kiln_Temperature	2	18	1.7	0.208	1.4	0.280	3.2	0.063	0.0	0.980	1	0.378	0.7	0.494
Harvest_Date*Kiln_Temperature	2	18	1.0	0.405	1.1	0.352	1.0	0.378	0.6	0.587	0.5	0.590	1.8	0.187
Temperature	2	51-53	40.0	<0.001	120.2	<0.001	142.7	<0.001	153.0	<0.001	2.6	0.084	112.5	<0.001
Harvest_Date*Temperature	2	51-53	0.8	0.472	2.6	0.081	1.8	0.183	5.3	0.008	1.1	0.327	3.9	0.026
Kiln_Temperature*Temperature	4	51-53	1.1	0.379	0.6	0.638	1.2	0.321	2.2	0.079	0.3	0.863	1.1	0.393
Harvest_Date*Kiln_Temperature*Temperature	4	51-53	0.4	0.782	1.8	0.146	0.7	0.623	0.8	0.531	1.7	0.177	1.4	0.238

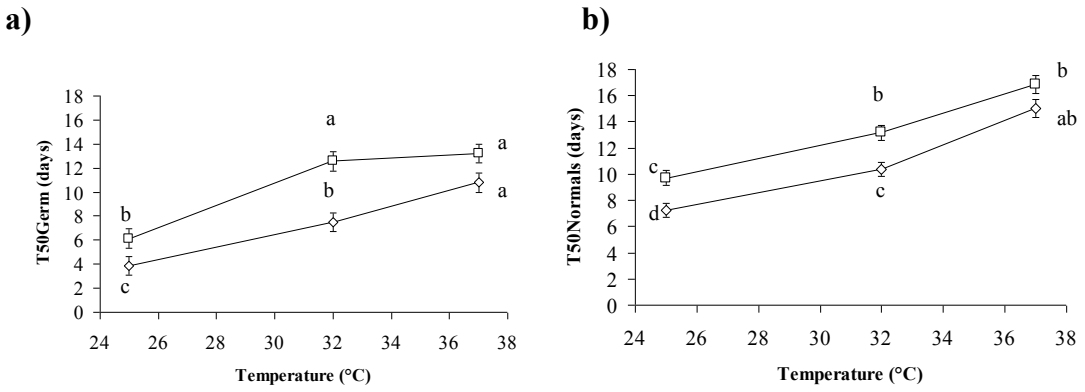


Figure 22. Least square means (±s.e.) for seeds harvested (◊ 11 and □ 13 months from commencement of flowering) for **a)** rate (T50) of germination (days) and **b)** rate (T50) of normal seedling development (days) and part of the kilning temperature study. Samples for the kilning experiment were harvested from the same six ramets from genotype 7335 (Otway race) which were used in the time of harvest trial. Samples were taken at the early (11 months elapsed from commencement of flowering) and commercial harvest (13 months elapsed) dates only.

Irrigation study

Irrigation treatments applied to the female tree did not have a significant effect on seed weight ($F_{2,10} = 0.9$; $P = 0.452$) or the number of seeds per capsule ($F_{2,10} = 3.9$; $P = 0.055$). The irrigation treatments did not affect the six germination traits and the effect of temperature was independent of irrigation treatments (Table 14). Inclusion of seed weight or the number of seeds per capsule in the mixed model analysis as covariates did not change this result.

