Breeding Systems of Some Cold Tolerant *Eucalyptus* Species

BY

WAYNE RUSSELL JONES

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PREFACE

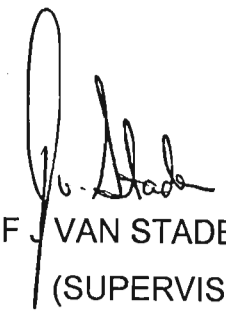
The experimental work described in this thesis was carried out at both, Sappi Forests Research, at the Shaw Research Centre near Howick and in the Research Centre for Plant Growth and Development, University of Natal Pietermaritzburg from February 1998 to December 2001 under the supervision of Professor J van Staden.

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

WAYNE RUSSELL JONES

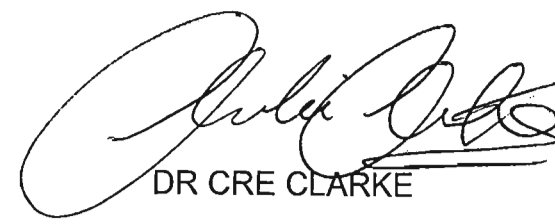
January 2002

We certify that the above statement is correct.



PROF J VAN STADEN

(SUPERVISOR)



DR CRE CLARKE

(CO-SUPERVISOR)

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ABSTRACT

Seasonal flowering times for *Eucalyptus nitens*, *E. dunnii*, *E. smithii*, *E. macarthurii and E. grandis* were evaluated in clonal grafted orchards located at the Shaw Research Centre (SRC) in KwaZulu-Natal, South Africa. The orchards are situated at 29 ^{0} 29 ' South, 30 ^{0} 11 ' East at 1100 m above sea level. The climate is cool (MAT 16.7 ^{0}C) with a January mean monthly maximum of 25.8 ^{0}C and July minimum of 4.4 ^{0}C. An estimated mean annual rainfall of 998 mm and median annual rainfall of 899 mm has been reported (PALLETT and MITCHELL 1993). It is evident that the different species flower consistently from one year to the next during the same period with similar mean flowering peaks. Long reproductive sequences where identified for all species relative to *E. grandis*, particularly *E. smithii* and *E. dunnii.* Paclobutrazol was used to initiate flowering to facilitate the study of the breeding systems of the different species. When applied as a soil drench during early summer an increase in the flower bud production in *E. nitens*, *E. smithii* and *E. grandis* was achieved.

The use of various cytochemical methods to test pollen viability, were shown to be mere indicators of potential viability and lack the reliability for adequate testing of stored pollen. From the range of *in vitro,* pollen viability studies the most successful media for all species tested was 30 % sucrose with 150 mg l^{-1} boric acid. Without boric acid in the media, the response after 24 h was significantly poorer ($p<0.001$). Significant differences ($p<0.05$) in the area of pollen grains were found between and within species. There was no significant difference between *E. dunnii* and *E. macarthurii* at the species level. Pollen of *E. smithii*, *E. grandis* and *E. nitens* were significantly smaller than that of both *E. dunnii* and *E. macarthurii.* From isolation experiments which limited potential pollinators it is apparent that a reduction of pollinators not only leads to poorer capsule survival but also poorer seed set.

Following an initial survey of pollinators of *E. grandis*, very few insects were recorded relative to surveys conducted in the natural habitats with indications that an association does exist between the presence of active pollinators and temperature.

The potential of flowers to set seed is clearly demonstrated by the difference between open pollinated flowers and controlled pollinated flowers following intra-specific crosses where differences in seed yield per capsule are very often more than double for species such as *E. nitens* and *E. macarthurii*. Similarly with inter-specific crosses, higher seed yields are extracted from crosses between closely related species. An extensive survey of orchards clearly demonstrates that *E. nitens* has the lowest clean seed recovery (13.8 %) significantly less than that of *E. smithii* (18.0 %) and both *E. macarthurii* and *E. dunnii* at 26.1 % and 26.0 % respectively.

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COMMON TERMS AND ABBREVIATIONS

BLUP	Best linear unbiased prediction
GA	Gibberellic acid
MAP	Mean annual precipitation
MAT	Mean annual temperature
NIRA	Near infra-red analysis
Paclobutrazol	Growth retardant
SRC	Shaw Research Centre

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Chapter 1

INTRODUCTION

1.1 Forestry in South Africa

Natural forests in South Africa consist of a narrow broken belt of closed canopy forest, which occur along the southern and eastern seaboards and open canopy savannah woodlands in the northeastern interior of the country. Compared to the world mean in excess of 30 %, South Africa only has 0.5 % of closed canopy forest and 19 % of which are savannah woodlands. This relatively poor forest resource is largely due to the low annual mean rainfall for the country. In order to conserve this small resource and supply timber to the country an ambitious man-made forestry enterprise was established (OWEN and VAN DER ZEL 2000).

In the southern hemisphere Southern Africa has the third largest area following Brazil and Chile and one of the oldest plantation resources. In South Africa forestry encompasses a total plantation area of 1.5 million hectares consisting of equal proportions of softwood and hardwood species of comparative yields. The product focus is softwood saw timber and strategically developed softwood and hardwood pulp and pulp products. Seventy percent of the plantation area and 90 % of the processing capacity is managed by private growers and processors, ranging from a few large companies, to many individual growers and processors organised by co-operatives. The plantation forestry sector is an important primary employer of people in rural areas while the forest products sector is the fourth largest manufacturing sector in South Africa (OWEN and VAN DER ZEL 2000). With only 0.07 % of the worlds productive forest area South Africa produces 1.2 % of the global industrial output. The mean annual increment (volume) is between 13-15 m^3/ha/year with the potential in excess of 20 m^3/ha/year.

Up to the 1950s and 1960s South Africa was regarded as the world leader in plantation forestry with the necessary research and development supporting the industry. With the expansion of plantation forestry in the southern hemisphere, new centres of excellence have developed particularly in New Zealand, Australia, Chile and Brazil. Initially, nearly all research and development was conducted and funded by government represented by the South African Forestry Research Institute and Universities. Private sector programmes effectively began with the establishment of the Wattle Research Institute in 1947, which later expanded its focus to include eucalypts and pines, and in 1984 became the Institute for Commercial Forestry Research. Today there has been a shift away from the state as primary sponsor of research and development to more funding from the private sector through co-operatives, contract research and through investment in their in-house capacity (DYER 2000).

Commercial forestry and certain aspects of national policy have been driven by research findings throughout the last century. Through tree breeding, volume production has increased from 10-30 %. Improvements through cloning have brought about a 46 % volume improvement in *E. grandis* clones. Research in wood properties laid the basis for the grading of sawn boards, and establishment of national standards. Plantation management practices in both silviculture and harvesting have been developed. Understanding the hydrological effects of afforestation and other land uses, which can now be calculated, are but a few examples (DYER 2000).

Today the global focus is on sustainability of forests, this increased emphasis has identified the need for research priorities in the area of sustainable forest management in South Africa (DYER 2000). Equally important has become the quality of the end product for the consumer in the global market. This is only achieved through an integrated supply chain from the forest to the mill.

1.2 Cold Tolerant *Eucalyptus* Species

Eucalyptus is a large genus of plants, which includes over 500 species. More than 200 of these occur in southeastern Australia occupying many different habitats. In this region they are absent from the shrublands of the dry interior and high alpine peaks where conditions are too severe for survival. In the most recent classification, PRYOR and JOHNSON (1971) recognised seven major subgenera based on the association of many morphological characters. The seven distinct groups were also substantiated by the breeding incompatibility between them. These subgenera were further subdivided into sections and series (BROOKER and KLEINIG 1983) (Table 1.1)

Table 1.1: Classification of the genus *Eucalyptus (Myrtaceae)* (BOLAND *et al.* 1980).

Subgenus	Section
BLAKELLA	LEMURIA
CORYMBIA	RUFARIA
	OCHRARIA
EUDESMIA	QUARARIA
	APICARIA
GAUBAEA	CRUTISARIA
IDIOGENES	GYMPIARIA
MONOCALYPTUS	RENANTHERIA
SYMPHYOMYRTUS	EQUATORIA
	TINGLERIA
	TRANSVERSARIA
	BISECTARIA
	DUMARIA
	EXSERTARIA
	MAIDENARIA
	HOWITARIA
	ADNATARIA
	SEBARIA

Many species of eucalypt are fast growing and produce high value timber with particular qualities. Consequently huge industries, particularly in the southern hemisphere have introduced and developed the technologies to support plantation forestry with eucalypts to serve as the raw material for the pulp, paper and solid wood markets. The origins of this industry go as far back as 1770 when Joseph Banks and Daniel Solander at Botany Bay made the first known collections of *E. gummifera*. In 1777 David Nelson a member of Captain James Cook's third expedition collected a specimen of *E. obliqua* at Bruny Island, southern Tasmania. It was from this collection that the French botanist Charles Loius L'Heritier de Brutelle described and illustrated an eucalypt for the first time (BOLAND *et al.*1980).

E. globulus was the first of the eucalypts to become widely known outside Australia. The first account of introductions of *E. globulus* in the Cape Colony was in 1828. By 1865 in the report by the Colonial Botanist more than twenty one species of eucalypt had been introduced into the Cape. In 1883 fourteen eucalypts were available from nuseries at Tokai and Worcester in the then Cape Colony for the purposes of fuel wood plantations. From 1881 the eucalypts spread rapidly from the Cape to other parts of the country with plantings recording impressive growth from the then Orange Free State, Natal, Transvaal and Lesotho. From the time of the Union in 1910 up to 1930 many species of eucalypt were tested in species trials. However, the information regarding seedlot origin, provenance and collection site details were inaccurate (POYNTON 1979).

Pioneers such as Fourcade and Hutchin recognised the importance of matching climatic conditions from different parts of the world with that of South Africa when using exotic species. "Climatic Fitness" a term used by Hutchin, has a striking similarity with the view of Charles Darwin. This insight fits into the modern day framework of the genetic adaptation of species to different environmental conditions. Through time species have evolved a genetic make-up, a *genotype*

that would be suitable for planting on similar sites no matter how far apart (VAN WYK and VERRYN 2000). Since 1930 most seed was supplied by various State Forestry Departments and other organisations in Australia with the appropriate collection and site details well documented. This allowed for a more scientific approach to species testing in South Africa which laid a good foundation for tree breeding programmes in South Africa (POYNTON 1979).

In Australia, only about sixty eucalypts out of the full complement of species are classed as being economically important producers of timber, though many of the rest yield useful timber (POYNTON 1979). Species trials have clearly identified the most important commercial eucalypts world wide, the bulk of which come from the subgenus *Symphomyrtus* (POTTS and DUNGEY 2001) (Table 1.2). Several species that were previously regarded as important have fallen into disfavour. This has been largely due to attack by pests and diseases or poor environmental adaptation, poor growth performance and poor wood properties (LOW and SHELBOURNE 1999; CLARKE 2000). In South Africa this is particularly true of species that fall into the subgenus *Monocalyptus*. Species such as *E. fraxinoides*, *E. regnans*, *E. fastigata*, *E. oreades* and *E. elata* have poor survival in the summer rainfall regions due to attack by *Phythophthora* (CLARKE and JONES 1998). The objectives of growing eucalypts have now polarised more definitely into growing short-rotation pulpwood for (LOW and SHELBOURNE 1999; CLARKE and JONES 1998) and dissolving pulp. Some species are also suitable for longer-rotation management for solid wood, (LOW and SHELBOURNE 1999; GRIFFIN 2001).

Eucalypt hybrids developed from the species listed in (Table 1.2) have become a significant component of plantation forestry, particularly in the sub-tropics, tropics and to a lesser extent in the more temperate zones. Historically hybrid development has focussed on F_1 hybrids to capture heterosis. However, developing hybrids that combined complementary traits now appears to be of greater concern (POTTS and DUNGEY 2001).

Table 1.2: The three main sections of *Symphyomyrtus* used in forestry and the associated species with commercial potential (POTTS and DUNGEY 2001).

Section	Series	Species
MAIDENARIA	VIMINALES	*dunnii* *nitens* *globulus* *macarthurii* *smithii* *cypellocarpa* *maidenii* *bicostata* *viminalis* *badjensis* *benthamii*
EXSERTARIA	TERETICORNES	*tereticornis* *camaldulensis*
	ALBAE	*urophylla*
TRANSVERSARIA	SALIGNAE	*grandis* *saligna* *pellita*

Historically, *Eucalyptus grandis* has been the most important hardwood for the South African forestry industry. However, an increasing demand for hardwoods particularly for the pulp and paper industry, has led to the expansion of hardwoods into the colder sites where *E. grandis* does not survive. Typically cold tolerant eucalypts are suited to sites above 1200 m above sea level, which are prone to frost and frequent snowfalls. The most common form of snow damage to trees is stem breakage, but trees can bend or be uprooted. Snow damaged plantations present a greater fire hazard and are prone to consequential damage

through pest and/or disease attacks. Snow damage to trees is strongly dependent on the interaction of meteorological conditions, topography as well as species and stand characteristics. In the Forestry areas in South Africa four major snow events have occurred in the past 30 years, on average a frequency of one event every 7.5 years (KUNTZ and GARDNER 2001). According to GARDNER and SWAIN (1996) *E. grandis* and *Acacia mearnsii* are the most susceptible commercial hardwood species with *E. nitens* being the most resistant to snowfalls (Table 1.3).

Table 1.3: Levels of resistance to snow damage for various commercial hardwood species.

Species	Resistance rating	Percentage damage
E. nitens	Very tolerant	≤ 5
E. fraxinoides, E. fastigata	Tolerant	5 - 20
E. smithii, E. badjensis	Moderately Tolerant	20 - 35
E. macarthurii, E. benthamii, E. dunnii	Slightly Tolerant	35 - 50
E. grandis, A mearnsii	Sensitive	50 - 100

Frost damage is equally severe in the Highveld of Mpumalanga and certain areas in KwaZulu-Natal, especially in the valleys and drainage areas. Most frost damage occurs in winter, following planting, in the form of tip scorching and or total scorching depending on the frequency and severity of the frost. Some species may be completely scorched and drop leaves but have the capacity to recover in the spring. This is typical of *E. macarthurii* one of the most frost-tolerant species planted in South Africa. Table 1.4 refers to the relative frost-tolerance of certain commercial species.

Table 1.4: Levels of resistance to frost damage for various commercial hardwood species.

Species	Resistance rating
E. macarthurii, E. benthamii	Very tolerant
E. nitens, E. badjensis, E.dorrigoensis	Tolerant
E. smithii, E. fraxinoides	Moderately Tolerant
E. dunnii, E. saligna	Slightly Tolerant
E. grandis, A mearnsii	Sensitive

In its natural habitat *Eucalyptus nitens* occurs between 600 and 1200 m elevation in extensive populations in the Victorian Alps, eastern Victoria and southern New South Wales provinces of Australia. Two distinct populations are also found at Barrington Tops and Ebor in northern New South Wales, at altitudes of up to 1600 m, with overall latitude range from 30° to 38° South (BOLAND *et al.* 1989) (Fig. 1.1). The mean maximum and minimum temperatures of the hottest and coolest months are 26 °C and –5 °C. Frosts are frequent and severe, and snow is common (SWAIN *et al.* 1998). In South Africa this species ideally suited to cooler sites in the summer rainfall regions of the country with a MAT not greater than 13 °C and MAP should be between 850–900 mm for optimum growth (HERBERT 1993).

Eucalyptus smithii occurs naturally along the eastern edge of the tablelands of southeastern New South Wales and adjacent coastal escarpment and lowlands. Scattered populations are also found in the eastern Gippsland district of Victoria. The altitude varies from 50-1150 m with overall latitude range from 34 ° to 38 ° South (BOLAND *et al.* 1989) (Fig. 1.2). The mean maximum and minimum temperatures of the hottest and coolest months are 28 °C and –2 °C. Frosts vary from few to frequent and snowfalls are light (SWAIN *et al.* 2000). *Eucalyptus smithii* is ideally suited to deep well drained soils on cool sites in the summer rainfall regions of South Africa with a MAT not greater than 15 –19 °C

(SCHÖNAU and GARDNER 1991) and MAP should be between 850–950 mm for optimum growth (HERBERT 1993).

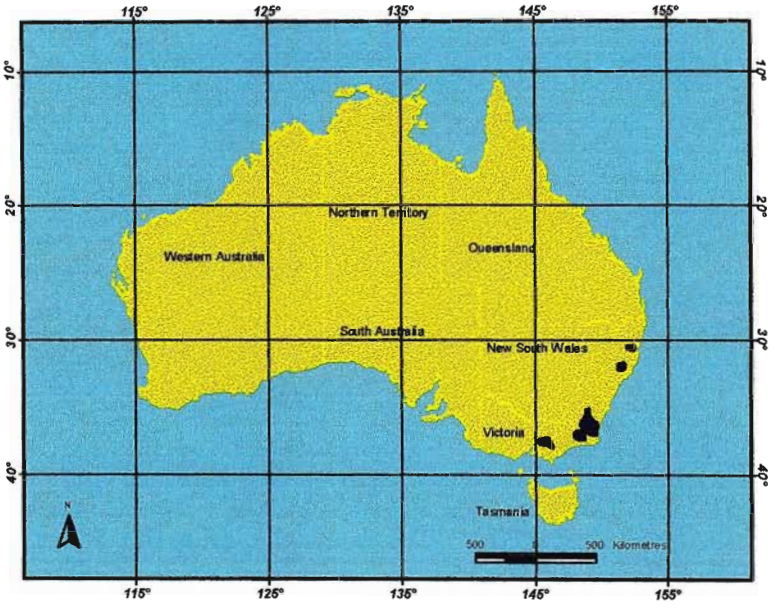


Figure 1.1: Natural distribution map of *Eucalyptus nitens* (Deane & Maiden) Maiden (BOLAND *et al.* 1989).

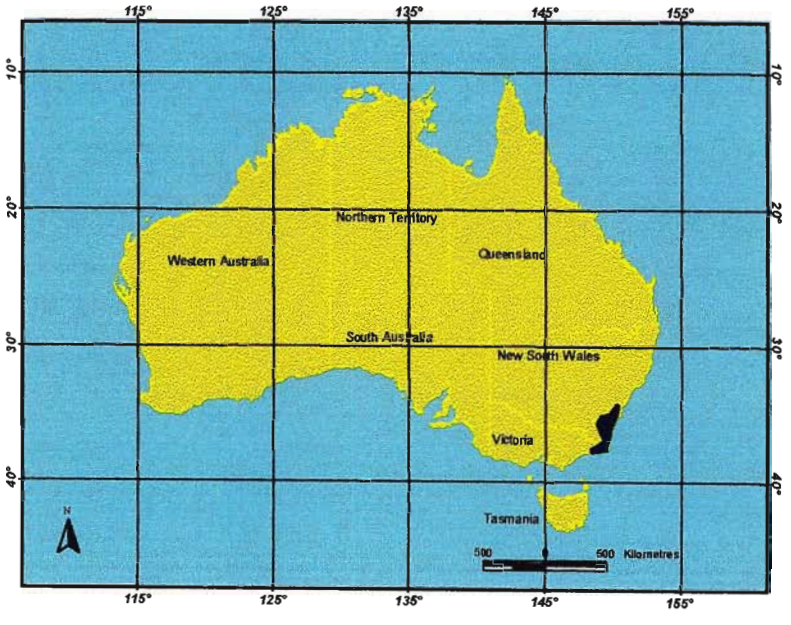


Figure 1.2: Natural distribution map of *Eucalyptus smithii* R. T. Baker (BOLAND *et al.* 1989).

Eucalyptus dunnii has a restricted natural occurrence in northeastern New South Wales extending into southeastern Queensland. The distribution covers approximately 250 km from west of Coffs Harbour in New South Wales northwards to the McPherson range. The altitude varies from 300–750 m with overall latitude range from 28 ° to 30 ° South, (Fig. 1.3). The mean maximum and minimum temperatures of the hottest and coolest months are 30 °C and 0 °C. Frosts vary from 20-60 every winter (BOLAND *et al* 1989). *Eucalyptus dunnii* grows better than *E. grandis* on cooler sites with more frost and snow tolerance. It is ideally suited to sites in the summer rainfall regions of South Africa with a MAT of greater than 15.5 °C (SCHÖNAU and GARDNER 1991) and MAP should be between 850–900 mm for optimum growth (HERBERT 1993).

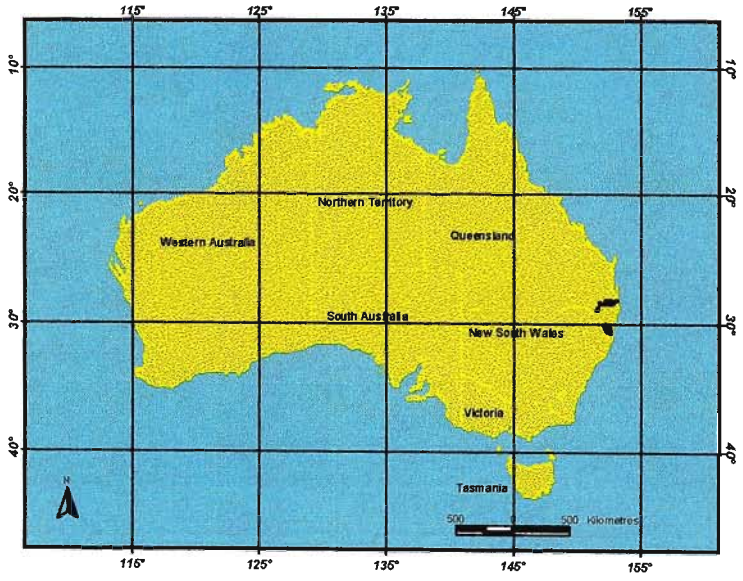


Figure1.3: Natural distribution map of *Eucalyptus dunnii* Maiden (BOLAND *et al.* 1989).

The natural occurrence of *E. macarthurii* is restricted to the central and southern tablelands of New South Wales, from the Blue Mountains to Goulburn. The altitude varies from 500–1200 m with overall latitude range from 33 ° to 35 ° South (Fig. 1.4). The mean maximum and minimum temperatures of the hottest and coolest months are 25 °C and –1 °C. Frosts are severe and frequent with

regular light snowfalls (BOLAND *et al.* 1989). *Eucalyptus macarthurii* is the most frost tolerant species current grown in South Africa; it is ideally suited to sites in the summer rainfall regions of the country with a MAT of greater than 15.5 °C (GARDNER and SWAIN 1996). The MAP should be between 850–900 mm for optimum growth (HERBERT 1993).