Table 14. Effect of irrigation treatments and temperature on six germination traits

Traits tested included the proportion of germination (Germ/Sown), proportion of germinated seeds which developed into normal seedlings (Normals/Germ), proportion of normal seedlings developed (Normals/Sown), germination rate (T50Germ), rate of development from germination to normal seedlings (T50Normals-T50Germ), and normal seedling development rate (T50Normals). Proportion data were arcsine transformed and rate data were log transformed. F is the F value and P is the probability. Num d.f. is the numerator degrees of freedom and Den d.f. is the denominator degrees of freedom

			Proportion						Rate (days)					
	Num	Den	Germ/Sown		Normals/Germ		Normals/Sown		T50Germ		T50Normals-T50Germ		T50Normals	
Fixed Effects	d.f.	d.f.	F	P	F	P	F	P	F	P	F	P	F	P
Irrigation	2	11	1.4	0.287	0.9	0.451	1.1	0.366	1.5	0.277	0.4	0.695	1.2	0.326
Temperature	2	33	2.3	0.119	60.5	<0.001	20.3	<0.001	98.7	<0.001	5.9	0.007	100.5	<0.001
Irrigation*Temperature	4	33	2.2	0.094	0.8	0.567	2.6	0.055	0.3	0.886	0.5	0.746	0.2	0.920

Discussion

The negative effect of high temperatures during the germination of *E. globulus* seeds on all traits found in this study supports the findings from Chapters 2 and 3. The significant genotype effect on all traits and differential response of genotypes to temperature detected in this study support the findings from Chapter 3, showing that the germination and normal seedling development response of *E. globulus* seeds to high temperature stress are under genetic control to some extent.

The time of harvest of seeds can influence the size and number of mature seeds per capsule as well as seed germination properties including response to high temperature stress. It is possible that the lower number of seeds obtained in the early harvested seeds may be due to discarding of immature seeds during cleaning. This could have contributed to the larger mean seed weight of the early harvested seed, as it is likely that the immature seeds were small. Sasse *et al*. (2003b) found that seeds harvested less than about 320 days (~10.5 months) since flowering had a much more variable proportion of viable seeds which germinated than seeds harvested later, suggesting that the 11 month sampling may be at the limits of the time required for seed maturation. Later harvesting resulted in delayed germination and seedling development in the Otway genotype but not in the other two genotypes tested. Over all genotypes, early harvested seeds were less sensitive to a delay in germination rate up to 32°C than the commercial and late harvested seeds.

Delayed rates of germination at higher temperatures (e.g. 32°C) appear to be a property of seed maturation in *E. globulus*, which may have adaptive significance to prevent or slow germination in adverse conditions. In the present study this delay did not appear to be well expressed in the early harvested seed. It has been argued that seed harvested too early may have insufficient reserves and not possess tolerance to stresses (Sliwinska 2003). Acquisition of desiccation tolerance has been shown to be linked with later stages of seed maturation in *Trifolium ambiguum* (Hay *et al.* 2010).

The effect of harvest time and its interactions with genotype on the germination response may reflect differences in environmental conditions during seed maturation

(Gulden *et al.* 2004). For example, the heat sum during seed development has been shown to affect seed size, water relations, dormancy, germination rate and desiccation sensitivity of *Aesculus hippocastanum* (Daws *et al.* 2004). Seeds pass through three distinct phases during development: embryogenesis when cell division and all seed structures are formed, active biosynthesis of reserve material and accumulation of storage reserves which leads to a rapid increase in seed fresh and dry weight, and seed maturation which occurs when dry weight accumulation ceases (Adams and Rinne 1980; Egli *et al.* 2005; Ellis *et al.* 1987; Welbaum and Bradford 1989). These development processes are under the control of both genetic and maternal environment factors (Hay *et al.* 2010). The accumulation of seed dry weight ceases when the abscission layer forms within the maternal tissue, cutting off the supply of water and nutrients to the developing seed (Hay *et al.* 2010). Studies have suggested that there may be critical periods during seed development when seeds are more sensitive to temperature (Egli *et al.* 2005). Temperature affects the rate of physiological processes such as assimilate import into developing plant organs (Farrar 1988; Wardlaw 1990). Such processes may have been affected in the present study due to the different heat sums seeds were exposed to, depending on the harvest time.

In the present study the genotypes selected were early, intermediate and late flowering. The different harvest dates may have given the seeds from different genotypes contrasting heat sums during stages of seed development. Air temperature during seed development can affect seed dormancy (Daws *et al.* 2004). Dormancy levels are typically inversely related to the heat sum (°C d) accumulated during development (Daws *et al.* 2004). Reduced developmental heat sum has been shown to result in smaller, less developed embryos of seeds of late-maturing annuals which shed seeds at an early developmental point (Wagner and Mitterhofer 1998). Variation in rates of germination and normal seedling development between harvest dates in the present study may be partly explained by the different heat sums imposed during seed development for the different genotypes. The early flowering Furneaux genotype was negatively affected by early harvesting for proportion of seeds germinated and normal seedlings developed and this may have been due to insufficient heat sum accumulation for seed development.

While the environment experienced by the female can impact on seed germination responses (Bryd and Delouche 1971; Daws *et al.* 2004; Egli *et al.* 2005), this was not evident in the irrigation study undertaken, arguing that the seasonal and site differences observed in Chapter 3 were not due to differences in water availability. Therefore the possibility that other factors such as temperature (Bryd and Delouche 1971; Daws *et al.* 2004; Egli *et al.* 2005) drive maternal environment effects of season and site need to be explored. Irrigation treatments were shown to have no significant effect on the six germination traits studied and no effect on seed weight or the number of seeds per capsule. Even though it has been shown by Suitor *et al*. (2010) that irrigation treatments significantly affect capsule set and vegetative growth, through induced changes in resource competition, there were no effects on seed germination or seedling development. It was shown by Suitor *et al*. (2010) that trees receiving full irrigation had higher capsule abortion than those receiving half or no irrigation. This would suggest reducing irrigation to maximise capsule retention, however seed set was reduced on the less-irrigated trees (although not significant). This would likely counter the benefit from increased capsule retention as seed production is the overall objective.

Despite seed extraction methods having been reported to have an effect on seed quality and germination traits in other species Samarah (2006), kilning temperature or its interaction with harvest time had no significant effect on any of the six germination traits included in the present study. Different seed extraction methods generally had no effect on germination of eggplant seeds (Sivaraj *et al*. (2008), and Nerson (2002) considers that seed maturity at harvest is the main factor determining seed germination. Kilning temperatures between 30°C and 50°C were tested for seed extraction in the present study. Kilning at temperatures as low as 30°C would not be recommended as more time was required at this temperature for the capsules to open and release seeds. This extended kilning time would reduce the efficiency of the commercial operation. It would be recommended to maintain commercial kilning at 40°C.

In summary, for the Strzelecki and Furneaux genotypes time of harvest did not affect the germination traits studied. For the Otway genotype, however, later harvesting slowed down the rates of germination and normal seedling development. Generally

there was a more uniform response of genotypes from early harvesting. Kilning temperature of capsules and irrigation treatments had no effect on any of the germination traits studied.

Chapter 6

General discussion

The objective of this thesis was to investigate seed production factors affecting the germination response of *E. globulus* seeds. This thesis can be divided into three main themes within the overall aim of improving germination and seedling development in *E. globulus*. Firstly (theme 1), *characterising the variation between commercial seed-lots* which aimed to improve the understanding of the germination response of genetically diverse seed-lots to temperatures below and above the optimum (Chapter 2). Secondly (theme 2), *genetic regulation* which covered maternal genetic regulation of the response of seeds to temperature at the race and genotype level and assessed the maternal versus paternal genetic influence on seed germination traits and response to high temperature stress (Chapters 3 and 4). Finally (theme 3), *agronomic factors* or maternal environment factors which may influence the germination response of *E. globulus* seeds (Chapters 3 and 5). The themes studied have not been investigated previously for effects on germination in *Eucalyptus*.