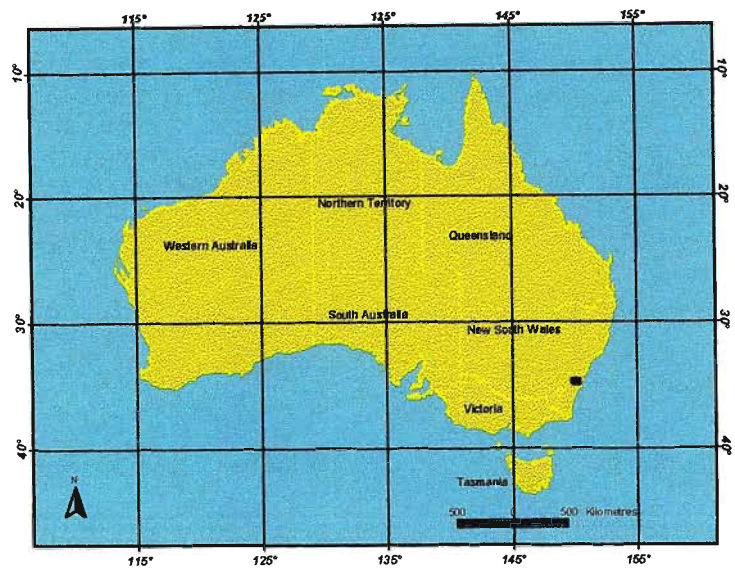


Figure 1.4: Natural distribution map of *Eucalyptus macarthurii* (Deane & Maiden) (BOLAND *et al.* 1989).

Finally the most widely planted eucalypt, *E. grandis* has a natural occurrence, which extends from Newcastle New South Wales to Bundaberg in Queensland. The altitude varies from 500–1100 m with overall latitude range from 25 ° to 33 ° South (Fig.1.5). The mean maximum and minimum temperatures of the hottest and coolest months are 30 °C and 3 °C in the south and 32 °C and 10 °C to the north. *Eucalyptus grandis* grows in humid to sub-humid conditions with a low incidence of frost (BOLAND *et al.* 1989). This species is ideally suited to sites in the summer rainfall regions of South Africa with a MAT of greater than 18 °C and the MAP should be greater than 900 mm for optimum growth.

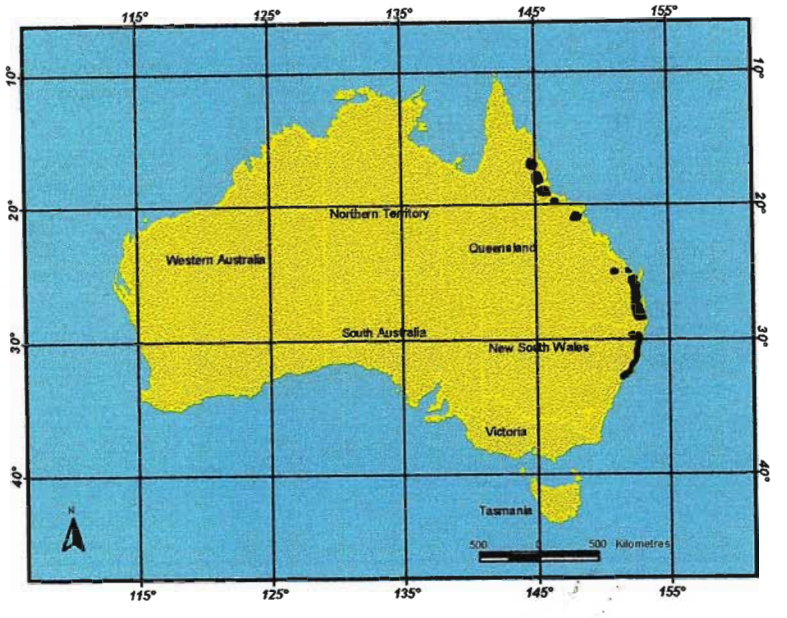


Figure 1.5: Natural distribution map of *Eucalyptus grandis* W. Hill ex Maiden (BOLAND *et al.* 1989).

1.3 Breeding Systems

Classical tree breeding depends upon the ability of breeders to combine characters from different parents, in order to develop superior new genotypes (SEDGLEY and GRIFFIN 1989). Two approaches are adopted to achieve this, firstly controlled pollinations and secondly mass pollination in seed orchards. The breeding system of a species dictates the limitations to any tree improvement programme and must be well understood to allow manipulation of flowers to facilitate the process of controlled crossing programmes. Both approaches depend upon manipulation of the reproductive biology and the outcome of both approaches is enhanced by information on the biological process under manipulation. The flowering behaviour of the plant and its breeding system are the two processes, which underpin classical tree breeding. Most eucalypts, which have hermaphroditic flowers, are protandrous, and the stigma does not become receptive until a number of days after dehiscence. This mechanism is

now well understood, and is particularly relevant in the development of hybrid genotypes (SEDGLEY 1996).

Eucalypts are a broad and diverse group, and many species and hybrids are utilised in the forestry industry. It would therefore be expected for eucalypts to show a variety of adaptations in the breeding systems suited to particular habitats, pollinators and life strategies. Despite this diversity, there are a number of important unifying features within the genus, which relate to the breeding systems. Gross floral morphology is very consistent across taxonomic groups, but there are differences in the size of the flower, ovule and seed number per flower, seed weight and the number of flowers per inflorecence, all of which have implications for resource allocation in reproductive output (ELLIS and SEDGLEY 1992). More specifically most species within the subgenus *Symphyomyrtus* have blunt or pinhead-shaped stigmas with a heavily cutinised stylar canal except that of *E. deglupta* and *E. micrcorys*, which have mop-like stigmas with long papillae. *Monocalyptus* species have blunt stigmas with few papillae and hollow styles and form a cohesive taxonomic group. On the basis of stigma and style morphology *Angophora* are more similar to *Corymbia* than to *Blakella* (SEDGLEY 1996).

A number of studies indicate that various members of the genus show preferential outcrossing and will form selfed seed only if outcrossed pollen is not available (GRIFFIN *et al.* 1987; SEDGLEY *et al.* 1989). Therefore no classical self incompatibility mechanism exists to avoid the formation of selfed seed and thus a mixed mating system under strong genetic control determines the genetic makeup of the seed produced. In terms of pollen tube growth and seed set a number of investigations have not identified any taxonomic pattern to this mechanism (SEDGLEY 1996). *Eucalyptus regnans* shows preferential outcrossing, following controlled self- and cross pollination, with postzygotic abortion of ovules occurring 16 weeks after pollination (SEDGLEY *et al.* 1989). In the case of *E. woodwardii* self pollination resulted in lower capsule retention and seed development than cross pollination. In instances of both self and cross

pollination pollen tubes grew down the style to the ovary but fewer ovules were penetrated following self- compared to cross pollination. Thus the outcrossing mechanism in *Eucalyptus woodwardii* was prezygotic and postzygotic seed abortion is only of minor importance. *Eucalyptus spathulata*, *E. cladocalyx* and *E. letophylla* favour outcrossing and set very little selfed seed. Studies of *E. camaldulensis* suggest that many, perhaps most mass flowering, high fecundity eucalypts are mainly self pollinated, and that efficient post-zygotic seed selection mechanisms allow them to be secondary outbreeders (SAMPSON *et al.* 1995).

1.4 Genetic Improvement

A major development in improving the quality of the end product has been by improving the effectiveness of the breeding programmes through the clarification of the breeding objectives for solid- and or pulpwood production. More specifically according to BORRALHO (2001) wood density, pulp yield and volume are the key traits that influence the economics of pulpwood production. Two of these variables, wood density and particularly pulp yield, have rarely been included in selection programmes in the past, yet they are shown to account for 70 % of the potential benefits from breeding for pulp production. Better and cheaper methods to measure these variables have been developed. These include Pilodyn, Near- infra red spectra analysis (NIRA) and pyrolisis, which will result in dramatic improvements in breeding. The next step is to extend into paper and determine if biological variables are important. Breeding programmes are an investment in the development of new technology, resulting in improved genotypes for clearly defined end products. Through breeding, considerable effort should be made to convert unimproved plantations to new ones with superior genotypes. Successful breeding will depend on the economic benefits associated with the improved planting stock, the deployment of the planting stock and longterm customer focus.

The processes involved in breeding and tree improvement are cyclic (generation). At each cycle the composition of each population differs due to improvements that are obtained in the breeding process. Genetic improvements in each cycle will be determined by breeding strategies employed in the future. A *Founder Population* represented by original introductions and species identified in trials provides material for a *Base Population*. From this a *Select Population* (SP) is obtained for breeding purposes, with a subset selected from the (SP) to form the *Production Population,* which provides seed and or clones for commercial deployment. The Select Population is used to generate the Breeding Population (BP), which then becomes the new Base Population for the next generation. Introductions of new material may come from *Infusion Populations* designed to enrich the original material by adding new genes to Breeding Populations at any point in time (VAN WYK and VERRYN 2000).

Eucalyptus breeding programmes are focussed on the improvement of the pure species through a process of selection of plus trees in successive generations. The selection criteria include multiple traits, performance of family members (pedigree information) and data on economic importance of particular traits. Sophisticated statistical techniques such BLUP are used to construct selection indices that predict the breeding values of selected trees (VAN WYK and VERRYN 2000).

The breeding process culminates in the development of seed orchards which can range from open pollinated clonal seed orchards or seedling seed orchards and clonal seed orchards subjected to mass controlled pollination, each with its own level of realised gain relative to the base population.

The greatest advance in industrial plantation forestry of the past 20 years has undoubtedly come from clonal deployment of hybrid genotypes. The first hybrid genotypes were identified simply as good phenotypes, which satisfied the criteria of a vigorous screening program for coppicing, rooting, growth performance and wood properties. The most famous examples of these are PF1 and HS2 varieties

selected from spontaneous hybrids in the Congo and the *E. urophylla x E. grandis* clones from open pollinated orchards of Aracruz in Brazil (GRIFFIN *et al.* 2000). Breeders make use of inter-specific hybrids for one of three principal reasons: to combine desired traits of two species; to exploit hybrid vigour (heterosis); or to increase the adaptability of a eucalypt species to areas which are marginal for the parent species (VERRYN 2000). Hybrid vigour can be measured as the difference between the F_1 or the F_2 generations and the mid-parent values, although useful heterosis is sometimes referred to as the amount by which the F_1 exceeds the better parent line (FALCONER 1981). Heterosis is an expression of non-additive effects, as the additive effects would result in the hybrid being intermediate to the parents (VERRYN 2000). The development of hybrid programmes has shown that hybridisation is a good strategy to improve *Eucalyptus* raw material for the pulp and paper industry. This can be achieved by taking full advantage of heterosis, combined with vegetative propagation to achieve rapid gains in forest productivity. Some important characteristics of wood and pulp are under additive gene control and can be manipulated through the development of hybrids. Furthermore the rooting ability of cuttings is under some influence of maternal effects, the inclusion of easy-to-root species in the hybrid programme, especially as the female parent is of fundamental importance to the propagation and deployment of superior genotypes (DE ASSIS 2000).

Eucalypts are still in the earliest stages of breeding and the major genetic changes that typically follow domestication have not yet been made. A genomic approach to more detailed understanding of important metabolic and physiological processes that result for example, in fibre formation and the identification of the genes that determine the major features of wood properties should eventually lead to new opportunities for direct genetic modifications of far reaching economic impact. The nature and extent of molecular interactions of genes and gene products in the final definition of phenotypes, represents the fundamental question in biology and by consequence in applied breeding. It should be kept in mind that focussed breeding strategies coupled with

quantitative genetics and breeders experience will continue to generate important genetic gains. Molecular breeding and more specifically genomics still has to move from promises to facts to finally prove its value in tree breeding (GRATTAPAGLIA 2001).

1.5 Seed Production

A major problem for the rapid progress of exotic forestry operations is the lack of high quality seed at a reasonable cost. This inevitably results in the use of inferior or improper seed with reduced productivity. The main objective of managed seed production is to obtain a reliable supply of *Eucalyptus* seed. At the same time an opportunity exists to improve desirable characteristics in the resulting commercial stands such as improved volume growth, form and fibre quality (BOLAND *et al.* 1980). The choice of seed source or orchard is of fundamental importance to the profitability of any commercial forestry enterprise. The development of high quality seed is complicated by changes in flowering and seed production due to environmental differences to the native habitat. This has been ascribed to a range of causes, such as latitude, elevation and rainfall (amount and timing). Temperature, an effect of latitude and altitude, is probably the most important factor in either inhibiting or promoting the trigger mechanisms for flowering (CHAMBERS *et al.*1997). Selection of a site where the species will produce abundant seed is essential for commercial exploitation. The exact location of the appropriate sites is identified by means of small trial plots or years of accurate observations of sites supporting good flowering and seed set (ELDRIDGE *et al.* 1994).

At the start of most *Eucalyptus* breeding programmes Seed Production Areas (SPA) are developed and managed to produce the first level of improvement. Seed Production Areas are any plantation compartments of known origin culled by mass selection and managed to produce seed. In most cases this seed will

never be deployed commercially, however due to cyclic flowering with some species shortfalls are always anticipated (GRIFFIN 2001). Seedling Seed Orchards (SSO) and Breeding Seedling Seed Orchards (BSO) are essentially open pollinated progeny trials that are thinned on the basis of within and between family variation. In the case of BSO's founders for the next generation will be identified, however SSO's don't serve this dual function. Clonal Seed Orchards (CSO) are a collection of grafted ramets of select genotypes rogued on the basis of progeny performance and managed for the production of open-pollinated seed. Finally the Mass Controlled Pollinated Orchard (MCPO) is an arboretum of selected, grafted, genotypes with known general and specific combining ability (GCA and SCA) (SANHUEZA and GRIFFIN 2001) managed for the production of full-sib seed for commercial deployment (GRIFFIN 2001).

All commercially important eucalypts have mixed but predominately outcrossed breeding systems (GRIFFIN 1989). Self-fertilisation results in inbreeding depression (SEDGLEY *et al.* 1989), therefore it is vital to design and manage seed orchards in ways that minimize inbreeding and maximise outcrossing. In order to achieve sustained production and reduce inbreeding depression, knowledge of the flowering times at the species, provenance, family, and within family level is the key to success. Furthermore, the species-flowering response to local climatic conditions increased flower production through the use of growth-retardants, orchard location, design and management and the impact of vectors must be clearly understood.

1.6 Aims of the study

Domestication of the Eucalypts as a method of capturing genetic gain has advanced over a number of years through well structured breeding programmes, implementation of new techniques and applied technologies. Major impediments remain when breeding with commercially important species due to a lack of

knowledge regarding their reproductive biology (MONCUR and BOLAND 2000). In South Africa species such as *E. dunnii*, *E. nitens*, *E. smithii* and *E. macarthurii* contribute not only volume but also specific qualities in the fibre supply chain chiefly for the pulp market (JONES and VAN STADEN 2001). The major objectives were therefore:

- To attempt to describe the full reproductive sequence of the above mentioned species relative to *E. grandis;*
- Classify the developmental stages by species and determine the time to seed maturity from the initiation of an inflorescence bud to the hard woody capsule containing the seed;
- Identify the peak of flowering windows by species and measure the variation from one season to the next, by provenance and by clone;
- Develop protocols for the successful collection and storage of pollen for all species and develop a suitable pollen germination media;
- Determine the variation in pollen grain morphological and size within and between species;
- Investigate the impact of pollen vectors on seed production relative to the time of year;
- Compare the differences in seed yield for inter-intraspecific crosses with the above species;
- Demonstrate the impact of growth retardants on flower production.
- Develop a set of recommendations in order to reduce the period from the selection of superior genotypes to seed production for the various orchard types and species.

Chapter 2

LITERATURE REVIEW OF THE BREEDING SYSTEMS OF EUCALYPTS

2.1 Introduction

LEWIS and JOHN (1972) defined the breeding system as all variables, with the exception of mutations, which affect genetic relationships of gametes that fuse during sexual reproduction. According to TIBBITS (1989) understanding these variables requires a sound knowledge of the reproductive biology, self and cross compatibility of a species. There is a great diversity between species in their reproductive structures and the size, shape and colour of flower buds, open flowers and fruit, and particular combinations of these are used in taxonomic classification of the genus (MIDGLEY 1988). The breeding system has been described by PRYOR (1976) as one of preferential outcrossing which is reinforced by a gene-controlled incompatibility mechanism which impedes or prevents selfing.

The reproductive cycle commences with initiation of an inflorescence bud, usually in the axil of a young leaf. The inflorescence bud consists of a single stalk bearing one to eleven buds surrounded by protective bracts (MIDGLEY 1988). Most *Eucalyptus* flowers are bisexual although the female structures are rarely reduced and nonfunctional as in the forms of *E. calophylla*, *E. ficifolia* and *E. haematoxylon* (BOLAND *et al.* 1980). Eucalyptus flowers produce nectar, which is important in attracting pollinators. These are mainly insects, although the flowers are not highly specialised for these vectors. Birds are important pollinators of some species, and small mammals may also play a role (CARR and CARR 1987).

Eucalyptus seed shed from the capsules consists of a mixture of viable seeds, derived from fertilised ovules, and chaff including unfertilised ovules and ovulodes. The fraction of viable seeds in such a mixture is usually less than 20 % (MIDGLEY 1988). Individual fruit usually have from 2 to 10 viable seeds depending on the species (BOLAND *et al.* 1980). The morphological development of buds, flowers and fruit leading to the development of mature seed takes place over a few months or can be extended to several years depending on the species (MIDGLEY 1988). According to CREMER *et al.* (1978), *E. regnans* takes three years from bud development to seed maturation with seed fall requiring a further two years. In exotic environments the phenology of many species can radically change from the distinct natural patterns of flowering and seed development to all-year occurrences of flowering (PRYOR 1976).

2.2 Reproductive Structures

Eucalypts belong to the plant family *Myrtaceae*, one of many that make up the Angiosperms, the flowering plants. Most Australian *Myrtaceae* produce flowers on or from shoots formed in the same season, flowers thus being borne near the tips of branches to provide a clear visual display to attract pollinators (BEARDSELL *et al.* 1993). The reproductive process takes place in the inflorescence, which is an arrangement of individual flowers on a stem. In eucalypts the inflorescence are usually simple and axillary such as *E. radiata* subsp. *radiata* (BOLAND *et al.* 1980) or *E. pauciflora* (BROOKER and KLEINIG 1983). *Eucalyptus tessellris* and *E. michaeliana* have compound and axillary flower configurations (BOLAND *et al.* 1980, BROOKER and KLEINIG 1983). *Eucalyptus polyanthemos* has a compound but terminal inflorescence (BOLAND *et al.*1980, BROOKER and KLEINIG 1983). Units of the inflorescence consist of single flowers in groups of one, three, seven and eleven or more borne on a single stalk the peduncle (BOLAND *et al.*1980, BOLAND *et al.* 1989, BROOKER

and KLEINIG 1983). The flower-bud clusters develop initially within protective bracts, which shed as the buds swell to reveal a cluster or umbel (BOLAND *et al.*1989, MIDGLEY 1988).

The flower bud may be stalked or sessile, the base of which consists of a hollow receptacle the hypanthium. This is surmounted by a calyptra (operculum), a cone-like structure formed by a fusion of sepals or petals or both (Fig. 2.1). The operculum is either a single-layered (*Monocalyptus*) or double-layered structure (*Symphyomyrtus, Corymbia*) (BOLAND *et al.* 1980). Eucalypt flowers never have typical petals and their colour is largely due to the stamens (BOLAND *et al.* 1989, MIDGLEY 1988). Internally the bud includes stamens and an ovary, essential structures of a bisexual flower. The male reproductive structures, the stamens, consist of a filament and anthers, which are attached to a band of tissue, the staminophore. The stamens consist of a slender filament, which supports the pollen sacs, or anther. In some species the outer filaments are barren. These staminodes either have no anther or are non-functional. The anther consists of two chambers set on opposite sides of a longitudinal connecting tissue the connective. Numerous forms of anthers can be found from oblong to ovoid in shape, typically common with species such as *E. dumosa* and *E. baileyana* (BROOKER and KLEINIG 1983).

The female structure consists of the ovary, style and stigma. Radial walls (septa) divide the ovary into anything from two to six chambers or loculi. A central, vertical, basically attached column bears the placenta, one per loculus on which ovular structures are borne in rows, the number varies according to the species (MIDGLEY 1988). The summit of the ovary narrows to become the emergent style, which terminates in a prominent or obscure organ the stigma and together comprise the gynoecium (BROOKER and KLEINIG 1983). If the ovary is sunk in the hypanthium it is referred to as inferior, however if above the bud rim it is described as semi-inferior (BOLAND *et al.* 1980). The ovular structures are different depending on the position on the placenta. Those towards the base of

the placenta are the ones most likely to become viable seed, while those towards the top comprise sterile structures with little chance of being fertilised (MIDGLEY 1988). At anthesis the operculum is shed and the anthers unfold (Fig. 2.1). At this time the anthers have mature pollen, but the stigma does not become receptive until some days later. This was clearly demonstrated in *E. grandis* by HODGSON (1976a).

2.3 Flowering of Eucalypts

Eucalypts in their natural environment usually flower within distinct seasons. This pattern is often upset when eucalypts are grown in exotic environments with little or no correlation with the site of origin (BROOKER 1985, SEWARD 1986). Natural flowering in eucalypts is highly irregular and controlling factors undefined, with the floral buds of many species developing during stem elongation. This usually occurs during the spring when temperature, day length and solar radiation are increasing following the less favourable growing conditions in the winter. *Eucalyptus regnans* (ASHTON 1975) and *E. melliodora* (MONCUR and BOLAND 1989) follow this paradigm. Thus it could be inferred that eucalypts require a period of stress induced by low temperatures and short days before initiating flowers. In contrast to many tree genera, floral development in eucalypts is not day-length dependant (SCURFIELD 1961; ELDRIGE 1968). However, there are reports of *E. occidentalis* flowering precociously at less than one year when grown under a long-day regime of 16 hours and longer (MONCUR 1992). There is some circumstantial evidence that water stress predisposes eucalypts to flower. In France, *E. viminalis* flowered 12 months after drought conditions compared to 30-36 months under well-watered conditions (MONCUR 1992).

Flowering of individuals within a species at one locality generally occurs at the same time each year although some *Eucalyptus* species can flower erratically (GRIFFIN 1980). Variations in the time of flowering within a species have been

associated with genetic control, seasonal delays at cooler and higher elevations and differences in soil types (LAW *et al.* 2000, BARBOUR *et al.* 2000). The onset of reproduction does not usually occur in Eucalypts until after the appearance of the adult foliage (flowering phase) (PRYOR 1966). According to WILTSHIRE (1998) the vegetative and flowering phase changes of eucalypts are under independent genetic control. Similarly HASAN and REID (1995) demonstrated the physiological independence of the vegetative and flowering phase changes in *E. globulus*. Precocious flowering in *E. globulus* spp. *globulus* is under strong genetic control with heritabilities ranging from 0.41 to 1.0, however, favourable environmental conditions are important to promote early flowering (CHAMBERS *et al.* 1997). According to TIBBITS (1989) consistent flowering of *E. nitens* from various provenances suggest strong genetic control of flowering at least at the population level. Similarly BURROWS and BURROWS (1992) demonstrated that flowering was consistent from one year to the next with species such as *E. populnea* and *E. moluccana*. Large differences in peak flowering have been found for *E.globulus* spp. *globulus* with no overlap between subraces, with narrow sense heritabilities at both the subrace and family level of between 0.3 and 0.9. (APIOLAZA *et al.* 2001). In most F_1 hybrids of *Eucalyptus*, flowering time is intermediate or synchronous with either parent. According to LOPEZ *et al.* (2000) there are exceptions such as *E. ovata x E. globulus.* The pure species (*E. ovata* and *E. globulus* are synchronous in their flowering while the F_1 hybrid has delayed flowering with no overlap with the parent species. This delay is a consequence of the additive inheritance of the timing of flower bud development. *Eucalyptus globulus* spp. *globulus* takes one year between bud initiation and flowering whereas *E. ovata* requires two years.

Particular seasonal conditions are thought to control the intensity of flowering by influencing bud formation and development. According to LAW *et al.* (2000) the greatest response in terms of flowering for some eucalypt species occurs with high autumn rainfalls which resulted in prolific flowering in late spring. During times of drought some species flower poorly because of bud destruction, bud

dormancy or reduced bud formation. Previous cool temperatures are the most consistent triggers for flowering of a number of species. Similarly, MONCUR (1992) was able to experimentally induce floral bud formation in *E. lansdowneana* using low temperature treatments. According to MONCUR and BOLAND (2000) conditions that favour flower induction in *E. nitens* are cold winters followed by a warm spring. The complex interaction between the extremes of both temperature and moisture at any particular site and genetic inheritance contribute to the expression of the reproductive phases in eucalypts. This was demonstrated by MONCUR and BOLAND (2000) using *E. nitens,* in Canberra ACT (550 m elevation). Flower buds appeared in December, bract shed occurred 6-8 weeks later and flowering commenced in May. In contrast at Tallaganda NSW (a cooler site, 1000 m elevation, and similar latitude), flower buds appear in late December with bract shed being delayed until October and flowering occurring the following January. Overall an increase in altitude resulted in a delay in flowering across 23 sites for *E. nitens* in Tasmania. The date of flowering correlated with altitude (r^2=0.72) (MONCUR and BOLAND 2000). Similarly with *E. grandis* at an altitude of 1200 m above sea level the peak flowering season was from April to June. At a lower altitude and with less rainfall the flowering peak was earlier and peaked from February to March (VAN WYK 1981).

Within a population the main flowering periods for individual trees may be quite separate. Such situations have been observed for *E. deglupta* (ELDRIGE 1976) and *E. regnans* (ASHTON 1975). Outcrossing between populations may be restricted in the case of *E. regnans*, where some higher altitude populations have been found to flower later than adjacent groups at lower altitudes (ASHTON 1975).

The first process in flowering is the dehiscence of the operculum. The expanding stamens force the operculum from the summit of the bud whereafter unfold or spread. There are many references to the approximate time of flowering for

various species both in natural and exotic environments (Table 2.1). While it is evident that strong correlations do exist between the various references of observed flowering within countries some large differences occur between countries. Furthermore the effect of the local conditions on seed productivity, maturation and seed shed also appear to be affected by the local environmental conditions (SEWARD 1986).

Table 2.1: Approximate peak flowering times for some *Eucalyptus* species.

Species	Natural Habitat	South African Habitat	Other Habitats
E. grandis	June to August (POYNTON 1979); April to August (BOLAND *et al.* 1980); April to August (BROOKER and KLEINIG 1983); April to August (BOLAND *et al.* 1989); February to July (LAW *et al.* 2000)	February to July (POYNTON 1979); February to June (VAN WYK 1981); February to March (HODGSON 1977)	February to May in Zimbabwe (SEWARD 1986); February to May in Uruguay (HARBARD – *pers comm* 1998)
E. dunnii	March to May (BLAKELY 1965); March to May (BOLAND *et al.* 1980); March to May (BROOKER and KLEINIG 1983); March to May (BOLAND *et al.* 1989)	February to June (JONES *et al.* 2000); February to May (JONES and VAN STADEN 2001)	February to April in Zimbabwe (SEWARD 1986); April to May in Uruguay (HARBARD – *pers comm* 1998)
E. smithii	January to March (BLAKELY 1965); January to March (BEADLE *et al.* 1972); January to March (GOODMAN 1973); January to March (POYNTON 1979); January to March (BOLAND *et al.* 1980); January to March (BROOKER and KLEINIG 1983); January to March (BOLAND *et al.* 1989)	November to January (LOOCK 1970) August to November (JONES *et al.* 2000); July to October (JONES and VAN STADEN 2001)	June to August in Zimbabwe (SEWARD 1986); January to March in Chile (HARBARD – *pers comm* 1998)
E. nitens *E. nitens*	January to March (BLAKELY 1965); January to March (BEADLE *et al.* 1972); January to March (GOODMAN 1973); January to March (POYNTON 1979); January to March (BOLAND *et al.* 1980); January to March (BROOKER and KLEINIG 1983); January to	March to October (JONES *et al.* 2000); March to October (JONES and VAN STADEN 2001)	April to November in Zimbabwe (SEWARD 1986); January to February in Tasmania (TIBBITS 1989); December to February in Tasmania (LOPEZ *et al.* 2000); January to February in Tasmania (BARBOUR *et al.* 2000); December to January in Chile (HARBARD – *pers comm* 1998)

Species	Natural Habitat	South African Habitat	Other Habitats
	March (BOLAND *et al.* 1989); February to March (BROOKER – *pers comm* 1999); January to April (GARDINER – *pers comm* 1999); May in Canberra and January in Tallaganda (MONCUR and BOLAND 2000)		
E. macarthurii	February to April (BLAKELY *et al.* 1965); February to April (BEADLE *et al.* 1972); February to April (PRYOR 1962); September to October (POYNTON 1979); February to April (BOLAND *et al.* 1980); February to April (BROOKER and KLEINIG 1983); February to April (BOLAND *et al.* 1989)	October to December (LOOCK 1970); July to December (JONES *et al.* 2000); August to December (JONES and VAN STADEN 2001)	May to September in Zimbabwe (SEWARD 1986); July in Uruguay (HARBARD – *pers comm* 1998)
E. globulus spp. *globulus*	October (POYNTON 1979); September to December (BOLAND *et al.* 1980); September to December (BROOKER and KLEINIG 1983); September to December (BOLAND *et al.* 1989); October to February in Tasmania (LOPEZ *et al.* 2000); September to December in Tasmania (WILLIAMS and POTTS 1996) September to December in Tasmania (HINGSTON and POTTS 1998)	October to November (LOOCK 1970)	April to September in Zimbabwe (SEWARD 1986); August to November in Chile (HARBARD – *pers comm* 1998); July to November in Chile (HARBARD *et al.* 1999)
E. saligna	January to March (POYNTON 1979); January to April (BOLAND *et al.* 1980); January to April (BROOKER and KLEINIG 1983); January to April (BOLAND *et al.* 1989); March to June (LAW *et al.* 2000)	December to February (POYNTON 1979)	November to February in Zimbabwe (SEWARD 1986); February to April in Uruguay (HARBARD – *pers comm* 1998)

2.4 Flower Enhancement

The induction of early (precocious) flowering could increase the rate at which desirable traits are captured by providing a means of reducing the generation time of breeding stock (HASAN and REID 1995). According to MONCUR (1992) eucalypts require a period of stress, induced by low temperatures and or short days, before initiating flower buds. The application of gibberellin (GA) biosynthesis inhibitors such as paclobutrazol [2RS.3RS)-1-(4-chlorophenyl1)-4-4-dimenthyl-2-1,2,4-triazol-1-y1-pentan-3-O1 or PP333] has been used to induce precocious and abundant flowering without degrading seed quality (WILLIAMS *et al.* 1999). According to HASAN and REID (1995) the use of paclobutrazol resulted in an increase in the flower bud and final capsule production in *E. globulus,* reducing the generation turnover time significantly.

Paclobutrazol is a broad spectrum, xylem-mobile plant growth retardant that inhibits GA biosynthesis and hence reduces the rate of cell division and expansion with associated shifts in physiological activity. These include the partitioning of carbohydrates and responses to water stress (GRIFFIN *et al.* 1993). Paclobutrazol may be taken up through the roots or by means of stem injection directly into the xylem (SHEARING and JONES 1986). Paclobutrazol has long been used in the horticulture industry to enhance flowering and control vegetative growth in crops, its value to forestry began with the first trials conducted by the CSIRO in 1984 (MONCUR 1993). Paclobutrazol can persist in the soil for several years, as it is generally resistant to chemical and biotic degradation and mass movement (JACKSON *et al.* 1996). Until recently commercial plantations were established from seed from natural stands. The use of growth retardants however has increased the production of seed from orchards (MONCUR and BOLAND 2000) (Table 2.2). Efficient use of paclobutrazol as a tool for managing flower and seed production requires a detailed understanding of responses to variation in dose rate, time and method of

application for different species and types. Optimal prescriptions will vary with the growing system and environmental conditions (SHEARING and JONES 1986).

Table 2.2: The use of gibberellin (GA) biosynthesis inhibitors such as paclobutrazol on some *Eucalyptus* species.

Species	Foliar Spray	Soil Drench	Collar Drench	Stem Injection
E. dunnii		Effective on mature trees (JONES *et al.* 2000)		
E. smithii		Effective on mature trees (JONES *et al.* 2000)		
E. nitens	Effective on mature trees (GRIFFIN *et al.* 1993)	Effective on grafts (MONCUR 1993); Effective on grafts (JONES *et al.* 2000); Effective on juvenile and mature trees, further enhanced in combination with nitrogen (WILLIAMS *et al.* 2001)	Effective on mature trees (GRIFFIN *et al.* 1993); Effective for 2 and 3 years respectively at two locations on grafts (MONCUR *et al.* 1994); Effective on grafts exposed to cold temperatures (MONCUR and HASAN 1994); Effective on mature trees (HETHERINGTON and MONCUR 1994)	Effective on mature trees (GRIFFIN *et al.* 1993); Effective for 1 year on grafts (MONCUR *et al.* 1994)
E. macarthurii		Effective on mature trees (JONES *et al.* 2000)		
E. globulus spp. *globulus*	Effective on mature trees (GRIFFIN *et al.* 1993); Effective on seedlings producing buds at 19 months (HASAN and REID 1995)	Effective on mature trees (GRIFFIN *et al.* 1993)	Effective on mature trees for six growing seasons (GRIFFIN *et al.* 1993)	Effective on mature trees for six growing seasons (GRIFFIN *et al.* 1993); Effective on mature trees (HETHERINGTON *et al.* 1992)

2.5 *Eucalyptus* Pollen

Angiosperm pollen consists of two or three cells depending on the timing of the second haploid mitosis, which in turn depends on the species. For bicellular pollen, it may occur after pollination but before germination or immediately after germination on the stigmatic surface, as with most Angiosperms (NEPI and FRANCHI 2000). The anther is the teterasporangiate in *Eucalyptus*, with development of the anther microsporocytes and microspores taking place slowly. In the case of *E. melliodora* it takes 6 months from the development of the archesporium to maturation of the pollen. Sporogenous cells are tightly packed and meiosis takes place synchronously in each anther. The bicellular pollen grains are shed via longitudinal slits, however most members of the *Symphyomyrtus* shed pollen through pores (BEARDSELL *et al.* 1993). According to PATEL *et al.* (1984) very little variation could be found throughout the family, with pollen grains measuring 10-15 µm in the widest dimension in the nonhydrated state. Pollen grains are generally tricolporate with a smooth surface and either acute or obtuse corners.

The need to quantify the capacity of pollen to germinate arose with the cultivation of plants, especially those grown for their fruits and seeds. Failure to fertilise the ovules in the ovary and the resulting failure of the ovary to become fruit may depend on many factors. The female part may be sterile, for various reasons, or there may be incompatibility mechanisms to prevent or block fertilisation at different levels (NEPI and FRANCHI 2000). *Eucalyptus regnans* shows high levels of preferential outcrossing following self and controlled cross-pollination, via postzygotic abortion (SEDGLEY 1996). In the case of *E. woodwardii* the prezygotic abortion was higher with selfed pollen. In other cases fertilisation failure may be due to the pollen (male sterility). This may be partial or total, due to genetic (hybridism, polyploidy, mutations) and environmental factors (rainfall, temperature, relative humidity, dehydration) (NEPI and FRANCHI 2000).

With the increasing interest in the use of controlled pollinations and selection of the male parent in eucalypt breeding there is a greater need to understand eucalypt pollen physiology (POTTS and MARSDEN–SMEDLEY 1989). It is therefore important to be able to check pollen fertilisation capacity. Various cytochemical methods, which differ in conception and reliability have been developed for this purpose (Table 2.3).

Table 2.3: Cytochemical methods used to evaluate pollen germination.

Subgenus	Cytoplasmic Stains	Fluoro–Chromatic Reaction	*In Vitro* Germination	*In Vivo* Germination
Symphyomyrtus	*E. grandis* (RHEMAN *et al.* 2000)		*E. grandis* 20 % sucrose, hanging drop for best germination (HODGSON 1975); E. *grandis* 20 % sucrose only for best germination (RHEMAN *et al.* 2000); *E. globulus* spp. *glolulus* , *E.ovata, E. morrisbyi* and *E. unigera* 20 % sucrose and 200 mg l^{-1} boric acid for best germination and pollen tube growth (POTTS and MARSDEN-SMEDLEY 1989); *E. dunnii* 30 % sucrose and 200 mg l^{-1} boric acid (BARBOUR and SPENCER 2000); *E. globulus* spp. *globulus, E.ovata, E. gunnii* and *E. unigera* 0-5 % agar with 20 % sucrose,150 mg l^{-1} boric acid (GORE *et al.* 1990); *E. dunnii* 30 % sucrose and 0.8 % agar (SOUSA 1990); *E. dunnii* 30 % sucrose and 0.8 % agar (SOUSA and PINTO 1992)	*E. grandis* (HODGSON 1975); *E. platypus, E. spathulata* (SEDGLEY and GRANGER 1996); *E. spathulata, E. cladocalyx* (ELLIS and SEDGLEY 1992); *E. nitens* and *E. globulus* spp. *globulus* (GORE *et al.* 1990)
Monocalyptus			*E. regnans* 30 % surcrose and 1.5 mg l^{-1} boron (GRIFFIN *et al.* 1982)	*E. regnans* (GRIFFIN *et al.* 1982); *E. regnans* (SEDGLEY *et al*. 1989)

Subgenus	Cytoplasmic Stains	Fluoro–Chromatic Reaction	*In Vitro* Germination	*In Vivo* Germination
Corymbia		*E. calophylla* (EGERTON-WARBURTON *et al.* 1993)	*E. calophylla* 20 % sucrose and 200 mg l^{-1} boric acid (EGERTON-WARBURTON *et al.* 1993)	*E. calophylla* (EGERTON-WARBURTON *et al.* 1993)
Reported as all species			Agar plate with suitable sucrose content (PRYOR 1976); 1.5 % agar with 35 % sucrose and 100 - 250 mg l^{-1} boric acid (VAN WYK 1981); 30 % sucrose and 150 mg l^{-1} boric acid (MONCUR 1995)	

The simplest germination tests are based on cytoplasmic stains, such as lactophenol Cotton Blue and Alexander stain and the concept that pollen devoid of cytoplasm is dead. Dead pollen grains do not stain or stain differently from those with cytoplasm. These methods are easy to use but don't give reliable results. Pollen grains, which remain unstained, lack a protoplast and are definitely aborted, but those with cytoplasm are not necessarily fully fertile, this means an overestimation of pollen viability (NEPI and FRANCHI 2000).

Other techniques involve using the enzyme method, which is based on the colour reactions induced by enzyme activity in the pollen grain. The most widely used method is that proposed by HESLOP-HARRISON and HESLOP-HARRISON (1970). This technique is known as FCR (Fluoro-chromatic reaction), it is base on entry of the nonpolar substrate fluorescein diacetate into the vegetative cell where it is hydrolyzed by esterase to a polar product (fluorescein) which is retained by the cell membrane. Pollen with an integral plasmamembrane will flouresce and is considered viable and non-flourescent pollen grains are eliminated. This method tends to overestimate viability due to certain cytological events such as the failure of first haploid mitosis and degeneration (NEPI and FRANCHI 2000). The fluoro-chromatic reaction stain procedure was able to detect differences in pollen viability from two collection sites for *E. calophylla* (EGERTON-WARBUTON *et al.* 1993).

More realistic values can be obtained by germinating pollen under appropriate conditions and calculating the percentage germination of germinating grains. When pollen germinates, a pollen tube is formed, the length of which is measured in diameters, namely pollen diameter. A pollen grain is regarded as having germinated when the pollen tube length exceeds one diameter. *In vitro* germination is controlled by temperature using a culture medium containing salts (the most widely used for *Angiosperms* is that of Brewbaker and Kwack 1964) with 5-15 % sugar (NEPI and FRANCHI 2000). The most successful germination media for the *Eucalyptus* species tested, ranges from 20 – 30 % sucrose with some level of boric acid, (Table 2.3). Grains that germinate are visible under low magnification dissecting microscopes without any need for special techniques. Pollen may also be germinated *in vivo*, where the pollen tube rapidly penetrates the stigma and style. The tissues of the stigma and style must be removed to reveal the pollen tubes, at which stage pollen tubes can be detected by flourescence of callose using aniline blue or lactophenol Cotton Blue (NEPI and FRANCHI 2000).

In vivo germination has been achieved with a number of eucalypts for the purposes of understanding pollen tube growth and early seed development. In *E. regnans* pollen tubes were shown to grow between the cells of the transmitting tissue and not in the stylar canal (SEDGLEY *et al.* 1989). Similarly GORE *et al.* (1990) used *in vivo* germination to demonstrate the unilateral cross-incompatibility when attempting to develop hybrids between *E. globulus* spp. *globulus* and *E. nitens*. *Eucalyptus nitens* pollen tubes had only grown 6 mm, well short of the full length of the *E. globulus* style (9–10 mm). *In vivo* germination methods give the most realistic results, but forms of competition exist *in vivo* between pollen at various levels (NEPI and FRANCHI 2000). According to ELLIS and SEDGELY (1992) reductions in pollen tube numbers in the style is caused partly by competition for space, as the lower style has a lower carrying capacity for pollen tubes due to narrower transmitting tissue in species such as *E. spathulata* and *E. cladocalyx*. Pollen germination is also influenced by mass

effects, and irrespective of the number of ovules per ovary a "minimum pollen load is often necessary to ensure fertilisation (PACINI and FRANCHI 1999).

2.6 Pollinating Agents

Seed production in *Eucalyptus* is dependent on the pollen transfer between flowers. This is due to the absence of parthenocarpy in this genus (GRIFFIN *et al.* 1987), as well as barriers to pollen transfer between anthers and stigma of the same flower caused by protandry (PRYOR 1976). The Australian members of the Myrtaceae show a great deal of diversity in pollination mechanisms and pollinators (BEARDSELL *et al.* 1993). According to PRYOR (1976) eucalypts are pollinated by a diverse number of insects and birds. Individual species may attract Hymenoptera, Diptera, Coleoptera, Thysanoptera and Lepidoptera, others may attract birds, bats and a range of marsupials (ARMSTRONG 1979). Most species appear to be pollinated by insects that are attracted to the nectar secreted into the bowl shaped flowers of many species (BEARDSELL *et al.* 1993). There is little evidence to indicate that wind plays a major role in the pollination process although some species such as *E. tereticornis* and *E. blakelyi* may be partly wind pollinated PRYOR (1976).

The relative abundance of floral visitors is influenced by the variation in the floral morphology and rewards as well as the weather at the time of flowering (HINGSTON and POTTS 1998). Eucalypts with small flowers are predominantly entomophilous (insect pollinated) whereas species with large flowers are mostly ornithophilous (bird pollinated) (FORD *et al.* 1979). In the winter rainfall areas of southern Australia birds may be more important pollinators, when it is frequently too cold and wet for insect flight.

The most common pollinators of eucalypts are bees from the family Collectidae (ARMSTRONG 1979). Wasps are less likely visitors, however, thynnid wasps have been observed on eucalpt flowers (ASHTON 1975).

Table 2.4: The entomophilus and ornithophilous pollinators of eucalypts.