Seeds grown in nurseries for plantation forestry deployment have been susceptible to slow and uneven germination and seedling development. Particularly slow germination and variable seedling development has been linked with periods of high temperature. The response of germinating *E. globulus* seeds to a range of temperatures below and above the optimum was investigated (Chapter 2). The optimum temperature for maximum development of normal seedlings was between 21.2°C and 24.8°C and for rate of normal seedling development was between 24.8°C and 25.5°C. This was lower than the optimum of 28°C reported by Lopez *et al* (2000), however that study used seeds from a more narrow genetic base and did not define the cardinal temperatures for germination. The lower cardinal temperatures for development of normal *E. globulus* seedlings were between 9.1°C and 10.5°C at low temperature. Low temperature exposure delayed germination but caused less seed mortality than high temperature exposure. The delay of commencement of germination at low temperatures can most likely be explained by the physiological processes being slowed down by the reduced temperature (Kader and Jutzi 2002).

The duration of the second phase of water uptake is dependent on imbibition temperature, with lower temperatures lengthening the second phase and delaying radicle protrusion (Bewley and Black 1994; Kader and Jutzi 2002). It is unlikely that low temperatures are responsible for the germination problems reported in commercial nurseries, at least in mainland Australia, however temperatures above 30°C are common in Australian nurseries during the summer months when seedling production is most common.

Temperatures above 30°C generally delayed germination, reduced total germination percentage, caused a high proportion of seed death and suppressed the development of normal seedlings (Chapter 2). The upper cardinal temperatures for development of normal *E. globulus* seedlings were between 40.3°C and 41.4°C at high temperature. Rate of germination and seedling development were more sensitive to temperatures outside the optimum range than percentage germination or normal seedlings. This suggests that when temperatures are outside the optimum temperature range in the nursery, uniformity in emergence and development will be more negatively affected than percent emergence and development.

Seeds fail to germinate at high temperatures due to a range of factors including seed mortality, thermoinhibition and thermodormancy. Thermoinhibition could not have been the sole factor responsible for low germination at high temperature as exposure to high temperature resulted in a high proportion of seed mortality and was thus not reversible. The increased level of abnormal seedlings at high temperatures suggests that there was a negative effect on seedling development as well as on germination. The variation in response of commercial seed-lots studied (Chapter 2) to low and high temperatures could have been due to numerous factors including genetics, storage time of seeds and seed orchard environment.

By sampling from multiple randomised ramets of maternal genotypes growing in a common environment, the extent of maternal genetic regulation of seed germination responses was assessed (Chapter 3). Maternal genotype had a significant effect on most germination traits but a differential response to temperature was only detected for percent germination and rate of development from germination to normal seedlings (Chapter 3). The differential responses were caused by only one or two

genotypes. The maternal genotype effect could have been due to the contributions to the embryo nucleus or specific maternal genetic effects, or effects of the endosperm, seed coat, or effects of maternal provisioning during seed development (Bischoff and Muller-Scharer 2010; Donohue 2009; Schmuths *et al.* 2006). An endosperm dosage effect could be ruled out as eucalypts produce seeds with no endosperm at maturity (Boland *et al.* 1980). The maternal genotype effect could be explained by race of origin for two traits relating to normal seedling development (Chapter 3). Seeds from Strzelecki were found to be more tolerant to high temperature stress for proportion of germinated seeds which developed into normal seedlings. Given that Strzelecki has been shown to be relatively tolerant to changes in water availability and evaporative demand for tree growth (Costa e Silva *et al.* 2006) some aspect of the environment of the Strzelecki trees may have selected for increased tolerance to high temperature stress.

The maternal plant genotype may affect seed germination and seedling development through a direct additive genetic contribution to the nucleus of the embryo, as measured from the male general combining ability, as well as through direct and indirect maternal effects. By using a diallel crossing design the relative importance of parental additive genetic effect on the embryo and maternal genetic effects on the germination and development of *E. globulus* seeds and their response to high temperature stress were tested. This work showed that both the paternal and maternal parent can affect the germination response, arguing for at least some influence of the nuclear genotype of the embryo (Chapter 4). Averaged across the temperatures tested (25°C, 32°C & 37°C), the paternal additive genetic effect was significant for five out of six germination traits and consistent with the female effect in most cases. However, while there was no paternal interaction with temperature for any trait, the maternal parent caused a differential response of five of the germination traits to temperature. These results argue that while additive genetic effects cause variation in the average germination response, it is the maternal genetic effect that affects the response to high temperature stress. The maternal genetic effect could be due to cytoplasmic inheritance of mitochondria and plastids, an indirect effect of the female genotype, through effects on the seed coat and other maternal tissue surrounding the embryo, or through the effects of maternal provisioning during seed development (Bischoff and Muller-Scharer 2010).