ENTOMOPHILUS		ORNITHOPHILOUS
Apis mellifera	Other	Various
E. grandis (POYNTON 1979); *E. grandis* (DAVIS and TRIBE 1996); *E. nitens* (MONCUR *et al.* 1995); *E. globulus* spp. *globulus* (HINGSTON and POTTS 1998); *E. globulus* spp. *globulus* (MONCUR *et al.* 1995); *E. saligna* (POYNTON 1979); *E. cosata* (HORSKINS and TURNER 1999); *E. siderpholia* (MONCUR *et al.* 1991); *E. pellita* (MONCUR *et al.* 1995); *E. camaldulensis* (MONCUR *et al.* 1995); *E. alba* (MONCUR *et al.* 1995); *E. diversicolor* (MONCUR *et al.* 1995); *E. ficifolia* (NICOLSON 1994)	*E. globulus* spp. *globulus* - 71 species (HINGSTON and POTTS 1998); *E. cosata* - 76 species (HORSKINS and TURNER 1999); *E. grandis* - 5 insect taxa (DU TOIT 1987); *E. ficifolia* - 4 insect taxa (NICOLSON 1994)	*E. globulus* spp. *globulus* - 7 species (HINGSTON and POTTS 1998); *E. incrasata* - 1 species (NICOLSON 1994)

The major plantation species have small flowers and no apparent adaptation to particular vectors. Honeybees are attracted to the flowers by nectar and the pollen, which adheres to all parts of their bodies, are regarded as an effective substitute for natural pollinators in the exotic environment (ELDRIDGE *et al.* 1994). As many as 71 different insect species and 7 species of bird are responsible for the pollination of *E. globulus* spp. *globulus* in Tasmania (HINGSTON and POTTS 1998). Similarly HORSKINS and TURNER (1999) identified 76 species pollinating *E. cosata* in Northeastern Victoria. The most dominant species associated with the pollination biology of eucalypts in both the natural and exotic environments is *Apis mellifera* (Table 2.4). Honeybees are well adapted to assist in the pollination process and there are examples of the

beneficial effects when used in the management of seed production of forest species (Table 2.5).

The success of honeybees as the major pollinator of a particular species depends on the status of nectar and pollen, which provides all the necessary nutrition for laval growth and metamorphosis (DIETZ 1975). Pollen is virtually the only source of natural protein available to bees, influencing both breeding and longevity (MONCUR *et al.* 1995). Pollen with less than 20 % crude protein cannot sustain colony requirements for optimum production. Crude protein concentrations in pollen are between 11 and 40 % for eucalypts and 16-29 % for acacias. Both the quality and quantity of pollen protein can independently, and in combination, influence supply of essential amino acids, which ultimately become available to the bees. Often insufficient eucalypt pollen is available to meet the protein requirements necessary to maintain body protein during honey flows (MONCUR *et al.*1991).

Table 2.5: The effect of *Apis mellifera* on seed production in Australia and Indonesia (HORWOOD 1996).

Species	Location	No. Seeds per Capsule without Bees	No. Seeds per Capsule with Bees
E. alba	Java	6.60	9.90
E. camaldulensis	Northern Queensland	5.68	14.32
E. diversicolor	Western Australia	1.20	2.20
E. globulus	Tasmania	20.80	25.30
E. nitens	Tasmania	5.1	5.4

The volume of nectar availability decreases during the day due to evaporation and use according to HORSKINS and TURNER (1999) for *E. cosata,* and for both *E. cladocalyx* and *E. ficfolia* (NICOLSON 1994). According to HORSKINS and TURNER (1999) honeybees are the most frequent visitors to the flowers of

E. cosata, collecting pollen and nectar at all times of the day, with foraging commencing at temperatures of (11.9 °C ± 1.4) much cooler than native bees (21.0 °C ± 2.0). According to NICOLSON (1994) foraging by honeybees was the most intense during the morning, before a decline in nectar volumes. Honeybee foraging in litchi orchards followed a similar pattern with nectar collections highest in the morning, while pollen collection peaked at noon (DU TOIT and SWART 1995). Honeybees also exhibit a within-flower foraging behaviour that is indicative of any successful pollinator (HORSKINS and TURNER 1999), however PATON (1997) observed restricted foraging without movement between plants over several days. This can result in decreased levels of seed set due to a lack of pollen transfer to the stigma or incompatible pollen being transferred. Environmental factors such as drought or prolonged rainfall may affect the normal rhythm of flowering, furthermore the weather conditions during flowering could prevent foraging if cold, wet and windy (HINGSTON and POTTS 1998).

2.7 Breeding and Pollination in Eucalypts

Genetic improvement of a particular species can only be achieved with a clear understanding of the breeding system, a well defined breeding strategy and deployment options through seed or clones. To ensure continuous improvement of the population, controlled crosses can be made to concentrate the best alleles from a range of selected parent trees. To produce a controlled cross, pollen from anthers of a selected male parent is placed on the stigmatic surface of a female parent. The stigma is isolated from all other pollen sources. Pollen germinates on the stigma, grows down the style to the ovary and fertilizes the ovules (MONCUR 1995). Most of the commercially important eucalypts have mixed but predominantly outcrossed breeding systems (GRIFFIN 1989). Self-fertilisation results in inbreeding depression and must be minimised and avoided (SEDGELY and GRIFFIN 1989). Significant differences in vigour and straitghtness were evident in open pollinated progeny of *E. grandis* relative to outcrossed progeny

due to a high level of selfing (HODGSON 1976b). Similarly, BURGESS *et al.* (1996) measured lower growth rates and poorer survival of *E. grandis* families with lower outcrossing rates. Severe inbreeding depression in seed set and field growth following selfing in *E. globulus* spp. *globulus* has been reported (HARDNER and POTTS 1995, HARDNER and POTTS 1997). According to SEDGLEY (1996) a large variation in the stigma and style morphology can be found amongst the various eucalypts. A number of studies have indicated that some species show preferential outcrossing and will so form selfed seed only if outcrossed pollen is not available (GRIFFIN *et al.*1987, SEDGELY and GRIFFIN 1989). Thus there is no classical self-incompatibility mechanism, to ensure that no seed is formed following selfing, but a mixed mating system, which is under genetic control. Based on many studies in terms of pollen tube growth and seed set there is also no taxonomic pattern (SEDGLEY 1996).

Eucalyptus regnans shows preferential outcrossing with pollen tubes growing between the cells of the transmitting tissue and not in the stylar canal, ovule penetration commences 5 days after pollination (selfed or outcrossed). Even with ovule penetration by the selfed pollen tubes, postzygotic abortion takes place. A different pattern is observed in *E. woodwardii,* where self-pollination results in lower capsule retention and seed development relative to cross-pollination. Similarly (HODGSON 1976b) found a drop in seed yield following selfing in *E. grandis*. This is largely due to the failure of pollen tubes to penetrate the ovules, thus resulting in prezygotic abortion (SEDGLEY 1996) (Table 2.7). Male sterility has been observed in *E. luecoxylon*, with pollen breakdown following meiosis, such that the grains were collapsed and empty at anthesis (ELLIS and SEDGLEY 1993).

In eucalypts the male phase generally begins approximately 2 days after anthesis, with pollen shed occurring in response to temperature and other environmental factors. The female stage begins with the appearance of a sticky secretion on the stigmatic surface. The time period from anthesis to stigmatic

receptivity varies from one species to the next (Table 2.6). According to MONCUR (1995) receptivity may be as short as 1-2 days or as long as 7-10 days.

Table 2.6: The time period from anthesis to stigmatic receptivity.

Species	Stigma Receptivity (Days)	Reference
E. regnans	10	GRIFFIN and HAND (1979)
E. woodwardi	7	SEDGLEY and SMITH (1989)
E. urnigera	13	SAVVA *et al.* (1988)
E. spathulata	7-10	ELLIS and SEDGLEY (1992)
E. platypus	7-10	ELLIS and SEDGLEY (1992)
E. globulus	6-10	ESPEJO *et al.* (1996)
E. grandis	4-6	VAN WYK (1977)
E. dunnii	6	SOUSA and PINTO (1994)
E. nitens	9	TIBBITS (1989)

The sequence of traditional controlled pollination advances through eight stages, (Fig. 2.1). Mass production of seed by controlled pollination of selected individuals is an increasingly important method of capturing genetic gain from tree breeding. Alternative methods have been found with the development of the "One-Stop Pollination" technique (OSP) which eliminates repeated visits to flowers. Emasculation of the flowers at anthesis is followed by slicing the stigma at the top of the style to provide a site for pollen adherence. Pollen is applied immediately, followed by isolation of the style with tubing in *E. globulus* spp. *globulus* as opposed to the isolation bag (HARBARD *et al.* 1999).

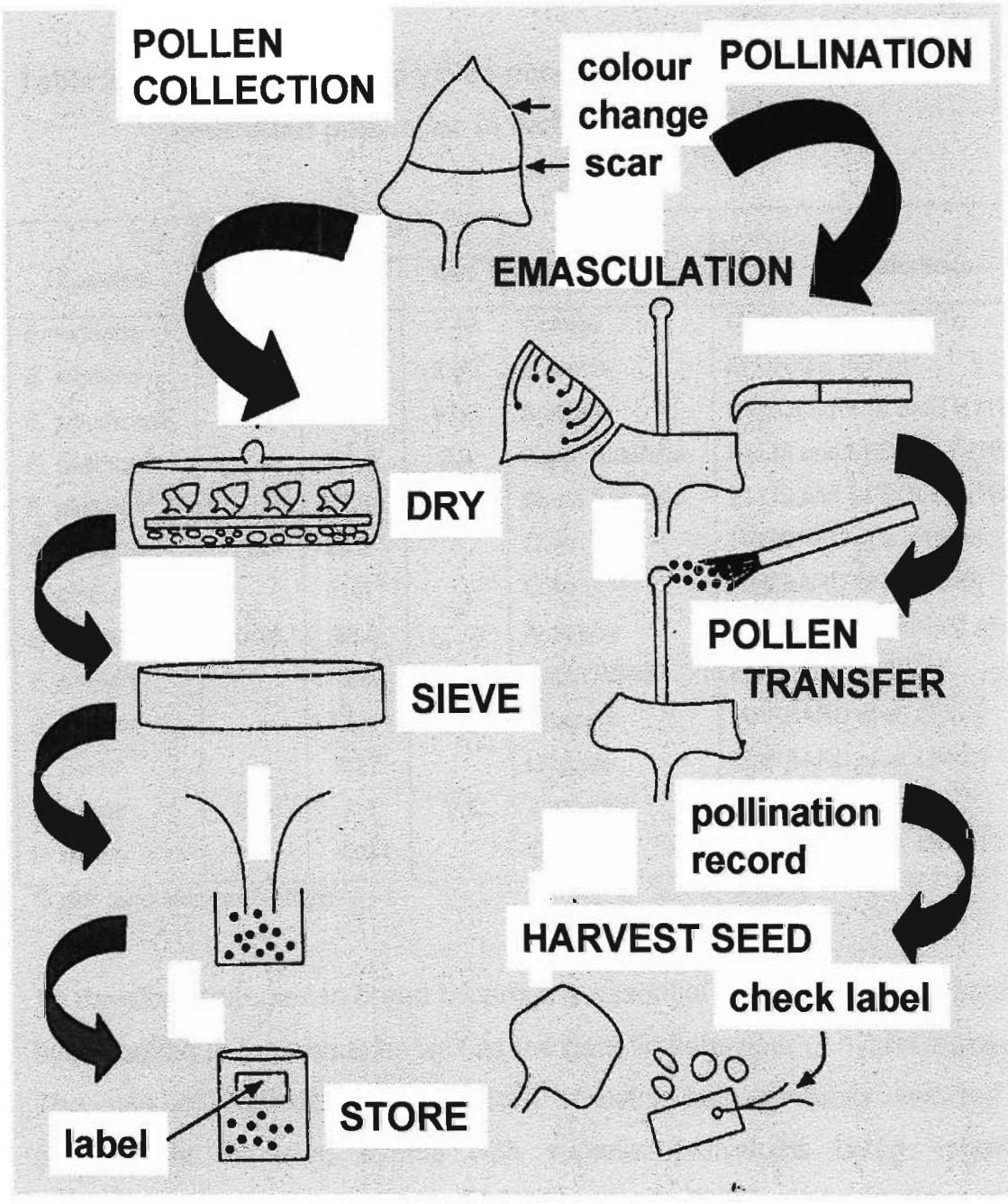


Figure 2.1: Techniques for pollen collection, emasculation and pollen transfer for controlled pollinations in eucalypts (MONCUR 1995).

Table 2.7: The relative seed yields per capsule following self and controlled pollination in various eucalypt species.

Species	Pollination			Location	Reference
	Open	Cross	Self		
E. regnans	2.14	1.82	1.19	Victoria	GRIFFIN *et al.* (1987)
E. regnans	4.1	4.2	2.60	Australia	ELDRIGE (1970)
E. woodwardi		18.2	6.80	Australia	SEDGLEY and SMITH (1989)
E. spathulata		21.9	2.2	South Australia	ELLIS and SEDGLEY (1992)
E. platypus		9.8	4.7	South Australia	ELLIS and SEDGLEY (1992)
E. globulus	11.6	25.7 *		Chile	HARBARD *et al.* (1999)
E. globulus		21.0 *		Chile	HARBARD *et al.* (2000)
E. globulus	10-20	38.0		Australia	VOLKER in MONCUR *et al.* (1995)
E. grandis		3.0	0.16	South Africa	HODGSON (1976a)
E. grandis		13.4 *		Uruguay	HARBARD *et al.* (2000)
E. dunnii		3.2 *		Uruguay	HARBARD *et al.* (2000)
E. nitens	3.8	7.9	2.2	Tasmania	TIBBITS (1989)
E. nitens		0.9 *		Chile	HARBARD *et al.* (2000)

* OSP (one stop pollination)

To develop strategies to breed hybrids it is essential to understand the barriers to hybridisation in the genus as well as the genetic behaviour of hybrid populations. The use of hybrids in commercial forestry has generally resulted from opportunistic crossing events with superior individuals being vegetatively propagated in large numbers (POTTS *et al.* 2000). Hybridization between species from the major eucalypt subgenera does not occur either naturally or artificially (PRYOR and JOHNSON 1971; GRIFFIN *et al.* 1988). The main reason for this, is the pre-zygotic barriers, the first of which is a unilateral structural barrier. According to GORE *et al.* (1990) the pollen tubes of small flowered species are not able to grow down the full length of the style. This is typical when crossing *E. nitens* males onto *E. globulus* females. The second pre-zygotic barrier to hybrid seed production is a physiological barrier that results in pollen tube abnormalities and pollen tube arrest in the pistil. The amount of pollen tube inhibition has been shown to increase with increasing taxonomic distance

between parents (POTTS *et al.* 2000). This broad trend for expression of the F_1 hybrid-inviability to increase with increasing taxonomic distance between parents has been demonstrated by comparing the percentage of normal progeny as a percentage of the seed sown. Intraspecific crosses of *E. ovata* and *E. globulus* gave 80 % and 84 % normal progeny respectively, however the hybrid *E. ovata x E. globulus* yielded only 41 % normal progeny (LOPEZ *et al.* 2000). Many intersectional F_1 hybrids within *Symphyomyrtus* are unsuccessful or exhibit high levels of inviability, although elite selections can be obtained (Table 2.8) (GRIFFIN *et al.* 2000). Abnormal phenotypes are more often encountered when crossing species from the section *Maidenaria,* with crosses between species from the sections *Exsertaria* and *Transversaria* being atypically successful (POTTS *et al.* 2000, DE ASSIS 2000).

Table 2.8: The proportion of viable F_1 hybrid combinations between *Symphyomyrtus* species from the three main sections used in forestry. The table shows the percentage (n) of species combinations for which no viability problems have been reported in manipulated crosses (POTTS *et al.* 2000).

Section	*Maidenaria*	*Exsertaria*	*Transversaria*
Maidenaria	80 % (27)	0 % (3)	9 % (11)
Exsertaria		100 % (5)	91 % (11)
Transversaria			100 % (7)

2.8 Seed Production

A common strategy in tree breeding is the establishment of species and provenance trials, followed by the selection of desirable individuals and the ultimate establishment of seed orchards from these trials. Seed orchards are by design a function of three main factors: level of domestication; time to production

and cost. Various types of orchards are used to produce seed for commercial deployment, each with its own level of genetic improvement over an agreed unimproved base (Table 2.9) (GRIFFIN 2001).

Table 2.9: Orchard options and levels of genetic improvement.

Code	Orchard Type	Level of Improvement	Species	Percentage Volume Gain	Reference
SPA	Seed Production Area	1	*E. nitens* *E. globulus*	4.9 5.0	JONES and CLARKE (2000); SIERRA (2001)
SSO	Seedling Seed Orchard	2	*E. globulus*	8.5	SANHUEZA and GRIFFIN (2001)
BSO	Breeding Seed Orchard	2	*E. dunnii*	9.0	JONES and CLARKE (2000)
CSO	Clonal Seed Orchard	3	*E. globulus* *E. globulus* *E. nitens* *E. globulus*	14.4 20.0 10-20 10-20	SANHUEZA and GRIFFIN (2001); SIERRA (2001); BOOMSMA *et al.* (2001); BOOMSMA *et al* (2001)
MCPO	Mass Controlled Pollinated Orchard	4	*E. globulus* *E. globulus*	26.0 25-35	SANHUEZA and GRIFFIN (2001); BOOMSMA *et al* (2001)

1 = lowest level of improvement; 4 = highest level of improvement

There are two main objectives with the deployment of genetic gain from managed orchards. Firstly, to ensure regular and sustained production and secondly, demonstrable gains relative to the level of improvement. There are however biological limitations to seed production, such as location, the diversity of flowering times, flowering intensity, diversity of pollen vectors and gene flow (MONCUR and BOLAND 2000).

In open pollinated seed orchards the ratio of viable seed produced relative to available ovules is low. Compared with open pollination, controlled pollination increases the number of seeds formed in each capsule (Table 2.7). This is a clear indication that insufficient pollen is reaching the stigma (MONCUR *et al.* 1995), or that foreign pollen or selfing is causing low seed set. Seed orchards

based on a number of provenances and families may comprise trees exhibiting a range of flowering thus resulting in poor synchronisation of pollination amongst trees. In *E. globulus* growing in northwest Tasmania, flowering occurred over five months with no pollen transfer between early and late flowering trees (MONCUR and BOLAND 2000). According to GROSSER *et al.* (2001) the parental contribution to progeny varied amongst clones in *E. nitens* suggesting that panmictic pollinations are not occurring. This may be due to differences in flowering times subjecting each clone to a different pollen pool. Distance between clones is important as the highest paternal contribution on a tree usually comes from the nearest neighbour. Similarly MONCUR and BOLAND (2000) suggest that if flowering intensity in the canopy is too high, genetic influence on neighbouring females will negate the benefits of sophisticated orchard designs. In a study by PATTERSON *et al.* (2001) differences in the rate of outcrossing between the top and the bottom of the canopy in *E. globulus* spp. *globulus* was significant. Trees with a tendency to be self-compatible consistently produce lower levels of outcrossed seed at the bottom of the canopy. Outcrossing rate ranged from 27-66 % at the bottom compared to 74-90 % at the top of the tree. The implication of this finding is that collection of seed for deployment where it is most accessible, may also be the most inbred.

Viable seed represents the dormant phase between generations. Extracted seed usually consists of a mixture of fertile seed and chaff (unfertilized ovules). In *E. alba*, a member of the *Symphyomyrtus*, fertile seed was produced towards the bottom of the placenta with two types of chaff, the elongated and cubic forms originating from different positions in the placenta region. By contrast members of the *Monocalyptus* produce fewer and less variable structures, with viable seed developing from the lowermost ovules (BOLAND *et al.* 1980).

The proportion by weight of viable seed in a seed lot may vary greatly from species to species, orchard location and from season to season (Table 2.10). Separating pure seed from chaff becomes a problem when the seed and chaff

have similar weights, sizes and colour, particularly with members of the *Monocalyptus* group.

Table 2.10: The percentage by weight of viable seed relative to total seed processed.

Species	Percentage Viable Seed	Location	Reference
E. diversicolor	16	Australia	BOLAND *et al.* (1980)
E. setosa	24	Australia	BOLAND *et al.* (1980)
E. globulus spp. *bicostata*	20	Australia	BOLAND *et al.* (1980)
E. torelliana	84	Australia	BOLAND *et al.* (1980)
E. grandis	20	South Africa	HODGSON (1975)
E. camaldulensis	40	Argentina	YACUBSON (1961)
E. viminalis	10	Argentina	YACUBSON (1961)

According to GROSE and ZIMMER (1958) most eucalypts should yield 3 - 20 % clean seed of the total collected. Separating the seed from the chaff is often difficult. Complete separation can be achieved with species such as *E. citriodora* and *E. maculata.* However in species such as *E. grandis* and *E. saligna* it is much more difficult. Loss of the very smallest seed in the sieving process is not serious because of slow germination and low vigour (BOLAND *et al.* 1980). Similarly WATSON *et al.* (2001) found that the larger *E. globulus* spp. *globulus* seed gave a significantly higher germination percentage and rate. HODGSON (1976a) found strong correlations between the number of seed per capsule and germination time, the latter was longer when there were many seeds per capsule (as after full-sib crossing), compared with capsules having few seeds (as in selfing). While grading seed into various classes is essential for automated sowing systems and good crop management, the question remains as to which grades of seed represent the highest genetic worth?

Chapter 3

FLOWERING OF THE EUCALYPTS

3.1 Introduction

Domestication of the eucalypts as a method to capture genetic gain has advanced over a number of years through well structured breeding programmes, implementation of new techniques and applied technologies. Major impediments remain when breeding with commercially important species due to a lack of knowledge regarding their reproductive sequences (MONCUR and BOLAND 2000). In South Africa species such as *E. dunnii*, *E. nitens*, *E. smithii* and *E. macarthurii* contribute not only volume but also specific qualities in the fibre supply chain chiefly for the pulp market.

In their natural environment, eucalypts usually flower within distinct seasons. This pattern is often upset when they are grown in exotic environments with little or no correlation with the site of origin (BROOKER 1985; SEWARD 1986). Flowering of individuals within a species at one locality generally occurs at the same time each year although some *Eucalyptus* species can flower erratically (GRIFFIN 1980). Variations in the time of flowering within a species have been associated with genetic control, seasonal delays at cooler and higher elevations and differences in soil types (LAW *et al.* 2000; BARBOUR *et al.* 2000). According to TIBBITS (1989) consistent flowering of *E. nitens* from various provenances suggest strong genetic control of flowering at least at the population level. Similarly BURROWS and BURROWS (1992) demonstrated that flowering was consistent from one year to the next with species such as *E. populnea* and *E. moluccana*. Large differences in peak flowering have been reported for *E. globulus* spp. *globulus* with no overlap between sub-races, having narrow

sense heritabilities of between 0.3 and 0.9 at both the sub-race and family level (APIOLAZA *et al.* 2001).

Eucalyptus macarthurii produces flowers before year five and any worthwhile seed before year 10. This has however, not been observed in species such as *E. nitens*, *E. dunnii* or *E. smithii*. Problems of biennial bearing or irregular flowering and high capsule abortion, characteristic of many eucalypt species, limit seed supply and restrict tree-breeding objectives. It is also difficult to vegetatively propagate these species by means of cuttings. For this reason, seed currently remains the most viable option for commercial establishment (JONES *et al.* 2000). The complex interaction between the extremes of both temperature and moisture at any particular site and genetic inheritance contribute to the expression of the reproductive phases in eucalypts.

The major objectives were therefore to identify the peak flowering windows for each species and measure the variation in flowering responses from one season to the next at the provenance level. Furthermore, to classify the developmental stages by species and determine the time required to reach seed maturity. Development of floral structures were monitored from the initiation of the inflorescence bud to the production of the hard woody capsule containing the seed. Based on the observations of the floral biology, reproductive sequence models were developed for *E. nitens*, *E. dunnii*, *E. smithii* and *E. macarthurii* in comparison to the subtropical *E. grandis* species.

3.2 Material and methods

3.2.1 Plant materials

All trials were conducted in clonal (grafted) orchards of *E. nitens*, *E. dunnii*, *E. macarthurii*, *E. smithii* and *E. grandis* planted at the Shaw Research Centre (SRC) in KwaZulu-Natal, South Africa. The orchards are situated at 29 ^{0} 29 '

South, 30 ^{0} 11 ' East at 1100 m above sea level. The climate is cool (MAT 16.7 ^{0}C) with a January mean monthly maximum of 25.8 ^{0}C and a July minimum of 4.4 ^{0}C. An estimated mean annual rainfall of 998 mm and median annual rainfall of 899 mm has been reported (PALLETT and MITCHELL 1993). The breeding populations for all species are made up of open-pollinated families from selections made in the South African land-race and from provenances in the natural range in Australia. The orchards have been established on level terrain at a wide espacement of 4x6 m. Each species was represented by varying numbers of clones and ramets per clone randomly established throughout the orchards.

3.2.2 Species descriptions

Eucalyptus nitens (Deane and Maiden) is a tall forest tree that has a good form, smooth bark throughout with a short basal stocking. The juvenile leaves are opposite, sessile, amplexicaul and ovate. Adult leaves are alternate, petiolate and lanceolate. The inflorescence is simple, axillary, 7-flowered with angular and somewhat flattened peduncles. Capsules are sessile, ovoid and often faintly ribbed (BOLAND *et al.* 1989). *Eucalyptus macarthurii* (Deane and Maiden) is a medium-sized tree with coarse, fibrous bark. The juvenile leaves are opposite, sessile, amplexicaul and lanceolate. Adult leaves are alternate, petiolate and narrow-lanceolate. The inflorescence is simple, axillary, 7-flowered with angular peduncles. Capsules are sessile or very shortly pedicellate and more or less hemispherical (BOLAND *et al.* 1989).

Eucalyptus dunnii (Maiden) is a medium-sized to tall forest tree with a rough brownish and flaky bark. The juvenile leaves are opposite, slightly alternate, petiolate, ovate or cordate. Adult leaves are alternate, petiolate and narrow-lanceolate. The inflorescence is simple, axillary, 7-flowered with flattened peduncles. Capsules are pedicellate and more or less hemispherical (BOLAND *et al.* 1989). *Eucalyptus smithii* (R T Baker) is a medium-sized forest tree with a

grey to dark brown bark. The juvenile leaves are opposite, sessile, amplexicaul and lanceolate. Adult leaves are alternate, petiolate and narrow-lanceolate. The inflorescence is simple, axillary, 7-flowered with angular or flattened peduncles. Capsules are pedicellate, globular or ovoid (BOLAND *et al.* 1989).

Eucalyptus grandis (Hill ex Maiden) is a tall to very tall forest tree with a smooth greyish to white bark with a short flaky stocking at the base. The juvenile leaves are alternate, petiolate and ovate. Adult leaves are alternate, petiolate and lanceolate. The inflorescence is simple, axillary, 7 to 11-flowered with flattened peduncles. Capsules are sessile or very shortly pedicellate, pyriform and slightly contracted at the rim (BOLAND *et al.* 1989).

3.2.3 Climate

Climatic conditions at the survey site (SRC) were collected by an automatic weather station from which the mean maximum and mean minimum temperature were calculated. Furthermore the actual daily rainfall was collected and mean monthly rainfall determined for the years 1993 to 2001. Understanding the effects of climate at a particular location are vital in order to correctly site future orchards and give breeders the opportunity to manipulate the onset of flowering.

3.2.4 Flowering assessments

Seasonal flowering times for *E. nitens*, *E. dunnii*, *E. smithii* , *E. macarthurii* and *E. grandis* were evaluated in clonal grafted orchards located at the site described above. All flowering observations were conducted on grafted, chemically induced (paclobutrazol applied as a soil drench) trees with the exception of *E. macarthurii*, which flowers adequately without chemical stimulation. Flowering patterns in some eucalypts grown in an exotic environment can vary appreciably from those of the same species growing in their natural habitat. It is therefore not advisable to use information emanating from studies in other countries, but rather

to determine flowering patterns independently (SEWARD 1986). With this in mind, a standardized scoring system was developed in order to build up reliable flowering records for various commercial species. The scoring system refers to the stage of individual umbels in the reproductive cycle, and is used to indicate the stage of advancement to maturity, of the inflorescence and capsules (JONES *et al.* 2001) (Table 3.1). For the purposes of evaluating the flowering in the orchards, the assessments were focussed on the most important buds (Stage 3 - operculum has fallen off, flower filaments and stigma are exposed). The percentage of the canopy in Stage 3 was evaluated every two weeks over three flowering seasons (Table 3.2).

Table 3.1: The reproductive stage scoring system.

Score	Description
Stage 1	Newly emerged umbels, with involucral bracts intact
Stage 2	Bracts have been shed, operculum protecting floral parts still intact
Stage 3	Operculum has fallen off, flower filaments and stigma are exposed
Stage 4	Floral parts have abscised, capsules have formed

Investigations were also conducted into the time required for development from Stage 1 to Stage 2 buds for all species. This experiment was conducted to determine whether there were differences between species, as all species under test produced Stage 1 buds within a narrow window, usually associated with the first spring flush.

Table 3.2: The percentage of the canopy in Stage 3 phase of development.

Score	Description
1	< 25 % of the buds at Stage 3
2	25 – 50 % of the buds at Stage 3
3	50 – 75 % of the buds at Stage 3
4	> 75 % of the buds at Stage 3

3.2.5 Data analysis

Where required, an ANOVA was applied or a two-way contingency Chi–squared table tests was used to evaluate the statistical significance of independence and t-tests were conducted on angular transformed data using the GENSTAT Statistical Programme.

3.3 Results

3.3.1 Flowering stage changes

With the appearance of the first Stage 1 buds in the orchards, at the ends of branches following the spring flush, (Fig. 3.1) an experiment was designed to evaluate the difference in the time required, in days, for the development from Stage 1 to Stage 2 (Table 3.1) for the species and clones within species. The experiment consisted of five species with three clones per species and 4 replications. The results indicated that *E. nitens* was significantly ($p<0.001$) slower than all other species, requiring up to 56.28 days for the inflorescence to develop from Stage 1 into to Stage 2. No significant difference could found between *E. smithii* and *E. macarthurii* both of which had the fastest development of 8.6 and 10.9 days respectively. *Eucalyptus dunnii* and *E. grandis* performed similarly. However, they were significantly different from the other three species requiring 19.51 and 25.50 days respectively (Table 3.2).

No significant differences could be found between the clones of *E. macarthurii*, *E. smithii* and *E. grandis*, for development time, in days, to form Stage 2 buds. However, there were significant differences ($p<0.05$) between the clones of *E. dunni* and *E. nitens*. Clone 3 in the *E. nitens* set was significantly different from clones 1 and 2. Similarly, in *E. dunnii*, clone 1 was significantly different from both clones 2 and 3 (Fig. 3.3).

Figure 3.1: Typical *E. nitens* Stage 1 buds in the process of bract-shed to form Stage 2 buds.

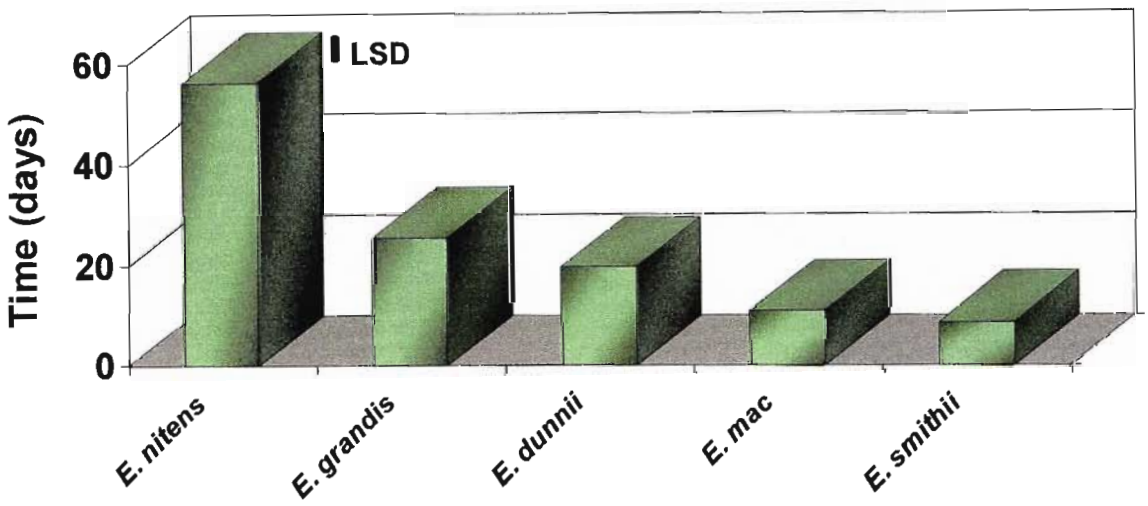


Figure 3.2: The development time in days from Stage 1 to Stage 2 buds for five eucalypt species at 1100 m above sea level, the climate is cool (MAT 16.7 ^{0}C) with a January mean monthly maximum of 25.8 ^{0}C and July minimum of 4.4 ^{0}C.

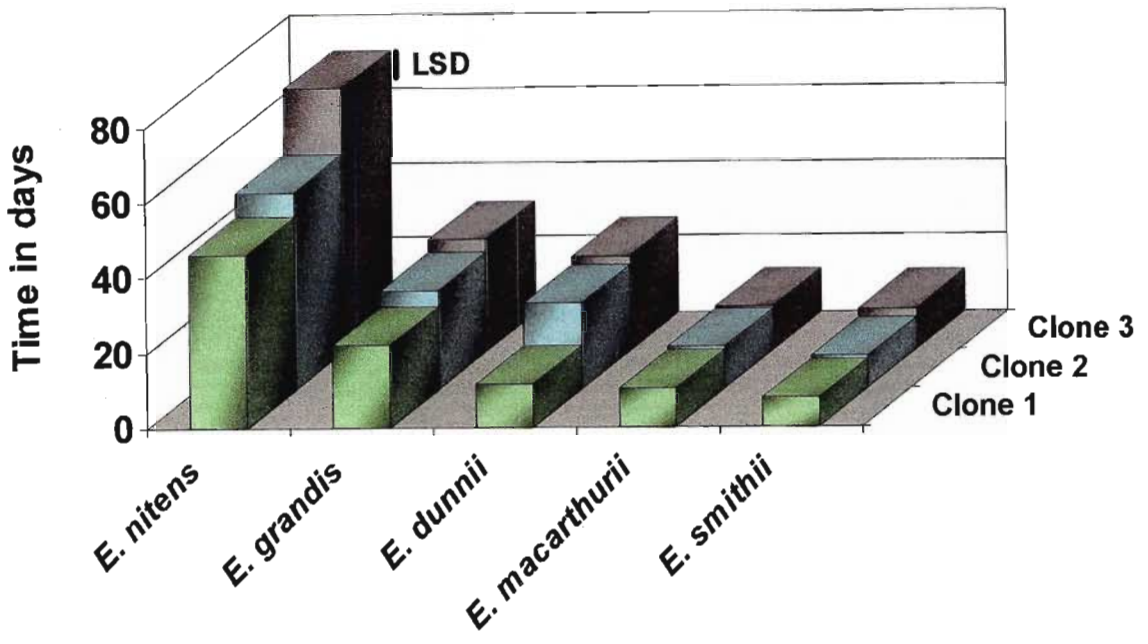


Figure 3.3: The floral development time in days from Stage 1 to Stage 2 of three clones from five eucalypt species located at 1100 m above sea level. The climate is cool (MAT 16.7 ^{0}C) with a January mean monthly maximum of 25.8 ^{0}C and July minimum of 4.4 ^{0}C.

3.3.2 Peak flowering times by species and seasonal effects

Climate is a fundamental determinant of the distribution of most terrestrial organisms and is measured using standardized techniques at thousands of localities in all parts of the world. From the survey data for all species in this study it is evident that the onset of Stage 3 flowering is under both strong genetic and environmental control. There was a highly significant ($p<0.001$) (327 $df = 33$) association between the months of flowering and individual species, consistent year on year flowering peaks for all the species (Fig. 3.5). In most cases there was a decline in the number of individual clones that produced buds (from one year to the next) due to the reduced effectiveness of paclobutrazol. It was evident that seasonal differences did not impact on the peak flowering time for the individual species.

More specifically if we consider *E. nitens* one of the most problematic flowering species, with few sites suited for growth in South Africa. A bioclimatic analysis of *E. nitens* estimates that this species grows where the approximate rainfall is 745 mm in southern New South Wales (SNSW) and in northern New South Wales (NNSW) where the estimated annual rainfall is 2160 mm. The mean annual rainfall for the whole natural range is 1269 mm and only 20 % of localities receive less than 843 or more than 1685 mm. In all provenances, the estimated average rainfall in the driest month amounts to about 5 % of the annual total (RICHARDSON and McMAHON 1991). Using the mean annual rainfall for the natural range (1269 mm), the rainfall during the driest month is estimated at 85 mm which is more than the accumulative total of the three driest months (June to August) at the survey site (Fig. 3.4).

Although *E. nitens* is grown successfully in South Africa as a commercial species, the bioclimatic analysis revealed no homoclimes in these regions. The most restrictive indices were driest month precipitation, driest quarter precipitation and coldest quarter precipitation. None of the potential *E. nitens*-growing areas in Southern Africa received between 39 and 91 mm of rainfall during the driest month, between 135 and 317 mm during the driest quarter or between 144 and 558 mm during the coldest quarter (RICHARDSON and McMAHON 1991). Sites where *E. nitens* is grown in South Africa are also much warmer, with an annual mean temperature of 15.1 °C versus 9.6 °C in the natural range. Similarly, higher annual mean maximum temperatures of 21.6 °C versus 15.1 °C, higher annual mean minimum temperatures of 8.6 °C versus 4.1 °C and higher average minimum temperatures for the coldest quarter, of 2.4 °C versus – 1.1 °C were recorded. These difference highlight the importance of locating sites as close to that of the natural range to improve the possibilities of flowering in shy flowering species such as *E. nitens*.

Environmental conditions that favour flower induction in *E. nitens* are cold winters followed by a warm spring, with spring and summer temperatures not exceeding

25 °C, or development is too rapid with flowering commencing in winter (MONCUR and BOLAND 2000). At the survey site flower buds appear from November, with bract-shed occurring 8-12 weeks later and flowering commencing from as early as the latter half of April through to October on a range of clones from different provenances. Similarly, in Canberra ACT at an elevation of 550 m above sea level flower buds appear in December, bract-shed occurs 6-8 weeks later, and flowering commences in May. In contrast, at Tallaganda NSW, a cooler site, 1000 m above sea level flower buds appear in late December, bract–shed is delayed until October and flowering occurs the following January (MONCUR and BOLAND 2000). From observations at altitudes that exceed 1500 m above sea level, further north of the survey site, between latitudes 25 °S and 26 °S, flowering extends into November with the peak during late winter and early spring. Increase in altitude delayed the onset of flowering across a range of sites in Tasmania (MONCUR and BOLAND 2000). At the survey site bract-shed for species such as *E. smithii* and *E. dunnii* was rapid relative to *E. nitens*, requiring 2-4 weeks opposed to 6-8 weeks. One would expect the development from Stage 1 to Stage 3 flowering for *E. smithii* and *E. dunnii* to continue fairly rapidly given the accumulated heat sums however the flower buds of these two species enter dormancy and only open 16-18 months later (Table 3.8). On the other hand, *E. nitens* begins to flower 3-5 months following bract-drop depending on the provenance and clone.

3.3.3 Reproductive sequence models

Eucalyptus dunnii clones flowered (Stage 3) from late February to May (Fig. 3.5). These results were comparable to the findings of BLAKELY (1965) and BOLAND *et al.* (1980) who observed that flowering in the natural range occurs from March to May. The Acacia creek and South Yabra provenances *of E. dunnii* flower over a shorter period (two months) as opposed to the Oaky Creek, Boomi Creek and Dead Horse Track provenances.

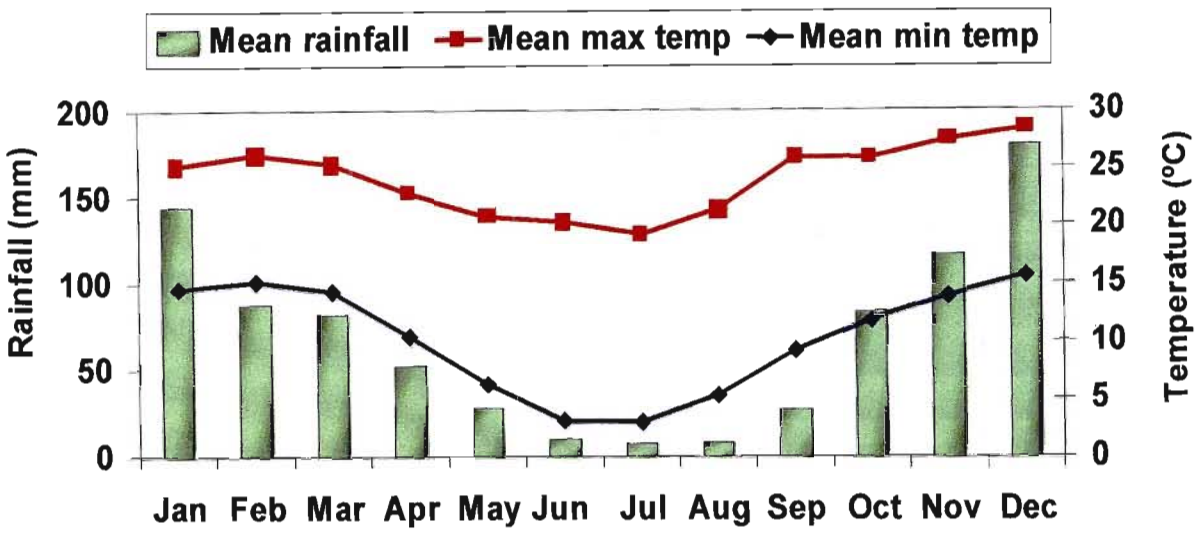


Figure 3.4: The mean maximum and minimum monthly temperatures and rainfall for the period 1993 - 2001 at the SRC, South Africa.

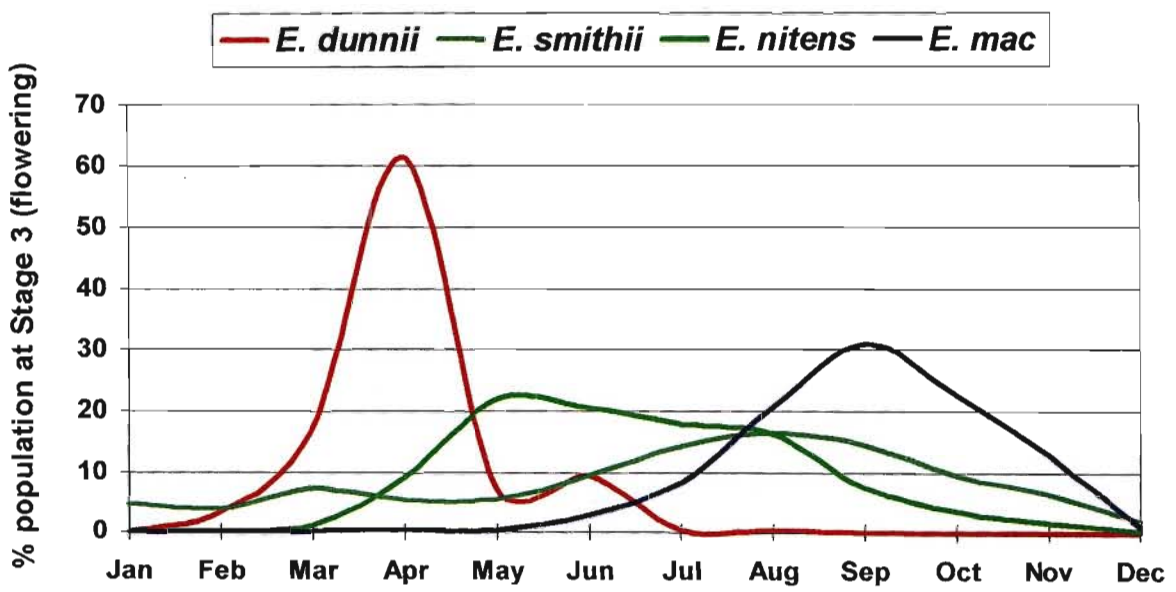


Figure 3.5: The peak Stage 3 flowering times for *Eucalyptus dunnii*, *E. macarthurii*, *E. smithii* and *E. nitens*. There was a significant association ($p<0.001$) between the peak flowering months and the different species.