The paternal nuclear effect indicated that there were nuclear genetic effects on germination traits, suggesting differences in the nuclear genes transmitted by the pollen of distinct paternal genotypes causing phenotypic differences among the progeny (Mazer and Gorchov 1996). However, the response to high temperature stress was more influenced by the maternal than paternal parent (Chapter 4). The seed coat is perhaps the most direct manner whereby the maternal genotype exerts control over germination (Donohue 2009) and it is possible that it regulates the response to high temperature in *E. globulus*. Another possibility for the maternal effect dominating the response to temperature is cytoplasmic inheritance including maternally transmitted plastids and their genomes and provisioning of seeds (Helenurm and Schaal 1996). Previous studies have shown that plastid inheritance is mainly from the maternal parent (Schmitz and Kowallik 1986) and have argued that this may cause maternal effects which influence germination and seed set (Husband and Gurney 1998). Mitochondrial DNA and chloroplast DNA are maternally inherited in *E. globulus* (McKinnon *et al.* 2001; Vaillancourt *et al.* 2004) and it could be that these impart tolerance to high temperature stress.

E. globulus has a mixed mating system and there can be some self-fertilisation when seeds are open-pollinated, making *E. globulus* susceptible to inbreeding depression (Hardner and Potts 1995). Some self-fertilisation can occur on mass-supplementary pollinated (MSP) flowers because the flowers are not emasculated (Patterson *et al.* 2004a), however contamination levels of up to 10% are considered acceptable by Harbard *et al.* (2000) for commercial seed production, particularly if the contamination is from males of high genetic quality which is the case in most arboreta where MSP is routinely used. The sensitivity of seeds derived from self-pollination to high temperature stress was compared to seeds derived from open-pollination or MSP. In a separate experiment (Chapter 4) comparing seeds from selfing, open-pollination and MSP averaged across temperatures the germination and development of selfed seeds was poorer than the open-pollinated and mass-supplementary pollinated seeds, and there was the suggestion that selfed seeds were more sensitive to high temperature stress. This study showed evidence for the early expression of inbreeding depression, with self seeds being negatively affected for proportion of normal seedlings developed and proportion of germinated seeds which developed into normal seedlings (Chapter 4).

One of the primary controls of germination and dormancy is through the maternal tissues surrounding the embryo (Mayer and Poljakoff-Mayber 1982). It is likely that the maternal genetic effects observed were due at least in part to differences in the maternal tissue. This conclusion is based on the finding that the maternal genotype effect on germination traits varied with sampling season and site (Chapter 3), suggesting interaction with environment and/or agronomic practices.

The effects of agronomic practices during seed production on *E. globulus* seed germination and response to high temperature stress were investigated (Chapter 5). The time of harvest of seeds was found to affect the rates of germination and seedling development (Chapter 5). Genotypes responded differentially to harvest date for all traits except the proportion of germinated seeds which developed into normal seedlings and rate of development from germination to normal seedlings. This could have been due to genetic based differences in flowering time or seed maturation time. Across all genotypes earlier harvested seeds were less sensitive to a delay in germination rate up to 32°C than the commercial and late harvested seeds (Chapter 5).