The limited variation in flowering time could be ascribed to the restricted occurrence of *E. dunnii* in Australia where the original material was collected (28 ° 23 ' to 28 °36 ' South and 152 ° 20 ' to 152 ° 42 ' East).

From the observations striking differences were apparent between *E. dunnii* and *E. grandis*. In the case of *E. dunnii* the development time from Stage 2 to Stage 3 extended for as long as 15-18 months (Table 3.3). *Eucalyptus macarthurii* flowers were observed from June to early December with peak flowering in August to September. According to BEADLE *et al.* (1972); PRYOR (1962) and BOLAND *et al.* (1980) flowering of *E. macarthurii* occurs from February to April in the natural range, representing a considerable difference to that observed at the survey site (Fig. 3.5).

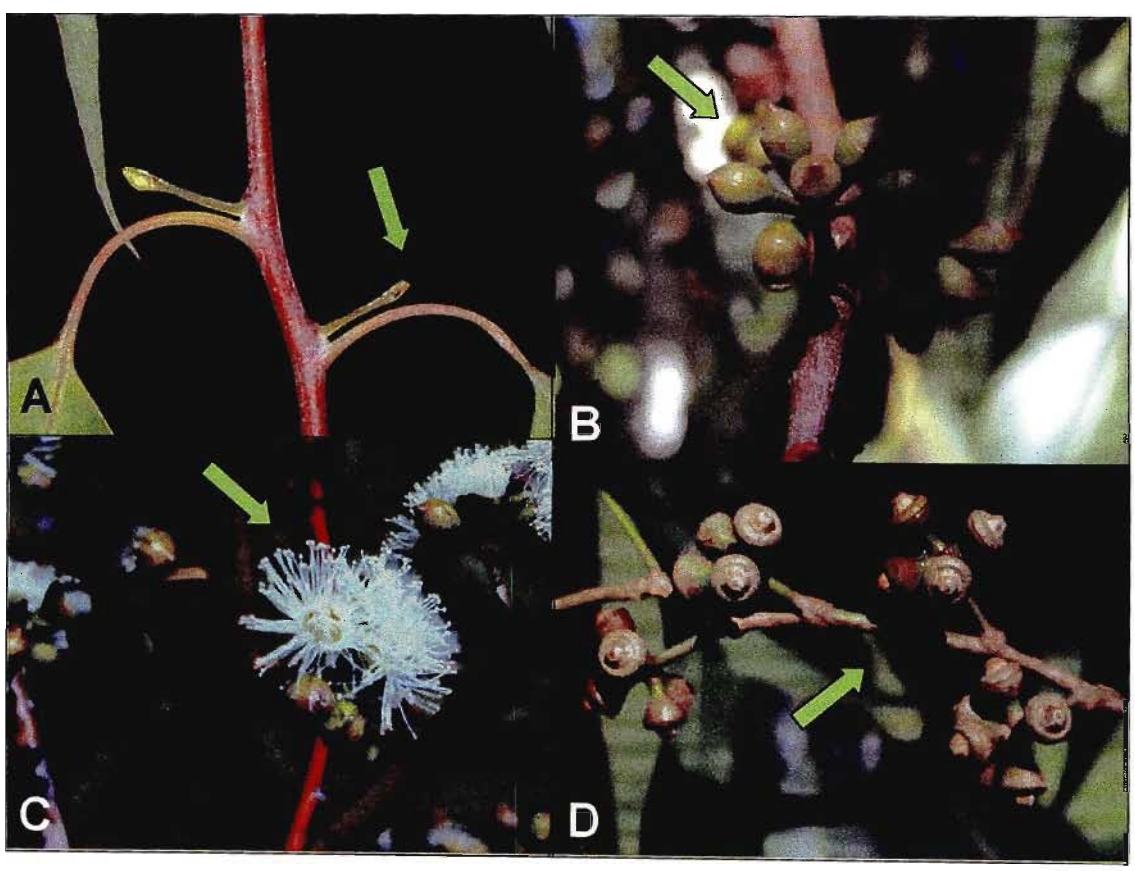
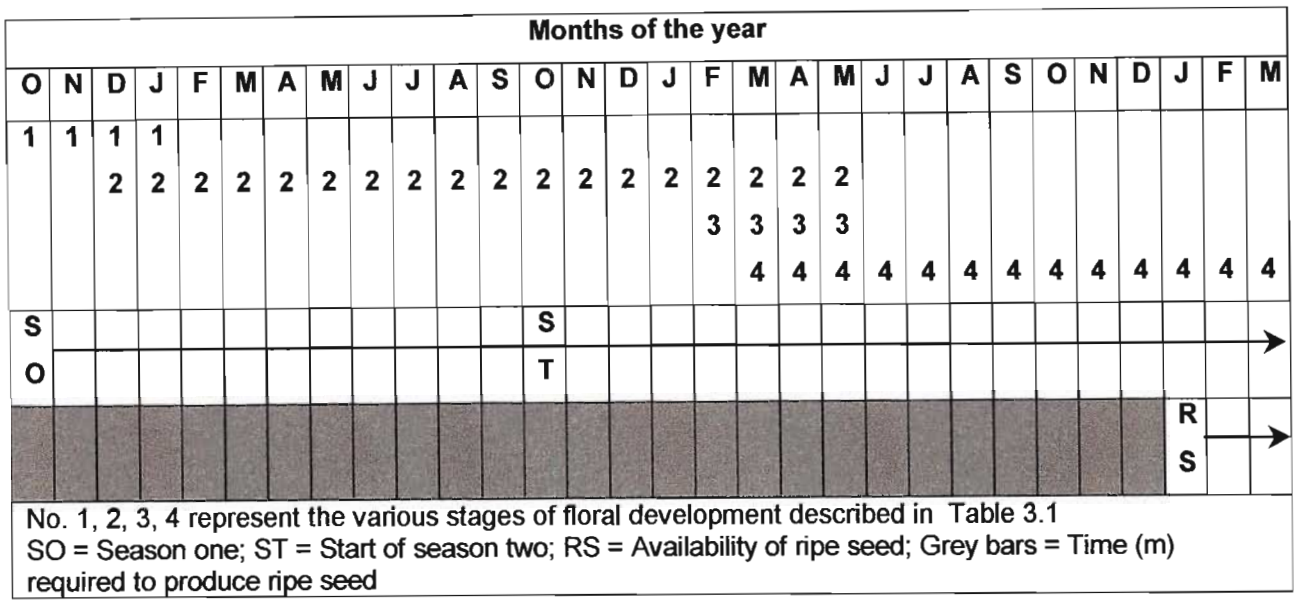


Figure 3.6: The reproductive sequence of *E. dunnii* (A) Stage1 (B) Stage 2 (C) Stage 3 and (D) Stage 4.

Table 3.3: The reproductive sequence of *E. dunnii*.



No. 1, 2, 3, 4 represent the various stages of floral development described in Table 3.1
SO = Season one; ST = Start of season two; RS = Availability of ripe seed; Grey bars = Time (m) required to produce ripe seed

The reproductive sequence model for *E. macarthurii* is short in comparison to that of *E. dunnii* due to a short Stage 2 bud phase, which extends from 8-12 months, representing a difference of approximately 6 months (Table 3.6).

Eucalyptus smithii flowers were observed from late winter to early summer, from July to October when the main flowering peak occurs. This is earlier than the peak flowering from January to March, in the natural range (BEADLE *et al.* 1972; GOODMAN 1973 and BOLAND *et al.* 1980) (Fig 3.5). Stage 3 flowering does however occur throughout the year depending on provenance and clone. Four groups have been identified based on flowering patterns and provenances have been allocated accordingly (Table 3.4). There is no obvious link between the origin of the parent material and the expression of flowering at the survey sight, however altitudes of below 500 m above sea level were common for all sites in group 1 and altitudes above 650 m above sea level were common for group 4. From the three year survey data the *E. smithii* flowering was consistent from one year to the next.

Table 3.4: Four flowering groups identified across the provenances of *E. smithii*.

Provenance	Location		Altitude (m above sea level)	Flowering habit
	Latitude (S)	Longitude (E)		
Group 1				
Bega	36 ° 35 '	149 ° 52 '	360	Flowering occurs from July to December with a definite break from January to June when little or no clones produce Stage 3 flowers
Mnt Dromedary	36 ° 18 '	150 ° 00 '	450	
Larry's Mountain	35 ° 51 '	150 ° 01 '	260	
Robertsons	34 ° 28 '	150 ° 41 '	500	
Group 2				
Tallaganda	35 ° 23 '	149 ° 37 '	950	Flowering occurs from March to September with a definite break from October to February when little or no clones produce Stage 3 flowers
Group 3				
Towamba	37 ° 05 '	149 ° 47 '	220	Flowering occurs with sporadic peaks in March and September
Group 4				
Wingello	34 ° 42 '	150 ° 12 '	700	Flowering occurs throughout the year with peaks in March and September
Wombeyan Road	34 ° 21 '	150 ° 10 '	650	
Nerriga	35 ° 05 '	150 ° 07 '	680	
Belimba Fire Trail	36 ° 04 '	149 ° 41 '	1040	

Eucalyptus smithii not only produces Stage 3 flowers over an extended period, but has an extended Stage 2 bud phase, in the order 15 –18 months, before becoming a mature Stage 3 flower. The reproductive sequence model for *E. smithii* is similar to that of *E. dunnii* due to the extended Stage 2 bud phase (Table 3.7).

Eucalyptus nitens flowers were observed from March to October (Table 3.8) with a few clones flowering as late as December. Flowering of *E. nitens* can be classified into three main groups of short medium and long, based on the flowering patterns of clones within provenances (Table 3.5). The provenances of

Tallaganda and Badja have been classified as short flowering, as the stage 3 is restricted to the period between April and September. The more northerly provenances of Barrington Tops, Ebor and Barren Mountain consisted of clones, which displayed a mediun flowering pattern from March to October. The final group consists of selected parent trees from the local landrace and displays a long flowering pattern from February to December. Irrespective of origin or provenance the peak flowering period remains consistent from one year to the next (Fig. 3.5). Flowering occurs during the winter months with main flowering peak from May to July when conditions are harsh with low rainfall and low temperatures. According to BEADLE *et al.* (1972), GOODMAN (1973), BOLAND *et al.* (1980) and TIBBITS (1989) flowering from January to March in its natural range which represent a considerable difference.

Table 3.5: Three flowering groups (short, medium and long) based on flowering patterns observed at a provenance level for *E. nitens*.

Provenance	Origin of parent material Location		Altitude (m above sea level)	Flowering habit Stage 3
	Latitude (S)	Longitude (E)		
Short				
Badja	36 ° 00 '	149 ° 36 '	900-1300	Flowering occurs from
Tallaganda	35 ° 48 '	149 ° 31 '	1280-1450	April to September
Jessievale*	26 ° 14 '	30 ° 31 '	1750	
Medium				
Barren Mountain	Unknown	Unknown	Unknown	Flowering occurs from
Barrington Tops	31 ° 55 '	151° 30 '	1450	March to October
Ebor	30 ° 23 '	152 ° 28 '	1400-1600	
Long				
Kalmoesfontein*	25 ° 19 '	30 ° 28 '	1700	Flowering occurs with
Gowan Brae*	29 ° 38 '	30 ° 08 '	1500	February to December

* Local land race provenance not known

Figure 3.7: The reproductive sequence of *E. macarthurii* (A) Stage1 (B) Stage 2 (C) Stage 3 and (D) Stage 4.

Table 3.6: The reproductive sequence of *E. macarthurii.*

Months of the year																												
O	N	D	J	F	M	A	M	J	J	A	S	O	N	D	J	F	M	A	M	J	J	A	S	O	N	D	J	F	M
1	1	1	1																										
		2	2	2	2	2	2	2	2	2	2	2	2																
								3	3	3	3	3	3	3															
									4	4	4	4	4	4	4	4	4	4	4	4									
S												S								→								
O												T																
																				R	→							
																				S								

No. 1, 2, 3, 4 represent the various stages of floral development described in Table 3.1
SO = Season one; ST = Start of season two; RS = Availability of ripe seed; Grey bars = Time (m) required to produce ripe seed

Figure 3.8: The reproductive sequence of *E. smithii* (A) Stage1 (B) Stage 2 (C) Stage 3 and (D) Stage 4.

Table 3.7: The reproductive sequence of *E. smithii.*

Month of the year																												
O	N	D	J	F	M	A	M	J	J	A	S	O	N	D	J	F	M	A	M	J	J	A	S	O	N	D	J	F	M
1	1	1 2	1 2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2 3	3 4	3 4	3 4	3 4	3 4	4	4	4	4
S O												S T																→
																											R S	→

No. 1, 2, 3, 4 represent the various stages of floral development described in Table 3.1
SO = Season one; ST = Start of season two; RS = Availability of ripe seed; Grey bars = Time (m) required to produce ripe seed

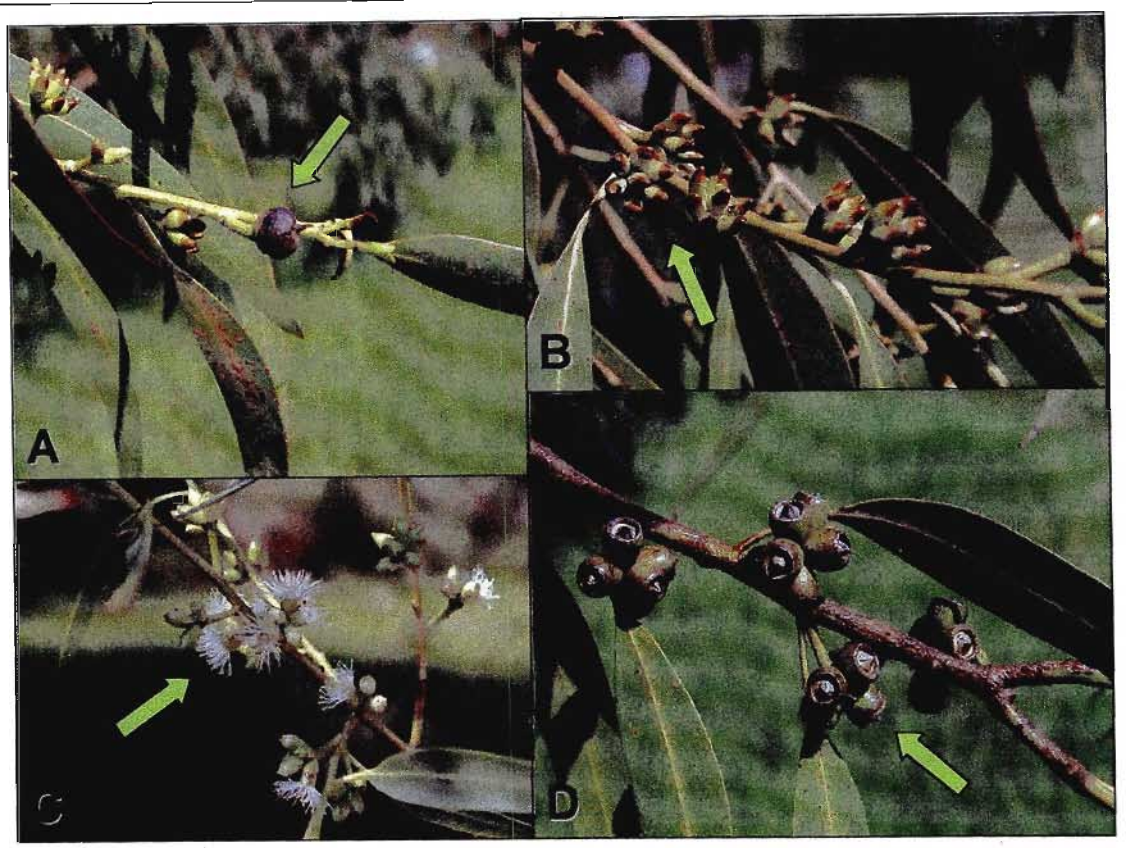


Figure 3.9: The reproductive sequence of *E. nitens* (A) Stage 1 (B) Stage 2 (C) Stage 3 and (D) Stage 4.

Table 3.8: The reproductive sequence of *E. nitens*.

Month of the year																												
O	N	D	J	F	M	A	M	J	J	A	S	O	N	D	J	F	M	A	M	J	J	A	S	O	N	D	J	F	M
1	1	1	1	1	1																								
		2	2	2	2	2	2	2	2	2	2	2																	
						3	3	3	3	3	3	3	3																	
							4	4	4	4	4	4	4	4	4	4	4	4	4	4									
S												S											→						
O												T																	
																	R	→	→										
																	S												

No. 1, 2, 3, 4 represent the various stages of floral development described in Table 3.1
SO = Season one; ST = Start of season two; RS = Availability of ripe seed; Grey bars = Time (m) required to produce ripe seed

It is evident that site and location affect the flowering times of the different species as is the case with *E. nitens* flowering in Canberra (550 m elevation), out of its natural range. Flowering started from May onwards in contrast with sites at Tallaganda NSW (1000 m elevation) tending to flower from January (MONCUR and BOLAND 2000). The reproductive sequences of *E. grandis* (Table 3.9) demonstrate the benefits of a short breeding cycle relative to the other species. It is evident that extended breeding cycles will impact on breeding and domestication of certain species (Table 3.10). More specifically *E. smithii* and *E. dunnii* have an extended breeding cycle, two to three times that of *E. grandis*.

The time (in days) from the first flower bud appearance (Stage 1) to flowering and seed maturation (Stage 4) at the survey site is reported in (Table 3.10). According MONCUR and BOLAND (2000) the time it takes for Stage 1 buds to develop into Stage 4 capsules is 346 days and a total of 3126 heat sums for *E. nitens* grown in Canberra ACT. At the survey site heat sums far in excess of those required at the Canberra site were generated, but the breeding cycle for *E. nitens* remained at 546 days. The most striking difference between the sites is the distribution of rainfall with Canberra having a more evenly spread rainfall as opposed to the survey site, which has a distinct dry period from June to August. In Canberra, the 38-year average rainfall for June, July, and August is 46 mm, 46 mm and 49 mm respectively (HALL *et al.* 1981). This is in direct contrast with the observations at survey site where an 8-year average rainfall for June, July, and August is 9.7 mm, 7.6 mm and 8.5 mm respectively.

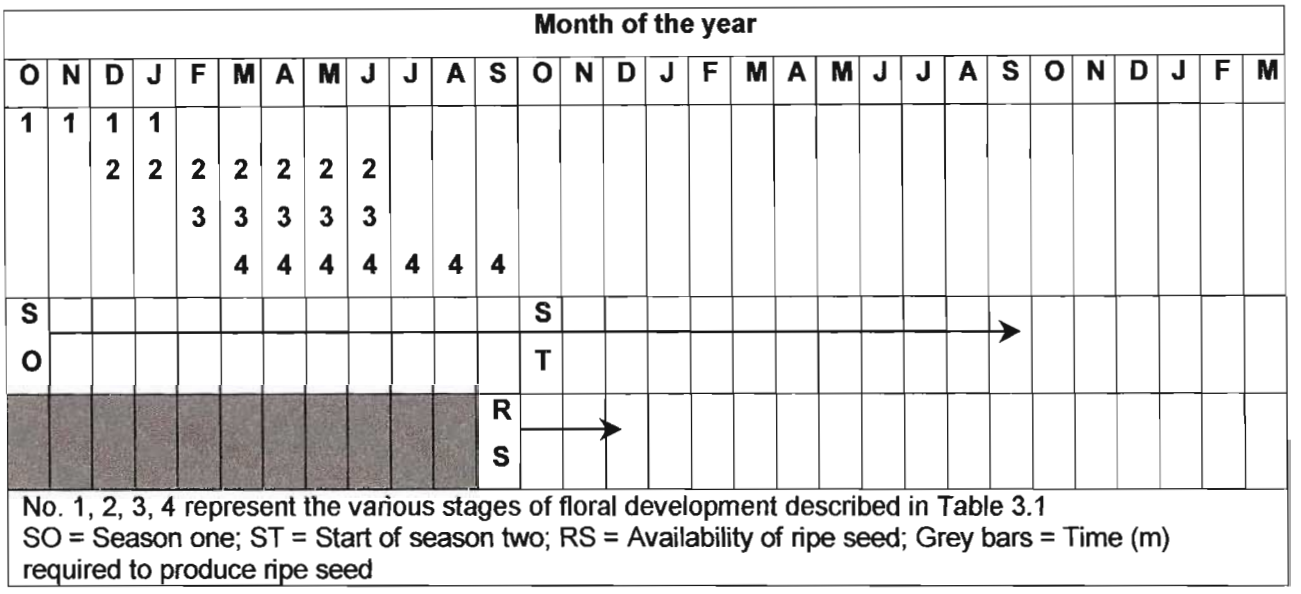
Once flower buds have been induced, non-stress conditions should prevail to ensure maximum bud retention. Moderate temperatures are required at flowering to ensure good pollination. After pollination, an increase in temperature and a non-stressed environment will speed up the seed maturation process in a species such as *E. nitens* (MONCUR and BOLAND 2000). The interaction between site, heat sums and moisture appear to be the important driving factor to shortening the breeding cycle of *E. nitens*. However, this may not be true with

E. dunnii and *E. smithii,* which have early bract-shed but have Stage 2 buds that remain dormant for an extended period from 15-18 months irrespective of an accumulation of heat sums.

Figure 3.10: The reproductive sequence of *E. grandis* (A) Stage1 (B) Stage 2 (C) Stage 3 and (D) Stage 4.

Ripening of the Stage 4 capsules usually takes from 7 to 12 months but differs from one species to the next. In the case of *E. dunnii* and *E. smithii* harvesting occurs 10-12 months after flowering, with *E. macarthurii* and *E. nitens* collections commence 7-9 months following peak flowering.

Table 3.9: The reproductive sequence model of *E. grandis*.



No. 1, 2, 3, 4 represent the various stages of floral development described in Table 3.1
SO = Season one; ST = Start of season two; RS = Availability of ripe seed; Grey bars = Time (m) required to produce ripe seed

Table 3.10: Number of days between the peak development of Stage 1 buds through to Stage 4 capsules and seed maturation for five commercial eucalypts at one site across a number of clones from a range of provenances.

Species	No. of Days		
	From Visible Floral Bud to Flowering (Stage 1 - Stage 3)	From Flowering to Seed Maturity (Stage 3 - Stage 4)	From Visible Floral Bud to Seed Maturity (Stage 1 - Stage 4)
E. dunnii	486-516	245-275	731-800
E. smithii	639-669	243-273	882-940
E. macarthurii	274-304	243-273	517-580
E. nitens	243-273	243-273	486-550
E. grandis	151-181	123-153	274-360

3.4 Discussion

Early and plentiful flowering are the most important factors for tree breeding programmes. The use of paclobutrazol in shy flowering species has enhanced breeding and seed production processes. An early summer, soil drench of paclobutrazol gave the best results in terms of bud production for *E. nitens* (JONES *et al.* 2000). Using this as a basis for application in commercial orchards major increases in seed yields of species such as *E. dunnii* and *E. macarthurii* have been achieved. Early flowering and seed production in shy flowering species such as *E. smithii* and *E. nitens* has also been attained. Furthermore, the flowering times and flowering windows have been clearly defined, giving more structure to the breeding programmes and scheduling of crossing programmes (JONES *et al* .2000).

It is evident that the different species flower consistently from one year to the next during the same period with similar mean flowering peaks. Similarly, TIBBITS (1989) found a good correlation in flowering from one year to the next and for trees within provenances of *E. nitens*, suggesting strong genetic control of flowering at least at the population level. Provenances tend to group together with the most variation at Stage 3 flowering, evident with *E. smithii*.

Environmental factors had the greatest impact on the bud development and flowering at the survey site as *E. nitens, E. dunnii* and *E. smithii* would not flower at an early age or produce viable crops without the use of paclobutrazol. This suggests that environmental conditions for bud production and conditions following are crucial for capsule retention and seed set. The heat sums model proposed by MONCUR and BOLAND (2000) can be applied to *E. nitens* although the heat sums generated do not appear to be a limiting factor. *Eucalyptus dunnii* and *E. smithii* also have dormant Stage 2 buds, extending the breeding cycle 2-3 times that of *E. grandis*. The reason for this extended bud dormancy in Stage 2 is not clearly understood. However, the interaction between the distinct dry and

warm winter days experienced in South Africa could be the limiting factor for *E. dunnii* and *E. smithii.*

It is therefore important to understand the factors that affect the onset of bud production and continued development. With the reproductive sequences established, the variation in flower expression can be correlated with environmental conditions before the onset of Stage 1 buds and followed through to Stage 4 capsules. By manipulation of environmental conditions through cultural practices such as grafting, growth retardants, watering regimes and fertilizer applications, it may be possible to advance or delay the breeding cycle. The correct siting of orchards and design must be both conducive to bud initiation and development but also facilitate good outcrossing between parent trees.

Chapter 4

FLOWER ENHANCEMENT

4.1 Introduction

Enhancement of bud and flower production is not only important for the production of open pollinated and full-sib seed but also in understanding the breeding system and flowering patterns in shy flowering species such as *E. nitens*, *E. smithii* and *E. dunnii.* Eucalypts require a period of stress, induced by low temperatures and or short days, before initiating flower buds (MONCUR 1992). The use of gibberellin (GA) biosynthesis inhibitors such as paclobutrazol has been used to induce precocious and abundant flowering without degrading seed quality (WILLIAMS *et al.* 1999). According to HASAN and REID (1995) the use of paclobutrazol resulted in an increase in the flower bud and final capsule production in *E. globulus,* reducing the generation turnover time significantly.

Paclobutrazol is a broad spectrum, xylem-mobile plant growth retardant that inhibits GA biosynthesis and hence reduces the rate of cell division and expansion with associated shifts in physiological activity. These include the partitioning of carbohydrates and responses to water stress (GRIFFIN *et al.* 1993). Paclobutrazol may be absorbed through the roots or by means of stem injection directly into the xylem (SHEARING and JONES 1986). Paclobutrazol can persist in the soil for several years, as it is generally resistant to chemical and biotic degradation and mass movement (JACKSON *et al.*1996).

4.2 Materials and Methods

4.2.1 Plant materials

Two experiments were conducted in clonal (grafted) orchards planted at the Shaw Research Centre (SRC) in KwaZulu-Natal, South Africa. The orchards are situated at 29 ^{0} 29 ' South, 30 ^{0} 11 ' East at 1100 m above sea level. The climate is cool (MAT 16.7 ^{0}C) with a January mean monthly maximum of 25.8 ^{0}C and July minimum of 4.4 ^{0}C. An estimated mean annual rainfall of 998 mm and median annual rainfall of 899 mm has been reported (PALLETT and MITCHELL 1993).

The first experiment was to determine the effects of paclobutrazol, as a soil drench, on flowering of *E. nitens* grafts. The resulting effects on flower-bud production were evaluated in a clonal grafted orchard as described above. Eight clones of similar age (1 to 2-years-old), originating from a range of commercial stands and progeny trials were used in the study (Table 4.1). The primary aim was to determine the optimum time of application to ensure graft survival and best bud production. The rate of application was 2.5 ml Cultar (250 g l^{-1} paclobutrazol) per centimeter of the collar diameter diluted in 5 ℓ of water. This solution was applied as a soil drench around the base of each tree. The applications were repeated every month on different ramets of the eight clones. Applications were initiated in March of year one until February of year two. Flower bud production was observed at the end of year two in December to January of year three (Table 4.2).

The second experiment was designed to determine the effects of paclobutrazol as a soil drench on the flowering of 14-month-old *E. smithii* and *E. grandis* grafts with a longterm objective of producing open pollinated hybrid seed. The effects of paclobutrazol on flower-bud production were evaluated in the clonal hedge, which consisted of two replications each composed of 3x3 tree square plots represented by one *E. grandis* clone (tree) surrounded by 8 *E. smithii* clones

(trees) in replication one. Similarly, in replication two, one *E. smithii* clone was surrounded by eight *E. grandis* clones. All the clones originated from selections conducted in commercial stands and progeny trials that were used in the study (Table 4.2). The rate of application was 2.5 ml Cultar (250 g l^{-1} paclobutrazol) per centimeter of the collar diameter diluted in 5 ℓ of water. This solution was applied as a soil drench around the base of each tree.

Table 4.1: List of the *E. nitens* clones included in the evaluation of the effects of paclobutrazol on flower induction.

Clone Number	Family	Provenance	Origin
12	32093	Barren Mountain	Dassport E88/03
53	LR A20	Ebor	Kalmoesfontein RP001T
48	LR A15	Ebor	Kalmoesfontein RP001T
63	LR	Not known	Gowan Brae ST001T
61	LR	Not known	Jessievale clone bank
70	LR A12	Ebor	Kalmoesfontein RP001T
9	32091	Badja State Forest	Jessievale E88/01
47	LR A20	Ebor	Kalmoesfontein RP001T

Table 4.2: List of the *E. smithii* and *E. grandis* clones included in the evaluation of the effects of paclobutrazol on flower induction.

Clone number	Species	Clone number	Species
EG45	*E. grandis*	1	*E. smithii*
1361	*E. grandis*	6	*E. smithii*
1362	*E. grandis*	7	*E. smithii*
1369	*E. grandis*	8	*E. smithii*
1376	*E. grandis*	12	*E. smithii*
902	*E. grandis*	20	*E. smithii*

4.2.2 Data analysis

Each experiment was analysed independently using χ^2 – tests to evaluate the statistical significance of treatment-related differences in flowering intensity scores.

4.3 Results

In Experiment one the application of paclobutrazol as a soil drench to grafted *E. nitens* trees (between 1 and 2-years-old) in the clonal orchard resulted in a significant response in bud induction. Historically flowering has not been observed in grafted *E. nitens* clones at this site younger than two years. The appearance of stage one buds and their survival are significantly ($p<0.05$) dependent on the months of application (Table 4.3). Paclobutrazol affected all clones with a visible stunting and shortening of the inter-nodes, in some instances treated clones had poorer survival. It was also evident that the late winter to early summer applications ensured the onset of bud break 12-14 months later during November to January (Fig. 4.1). The apparent failure of paclobutrazol to stimulate bud induction when applied during the late summer and early winter, may possibly be related to a drop - off in metabolic activity, drought and cold stress (Fig. 4.1).

In Experiment two paclobutrazol was applied as a once-off application in October following establishment of the grafts in the seed orchard in February of the second year (Table 4.4). The time after treatment with paclobutrazol (as a soil drench) had a significant effect ($p<0.01$) on bud break with the grafted *E. grandis* clones producing buds in year three while the *E. smithii* clones produced none. This resulted in subsequent flowering of *E. grandis* in year three. Bud-break was not independent of clone ($p<0.01$) with only some clones producing flowers at this early stage.

Table 4.3: Production of Stage 1 buds on clones of *E. nitens* over different months following treatment with paclobutrazol as a soil drench.

Month of Treatment	Original No. of Treated Trees	Surviving Grafts with Buds	Difference
March	8	1	7
April	8	2	6
May	8	3	5
June	8	1	7
July-August	8	3	5
September	8	8	0
October	8	5	3
November-December	8	4	4
January	8	4	4
February	8	5	3

χ^2 20.40404 (*df*=9) ($p<0.05$)

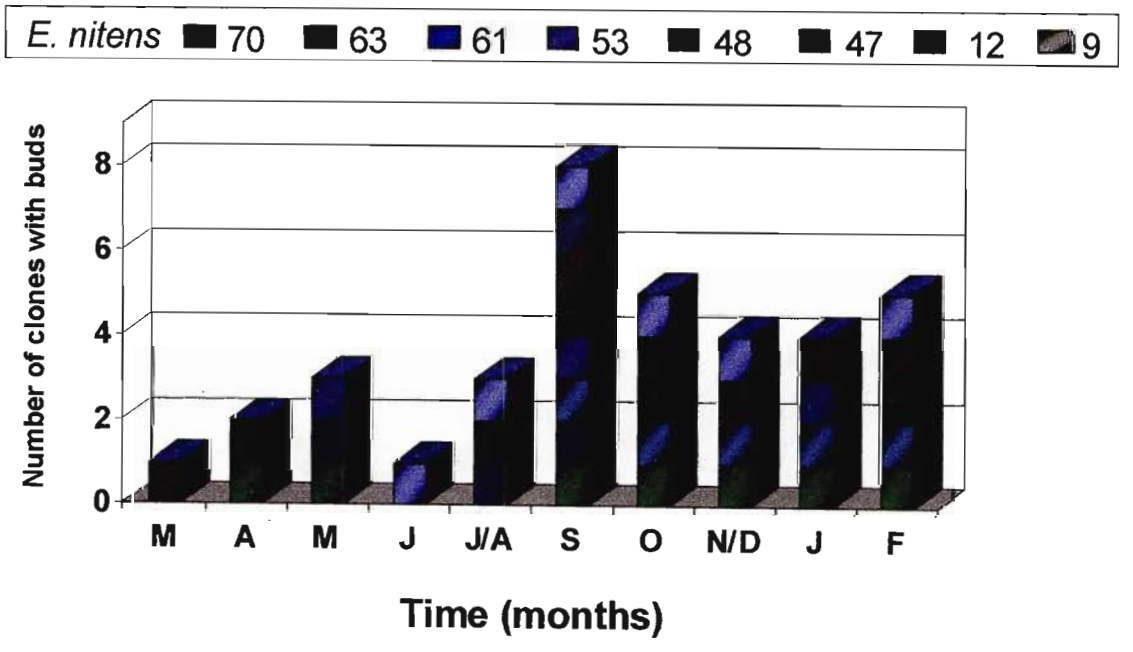


Figure 4.1: The effect of paclobutrazol application, as a soil drench, on flower bud production in 8 clones of *E. nitens* grafted trees. Applications were repeated every month from March in year 1 to February in year 2.

While there was clear evidence of stunting and shortening of the inter-nodes in *E. smithii*, the first Stage 1 buds only appeared in November of year three (Fig. 4.2). Fourteen months after treatment both the *E. grandis* and *E. smithii* clones produced Stage 1 buds with bud-break occurring independently of clone. Every clone, irrespective of the species produced Stage 1 buds.

Table 4.4: The sequence of events required to promote flowering: establishment, treatment and subsequent enhancement of flowering for both *E. grandis* and *E. smithii* clones.

Time of Event	Events Required to Promote Flowering	
	***E. grandis* Clones**	***E. smithii* Clones**
Year 1 (July-Sept)	Selection and grafting	Selection and grafting
Year 2 (Jan)	Establishment	Establishment
Year 2 (Oct)	Application of paclobutazol as a soil drench	Application of paclobutazol as a soil drench
Year 3 (Feb)	Prolific Stage 1 and Stage 2 bud production	No Stage 1 buds produced
	Appearance of buds not independent of clone at 4 months χ^2 19.26954 (*df* =5) **	
Year 3 (Feb-Jun)	Flowering (Stage 3) and capsule formation (Stage 4)	No buds
	Species has a significant effect on the appearance of buds following treatment; χ^2 16.83962 (*df*=1) **	
Year 3 (Nov)	Prolific bud production (Stage 1)	
	χ^2 4.736188 (*df*=5) Appearance of buds independent of clone at 14 months (ns)	
Year 4 (Feb-Jun)	Flowering (Stage 3) and capsule formation (Stage 4)	No Flowering only Stage 2 buds
Year 4 (Nov)	Prolific (Stage 1) bud production	Second set of prolific Stage 1 bud production on some clones

ns = non significant; ** = ($p<0.01$)

In year three both *E. grandis* and *E. smithii* produced Stage 2 buds, which represent the next phase in the chronological development of the flowers. *Eucalyptus smithii* produced no stage three buds by November of the fourth year, with flowering only expected from June to September in year five. *Eucalyptus*

grandis on the other hand completes a full cycle from Stage 1 to Stage 4 within a twelve month period.

Figure 4.2: Stage 1 buds of (A) *E. grandis* and (B) *E. smithii*, with shortened inter-nodes visible, particularly with *E. grandis*.

4.4 Discussion

It is evident from the results that it is possible to induce flowering using paclobutrazol, a broad spectrum, xylem-mobile plant growth retardant that inhibits GA biosynthesis. The use of paclobutrazol resulted in an increase in the flower bud production in *E. nitens*, *E. smithii* and *E. grandis* in relation to little or no flower production on young (1 to 2-year-old grafts) at the study site (SRC), particularly for *E. nitens* and *E. smithii*. The onset of early flowering not only allows for the production of seed but also facilitates the study of flowering

patterns and clonal differences. The implications for breeding programmes are that the use of growth retardants will reduce the generation-time significantly and increase seed production in orchards (MONCUR and BOLAND 2000). Following treatment all species produced increased crops of flowering buds in the second growing season after the soil drench treatments. *Eucalyptus grandis* did however respond in the first season four months after treatment but the effect was limited to a number of clones. The second growing season, following a September to October application, should yield the best response.

Paclobutrazol varies in effectiveness and persistence according to the mode of application. A transient response can be achieved with applications by stem injection when metabolism of the active ingredient lowers effectiveness. By contrast, in soil-drench applications only a small amount of paclobutrazol may be absorbed immediately. The remaining chemical remains in the soil until active roots reach these locations. Alternatively the chemical might become unavailable in dry soil but could be taken up following rain or irrigation (MONCUR *et al.* 1994). According to GRIFFIN *et al.* (1993) soil drench treatments of six-year-old *E. globulus* yielded significant improvements in floral bud production when applied during May, the winter rainfall period in Victoria. Furthermore a foliar spray on *E. globulus* in September was too late for an immediate floral bud response. HETHERINGTON *et al.* (1991) were able to increase the bud crop in the same year but in the next growing season in *E. globulus* using the trunk injection method applied in March. Similarly MONCUR and HASAN (1994) achieved flower bud induction with soil drench applications of paclobutrazol applied to *E. nitens* grafts grown in the winter rainfall areas of Tasmania, which are prone to low temperatures. This combination of reduced net biosynthesis of endogenous GA and exposure to cold temperatures produced floral buds. In experiments one and two the soil drench treatments applied in September and October have successfully induce floral buds, but in most cases only in the second season following application. Most references to the use of paclobutrazol have achieved similar results with applications in the winter rainfall months.

From experiment one, winter applications in the summer rainfall regions may delay the onset of floral bud induction due to a lack of moisture (Fig 3.4) if irrigation is not applied. Results indicate that the most reliable time for evaluation of a bud response is in the second season, 14 months following treatment. Paclobutrazol is persistent in the soil following soil drench treatments. GRIFFIN *et al.* (1993) found that the effect on growth of *E. globulus* and positive bud response remained for at least six growing seasons following treatment.

Although the mechanisms for floral induction are not known, trees growing in the natural environment will be exposed to a series of cold periods, changing day lengths and in many cases, variations in water status. The effects of these stimuli either singly or in combination, may, result in a flowering threshold being surpassed. The use of paclobutrazol allows tree breeders to produce improved seed, shorten the breeding cycle and most importantly understand and document the species specific breeding systems and flowering patterns in shy flowering species.

Chapter 5

EUCALYPTUS POLLEN

5.1 Introduction

With the increasing interest in the use of controlled pollinations and selection of the male parent in eucalypt breeding there is a greater need to understand eucalypt pollen physiology (POTTS and MARSDEN–SMEDLEY 1989). It is therefore important to be able to check pollen fertilisation capacity. Various cytochemical methods, which differ in conception and reliability have been developed for this purpose.

The simplest germination tests are based on cytoplasmic stains, such as Lactophenol Cotton Blue and Alexander's Stain, and the concept that pollen devoid of cytoplasm is dead. Dead pollen grains do not stain or stain differently from those with cytoplasm. These methods are easy to use but don't give reliable results. Pollen grains, which remain unstained, lack a protoplast and are definitely aborted, but those with cytoplasm are not necessarily fully fertile. This means an overestimation of pollen viability (NEPI and FRANCHI 2000).

Other techniques involve using the enzyme method, which is based on colour reactions induced by enzyme activity in the pollen grain. The most widely used method is that proposed by HESLOP-HARRISON and HESLOP-HARRISON (1970). This technique is known as FCR (Fluoro-chromatic reaction), it is based on entry of the non-polar substrate fluorescein diacetate into the vegetative cell where it is hydrolyzed by esterase to a polar product (fluorescein) which is retained by the cell membrane. Pollen with an integral plasmamembrane will fluoresce and is considered viable and non-fluorescent pollen grains are eliminated. This method tends to overestimate viability due to certain cytological

events such as the failure of first haploid mitosis and degeneration (NEPI and FRANCHI 2000). The fluoro-chromatic reaction stain procedure enabled detection of differences in pollen viability from two collection sites for *E. calophylla* (EGERTON-WARBUTON *et al.* 1993).

In vitro germination is controlled by temperature using a culture medium containing salts. The most widely used for *Angiosperms* is that of Brewbaker and Kwack (1964) supplemented with 5-15 % sugars (NEPI and FRANCHI 2000). A pollen grain is regarded as having germinated when the pollen tube length exceeds one times the diameter of the grain. Grains that germinate are visible under low magnification dissecting microscopes without any need for special techniques. Pollen may also be germinated *in vivo*, where the pollen tube rapidly penetrates the stigma and style. The tissues of the stigma and style must be removed to reveal the pollen tubes, at which stage pollen tubes can be detected by flourescence of callose using Aniline Blue or Lactophenol Cotton Blue (NEPI and FRANCHI 2000).

5.2 Materials and Methods

5.2.1 Plant Materials

A series of experiments were conducted in order to understand the viability of pollen using cytoplasmic stains, colour reaction and *in vitro* germination. The first experiment was to determine whether any morphological differences exist between species with particular reference to size. Five species from the subgenus *Symphyomyrtus,* one from the series *Salignae* (*E. grandis*) and four from *Viminales* (*E. dunnii, E. smithii, E. macarthurii* and *E. nitens*). All the experiments were conducted using pollen collected and processed from clonal (grafted) orchards planted at the Shaw Research Centre (SRC) in KwaZulu-Natal, South Africa. The orchards are situated at 29 ^{0} 29 ' South, 30 ^{0} 11 ' East at

1100 m above sea level. The climate is cool (MAT 16.7 ^{0}C) with a January mean monthly maximum of 25.8 ^{0}C and July minimum of 4.4 ^{0}C. An estimated mean annual rainfall of 998 mm and median annual rainfall of 899 mm has been reported (PALLETT and MITCHELL 1993). These clonal orchards are a collection of superior mother trees used in the breeding programme to advance the generations and for production of hybrid progeny.

Pollen was collected by placing excised branches into buckets of water, all opened flowers were removed leaving unopened flowers to open at room temperature under controlled conditions. On a daily basis opened flowers were collected, anthers cut and placed into desiccators until dry. Pollen was extracted by shaking the pollen through a sieve of < 50 μm, collecting and placing the clean pollen into plastic straws. Each clone was catalogued and stored separately (multiple straws) in airtight bottles containing silica gel at –10 °C for 0 -24 months.

5.2.2 Pollen morphology and size

Experiment one was designed to determine if any morphological or size differences exist between the species and whether any significant variation between clones within a species was apparent. For the experiment fresh pollen was collected and processed for each species (Table 5.1). The pollen was viewed under a Phillips XL30 Environmental Scanning Electron Microscope (ESEM). Eighteen photographs of each species were taken (at 1000x magnification), six per clone. The area (in μm^2) of ten pollen grains per image was calculated using an image analyser.

Table 5.1: Origin of pollen used to determine pollen size (area).

Species	Clone Number	Origin (Provenance)	Pollen Number
E. dunnii	D74	Dead Horse Track	10960
	D35	Dead Horse Track	10963
	D38	Dead Horse Track	10975
E. smithii	S8	Nerriga	10906
	S20	Wombeyan Road	10553
	S24	Belimba Fire trail	11086
E. grandis	G902	Bulahdelah	10991
	EG45	SAFRI	10981
	G1361	Local LR	10982
E. nitens	N47	Kalmoesfontein	11123
	N5	Tallaganda	11095
	N9	Badja State Forest	10905
E. macarthurii	H44	Local LR	11117
	M198	Local LR	11113
	H17	Local LR	10993

5.2.3 Determination of pollen viability using cytoplasmic stains and enzymatic colour reactions

Experiment two was designed to test the viability of pollen stored (3 -7 months) at a temperature range of between > 0 °C and < 4 °C using Alexander's stain (Table 5.2). Four species were selected for evaluation, with one clone per species, with the exception of *E. nitens* represented by two clones. The experiment was designed to have three replications with two viability counts per replication. Viability was expressed as a percentage of stained grains over the total in view. This stain differentially colours abortive and non-abortive pollen. Abortive pollen appears green and grains with protoplasm appear pink. There are some disadvantages to this technique, it assumes that the presence of protoplasm means a pollen grain is viable. Following preparation the pollen was

placed on a slide with the addition of 1-2 drops of stain. The slide was flamed for less than a minute and a coverslip positioned. After five min the pollen was evaluated under a light microscope (Olympus BH-2).