Delayed rates of germination in response to high temperature stress (e.g. 32°C) appear to be a property of seed maturation in *E. globulus*, which may have adaptive significance to prevent or slow germination in adverse conditions. In the present study this delay did not appear to be well expressed in the early harvested seed. Sasse *et al*. (2003b) found that seeds harvested less than about 320 days (~10.5 months) since flowering had a much more variable proportion of viable seeds which germinated than seeds harvested later, suggesting that the 11 month sampling may be at the limits of the time required for seed maturation. It is likely that the different heat sums imposed during development of seeds from the different harvest dates played a role in the variation in germination rate and seedling development and this could also explain seasonal and site effects and interactions reported in Chapter 3. Neither kilning temperature nor maternal tree irrigation affected any germination trait recorded.

This thesis highlights the complexity of the germination of *E. globulus* seeds and demonstrated that the germination response of *E. globulus* seed-lots to optimum and

high temperature stress extends back to the seed orchard. This study shows that in addition to maternal environment and maturation effects, there may be additive genetic as well as maternal genetic effects on seed germination responses of *E. globulus* which may impact on the proportion and synchrony of seed germination and development. Even if temperature is controlled within nurseries, so that seeds are not exposed to temperatures higher than 30°C, there are other factors which will affect the germination characteristics including tree selection and genetics, maternal environment and harvesting factors such as seed harvest site, season and age. Depending on these factors, seeds may be inherently variable in their germination characteristics, independent of temperatures in the nursery. By considering these factors it should be possible for seed producers to supply seed with confidence of its performance across a range of temperature conditions in the nursery.

One key question arising from this work is whether it is cost-effective for seed producers to take measures to improve the germination performance of seeds prior to dispatch, or if it is more viable to continue regrading variable seed-lots in the nursery. The answer to this will depend on how accurate the predictions of tolerance to high temperature stress are and prediction of genotype and other variation at optimum temperature and how predictable this is from year to year. Genotypes interacted with both season and site for all traits except rates of germination and development (Chapter 3) and this could not be explained by time of harvest. This suggests that even if time of harvest is consistent, the germination characteristics of genotypes may vary with season and site of sampling. In some cases, a large proportion of sensitive seed-lots may not germinate in the nursery and this loss can not be recovered by regrading. The only way to avoid this is to identify sensitive seed-lots prior to dispatch and avoid sending them to nurseries which experience high temperature conditions. One possibility is that seed producers charge a premium for seeds that are „tolerant' to high temperature stress, to recover the extra costs associated with maintaining seeds in single tree lots and increasing the time input for commercial germination testing.

The outcomes from the research contained in this thesis have significant relevance to industry. Seed-lots responded differentially to temperature in maximum percentage seedling development (Chapter 2), suggesting that suitable pre-screening should

allow the avoidance of seed-lots which are sensitive to high temperature exposure. It was also shown that it would be more useful if germination testing of seed-lots was undertaken at temperatures higher than 33°C, to allow identification of sensitive seed-lots. At the current temperature used in commercial germination testing (25°C) there is generally a high percent germination for most seed-lots (Chapter 2), however this temperature is not reflective of nursery conditions and does not provide information about the susceptibility of seed-lots to high temperature stress. Currently, commercial germination testing only assesses the percentage germination of seed-lots and this research shows that the rates of germination and seedling development should also be recorded as they are useful predictors of seed-lot performance at high temperatures.

Based on the findings from Chapter 3, if seed producers want to maximise uniformity in germination and seedling development of seed-lots and response to temperature it is recommended that seed-lots should be dispatched in individual genotype lots. If seed producers want an indication of genotype performance at high temperatures it is recommenced that scoring of germination rate and seedling development rate as well as percent germination should be included in commercial testing of seed-lots. If seed producers are interested in the response of several germination traits to high temperature then it is also recommended that consideration be given to the maternal parent of seeds prior to dispatch as the maternal parent caused a differential response to temperature for five out of the six germination traits studied (Chapter 4). Consideration should also be given to the selection of pollen for use in the MSP program, as the embryo nuclear genotype was also shown to influence several germination traits.

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