Experiment three was designed to compare the different methods of testing pollen viability and to identify a method that was both rapid and accurate. Two methods of testing were used a) Alexander's Stain as described in experiment two and b) *in vitro* germination of pollen on agar plates containing 20 % sucrose. In the latter treatment pollen was allowed to germinate for 48 h before evaluation. Included in experiment three were two levels of pollen status a) freshly collected and b) stored pollen (14–17 months < 0 °C) (Table 5.3).

Table 5.2: Species and clonal source of pollen used in the assessment of the Alexander Stain as a means of determining pollen viability.

Species	Clone number	Origin (Provenance)
E. dunnii	D36	Dead Horse Track
E. smithii	S64	Wingello
E. grandis	EG45	SAFRI
E. nitens	N61P	Jessievale
E. nitens	N9P	Badja State Forest

Two species were selected with one clone per species evaluated. The experiment was designed to have three replications with three counts per replication. Viability was expressed as a percentage of stained grains over the total in view for the stain treatment and percentage germination for the *in vitro* treatment. Pollen was considered germinated if the pollen tube length was greater than one times the diameter of the pollen grain (POTTS and MARSDEN-SMEDLEY 1989).

Table 5.3: Species and clonal source of pollen used to estimate viability using Alexander's Stain and sucrose-based media.

Species	Clone number	Origin (Provenance)
E. grandis	G1361 (fresh)	Local LR
E. grandis	G1361 (stored)	Local LR
E. nitens	N47 (fresh)	Local LR
E. nitens	N47 (stored)	Local LR

Experiment four was designed to compare *in vitro* germination with the fluorochromatic reaction (FCR test). The FRC test was prepared using 10 ml of freshly made 30 % sucrose solution placed in a transparent vial with the addition of 1-3 drops of flourescein diacetate / acetone solution. Pollen grains were stored on wet filter paper for 30 min to allow for membrane recovery. The FCR mixture was placed on a slide and pollen dusted onto the drops before covering with a cover slip. The slide was then placed in a petri dish lined with filter paper for 10 min to stain. Pollen was examined under a fluorescent microscope using a violet exciter filter that emits a beam of purple-blue light. Those pollen grains with bright green fluorescence were scored as viable. The *in vitro* germination was assessed after incubation in a solution of 30 % sucrose, containing 150 mg l^{-1} boric acid, for 48 h at 25 °C. Fresh pollen was collected and processed from two species, *E. smithii* and *E. macarthurii,* represented by one clone per species (Table 5.4).

Table 5.4: Source of pollen used in the FCR test and on the *in vitro* sucrose-based media to determine viability.

Species	Clone number	Origin (Provenance)
E. smithii	S334 (stored)	Tallaganda
E. macarthurii	H51 (fresh)	Local LR

5.2.4 Optimal sucrose concentrations for pollen viability testing

The final experiment was a set of *in vitro* 3x3x2 factorials with clone, sucrose level in the medium to a maximum of 30% and the presence or absence of boric acid as independent factors. A maximum level of 30% was set based on the findings of (POTTS and MARSDEN-SMEDLEY 1989) who found optimal sucrose levels for other *eucalyptus* species ranging between 20-30%, over a range of boric acid levels between 0 and 100 mg l^{-1} . The objective was to identify a suitable *in vitro* pollen germination medium for the five commercially important species (Table 5.5). Each species was tested separately using fresh pollen (< 2 months old, stored at –10 °C). The experiments were designed having 6 replications with a total of 50 grains counted per treatment. Viability was expressed as a percentage of germinated grains over the total of 50 grains evaluated. Pollen was considered germinated if the pollen tube length was greater than one times the diameter of the pollen grain (POTTS and MARSDEN-SMEDLEY 1989).

Table 5.5: Source of pollen used in the *in vitro* germination tests using different levels of sucrose and boric acid.

Species	Clone number	Origin (Provenance)
E. dunnii	D74;D35;D38	Dead Horse Track
E. smithii	S72;S334	Tallaganda
	S7	Wombeyan Road
E. grandis	G902	Bulahdelah
	EG45	SAFRI
	G1361	Local LR
E. nitens	N47	Local LR
	N5	Tallaganda
	N9	Badja State Forest
E. macarthurii	M60;M198;M1	Local LR

5.2.5 Data analysis

For experiment one the analysis was done using GLM (General linear models) in SAS (SAS 1998) due to the unbalance caused by an unequal number of observations. In some instances images could not be accurately measured due to their positioning on the photograph. A Tukey's Studentized range test was used to detect differences between and within species. For the remaining experiments an analysis of variance was performed using the GENSTAT Statistical Programme. The ANOVA was conducted on angular transformed data and the least significant difference (LSD) was calculated.

5.3 Results

5.3.1 Pollen morphology and size

From experiment one it was evident that *Eucalyptus* pollen has a distinct, triangular shape with very little variation between species (Fig 5.1). Similarly, PATEL *et al.* (1984) found little variation throughout the family, with pollen grains measuring 10-15 μm in the widest dimension in the unhydrated state. Pollen grains are generally tricolporate with a smooth surface and either acute or obtuse corners resulting in a distinct triangular shape.

When comparing five species from the subgenus *Symphyomyrtus,* one from the series *Salignae* (*E. grandis*) and four from *Viminales* (*E. dunnii, E. smithii, E. macarthurii* and *E. nitens*) significant differences in the area of pollen grains were found between and within species. The adjusted mean surface area of the pollen grains ranked *E. nitens* as having the smallest sized pollen grains (243.378 μm^2) and *E. dunnii* the largest pollen grains measuring 357.654 μm^2 (Table 5.6). There was no significant difference between *E. dunnii* and *E. macarthurii* at the species

level. *Eucalyptus smithii*, *E. grandis* and *E. nitens* were significantly smaller than both *E. dunnii* and *E. macarthurii* (Table 5.6).

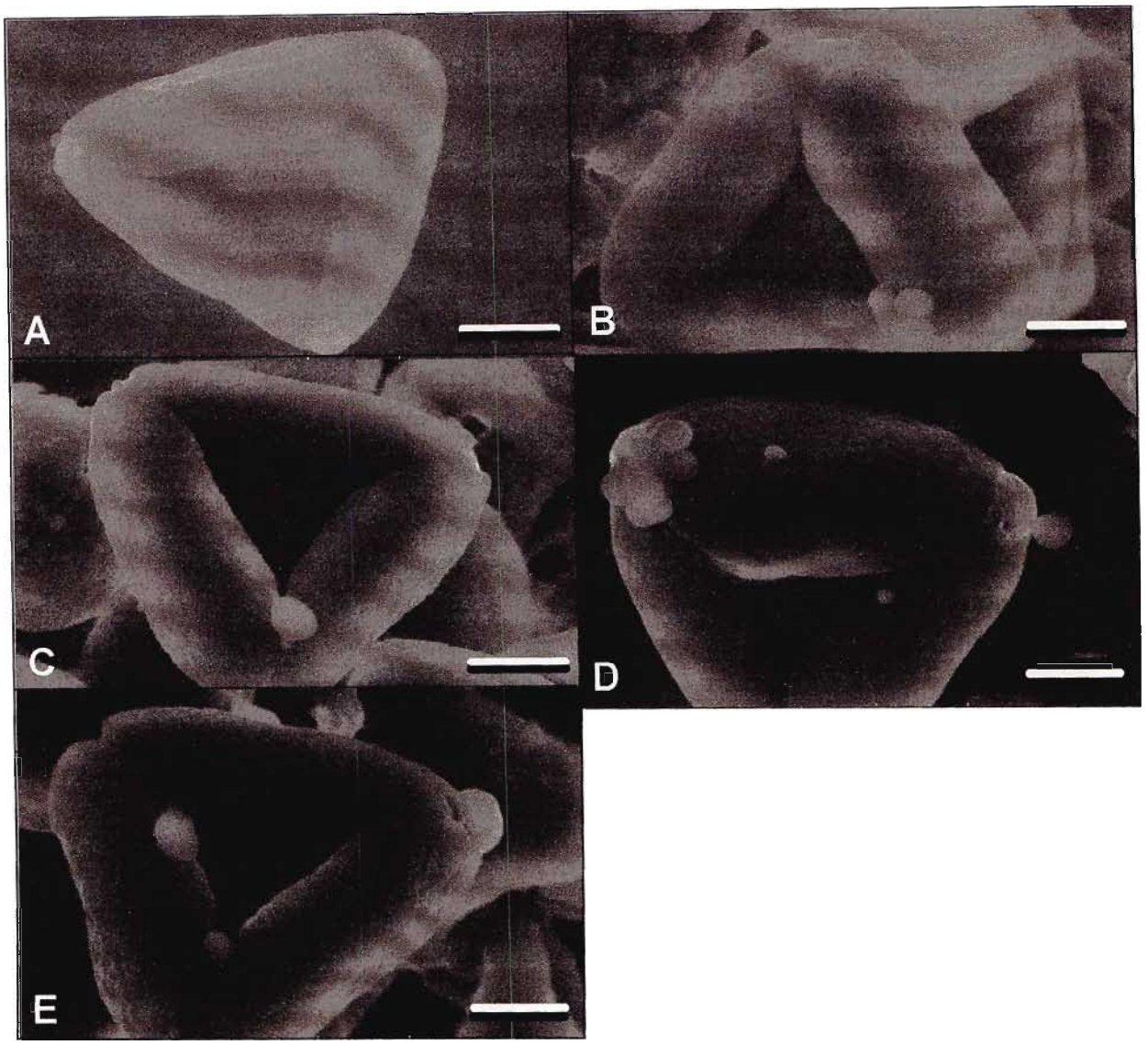


Figure 5.1: Pollen grains of a range of *Eucalyptus* species. (A) *E. nitens*, (B) *E. smithii*, (C) *E. macarthurii*, (D). *E. grandis* and (E). *E. dunnii*. (Bars represent 5 μm).

Eucalyptus nitens pollen was significantly smaller than that of all other species, although some clones within species were not significantly different from clones of other species (Table 5.7). The largest variation was found in *E. smithii* with clone 8 having the largest pollen grains (408.369 μm^2) and clone 20 the smallest

(269.168 μm^2). Significant differences could be detected between clones within species, this could relate to the origin of the parent trees and or sample size (Table 5.7).

Table 5.6: The adjusted mean surface area of *Eucalyptus* pollen grains.

Rank	Species	Area (μm^2)
1	*E. dunnii*	357.654 (ns)
2	*E. macarthurii*	346.089 (ns)
3	*E. smithii*	324.453 **
4	*E. grandis*	289.574 **
5	*E. nitens*	243.378 **

** = Values significant at ($p<0.05$)
ns = Non significant

Table 5.7: Pollen size per clone within each species tested.

Species	Clone number	Rank	Area (μm^2)
E. dunnii	D35	1	372.371 (ns)
	D38	2	365.733 (ns)
	D74	3	334.859 **
E. macarthurii	M198	1	366.861 (ns)
	H17	2	362.082 (ns)
	H44	3	309.322 **
E. smithii	S8	1	408.396 **
	S24	2	295.793 (ns)
	S20	3	269.169 (ns)
E. grandis	G1361	1	302.680 **
	EG45	2	284.795 (ns)
	G902	3	281.245 (ns)
E. nitens	N47	1	269.794 (ns)
	N5	2	266.020 (ns)
	N9	3	194.321 **

** = Values significant at ($p>0.05$)
ns = Non significant

5.3.2 *Determination of pollen viability using cytoplasmic stains and enzymatic colour reactions*

The use of Alexander's Stain to determine the potential viability of pollen in experiment two by calculating the percentage of abortive and non-abortive pollen grains based on the presence of protoplasm, identified significant differences between the clones under test. From the analysis of variance *E. grandis,* clone EG45, performed the worst with 11.3 % potential germination while *E. nitens* responded the best with 81.2 % potential germination (Table 5.8). Highly significant differences ($p<0.001$) in clonal responses were observed reflecting a wide range of potential levels of viability. However, the true germination potential is not known.

Table 5.8: Potential germination ability of *Eucalyptus* pollen using Alexander's Stain.

Species	Clone number	Rank	Potential viability	
			Percentage	Transformed[1]
E. nitens	N9P	1	81.2	65.3
E. dunnii	D36	2	76.0	61.0
E. smithii	S64	3	72.8	58.7
E. nitens	N61P	4	55.5	48.7
E. grandis	EG45	5	11.3	18.4
LSD				10.95

[1] angular transformation of percentage data

In order to test the validity of the use of the Alexander's Stain as a quick indicator of potential pollen viability, the staining technique was evaluated in comparison to *in vitro* germination on sucrose-based medium. Two species (*E. grandis* and *E. nitens*) and two pollen types (fresh and stored) were tested. From the analysis of variance of the data in this experiment, highly significant differences ($p<0.01$) were found between species, media and pollen type. There were also significant species- pollen type, species-media type and pollen-media type interactions.

Eucalyptus nitens (Fig. 5.2) pollen germinated significantly better ($p<0.01$) than that of *E. grandis* (Fig. 5.3). Overall, fresh pollen gave a significantly better ($p<0.01$) response than stored pollen. Pollen viability in terms of the Alexander's Stain was significantly better ($p<0.01$) than *in vitro* pollen germination. No significant difference could be found between fresh and stored pollen for both *E. nitens* and *E. grandis* using Alexander's Stain. In contrast germination of fresh pollen *in vitro* was significantly better ($p<0.01$) than stored pollen for the same *E. nitens* and *E. grandis* clones.

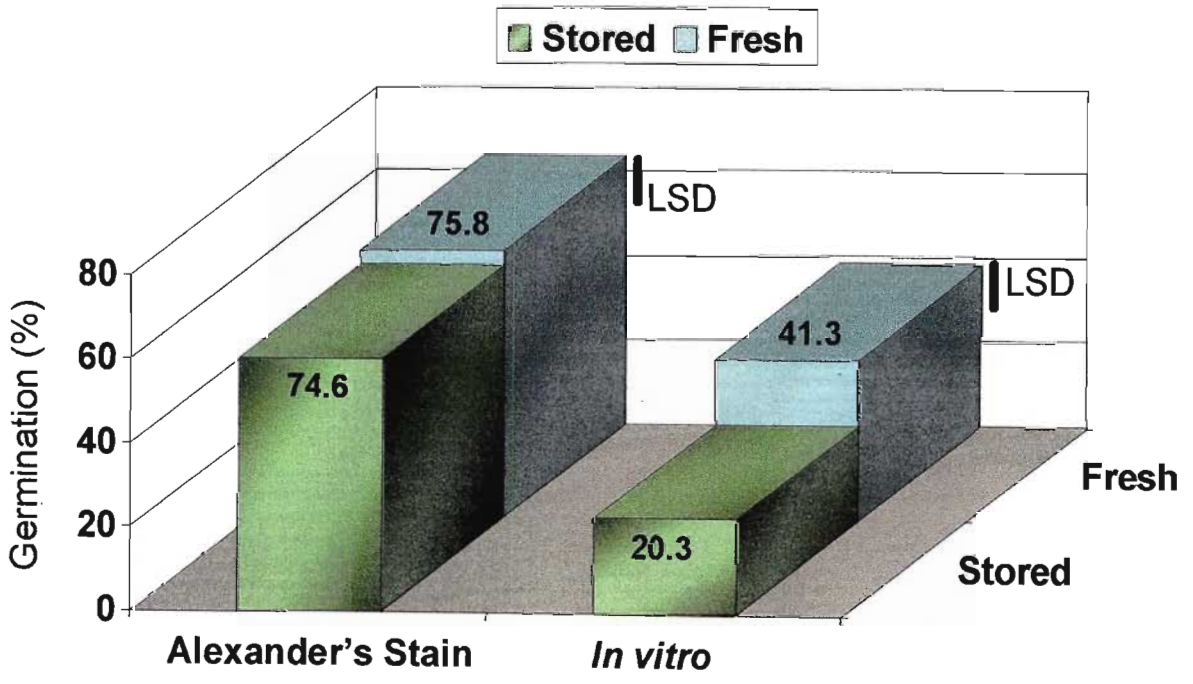


Figure 5.2: Comparison of potential pollen germination of *E. nitens* (angular transformed data) using Alexander's Stain and *in vitro* sucrose-containing medium. Values on the bars represent actual germination percentage (LSD, $p<0.05$).

Alexander's Stain also gave significantly higher estimates of germination potential for both stored and fresh pollen with the exception of one combination. No significant difference could be detected between Alexander's Stain and the *in*

vitro sucrose medium when comparing fresh *E. grandis* pollen (Fig. 5.3). It is evident that fresh pollen was superior to that of stored pollen for both species and that the use of Alexander's Stain overestimated the true germination potential of the pollen, particularly for stored pollen.

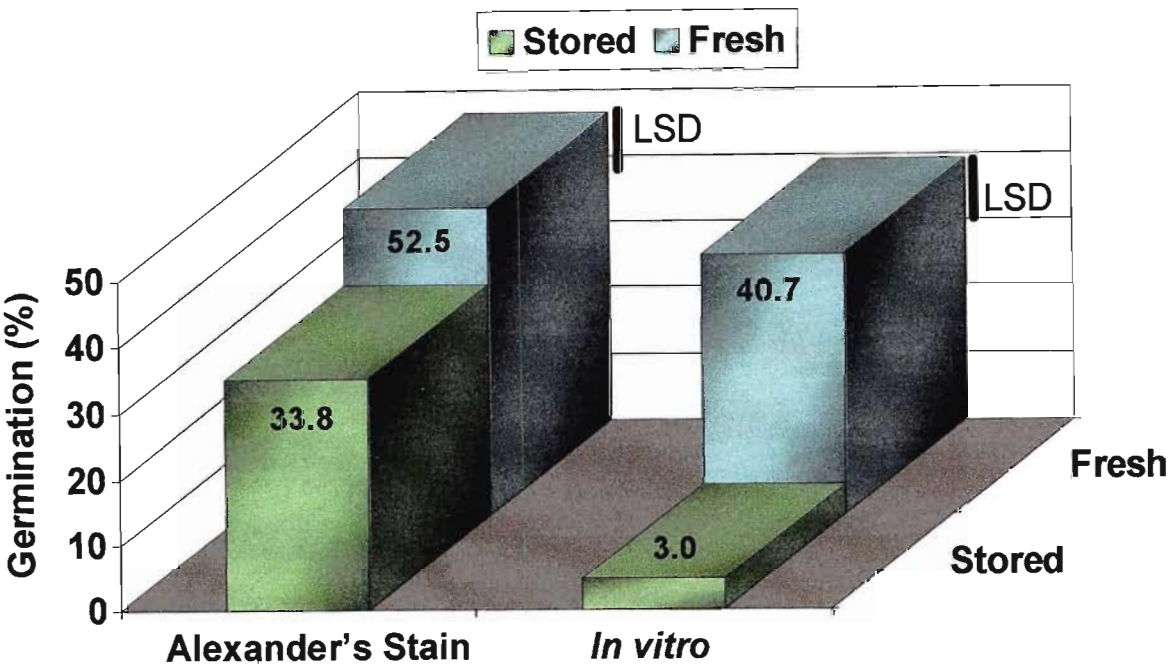


Figure 5.3: Comparison of potential pollen germination of *E. grandis* (angular transformed data) using Alexander's Stain and *in vitro* sucrose-containing medium. Values on the bars represent actual germination percentage (LSD, $p<0.05$).

In the fourth experiment non-orthogonality of the data reduced the precision of the trial but offered some insights into the use of different techniques. The FCR test gave similar results to that of the previous trial using Alexander's Stain. The FCR test was significantly better ($p<0.01$) than *in vitro* germination. *Eucalyptus smithii* gave significantly higher ($p<0.001$) germination than *E. macarthurii* both with the FCR test and *in vitro*. The FCR test overestimated the potential viability of the pollen but there was a weak, positive correlation of ($r = 0.1769$) for the

E. smithii clone between the two media types suggesting that the FCR test may be used to predict potential viability.

Table 5.9: Potential germination ability of *Eucalyptus* pollen comparing the FCR and *In vitro* (sucrose media) tests.

Species	Clone number	Test	Potential viability		
			Percentage	Transformed[1]	
E. smithii	S334	FCR	77.7	*62.1*	**Mean**
E. macarthurii	H51	FCR	53.0	*46.4*	55.72
E. smithii	S334	*In vitro*	54.3	*47.7*	
E. macarthurii	H51	*In vitro*	16.4	*23.7*	35.17
Significant difference between FCR and *In vitro* germination				*(t=3.30) (df=22)*	(p<0.003)
Significant difference between clone S334 and H51				*(t=4.71) (df=22)*	(p<0.001)

[1] = Angular transformation of percentage data

5.3.3 Optimal sucrose concentrations for pollen viability testing

Variations in sucrose concentrations in the presence or absence of boric acid yielded highly significant ($p<0.001$) results for all species tested. *Eucalyptus smithii* gave the best germination followed by *E. nitens*, *E. dunnii*, *E. macarthurii* and unexpectedly *E. grandis* was worst. The level of sucrose in the media was important with 30 % being best for all species. The presence of boric acid in the medium gave significantly better germination. The species sucrose interaction gave no significant differences between species with 5 % sucrose added to the media, at 15 % sucrose *E. dunnii* was significantly better ($p<0.05$) than *E. grandis*, *E. smithii*, *E. nitens* and *E. macarthurii* with the overall germination increasing from 3 to 5.2 %. At 30 % sucrose a marked change was apparent with *E. smithii* being significantly better ($p<0.05$) than all species followed by *E. nitens*, no significant difference was detected between *E. dunnii* and

E. macarthurii with *E. grandis* being worst. The overall germination increased significantly ($p<0.05$) from 5.2 % to 16.3 % (Fig 5.4).

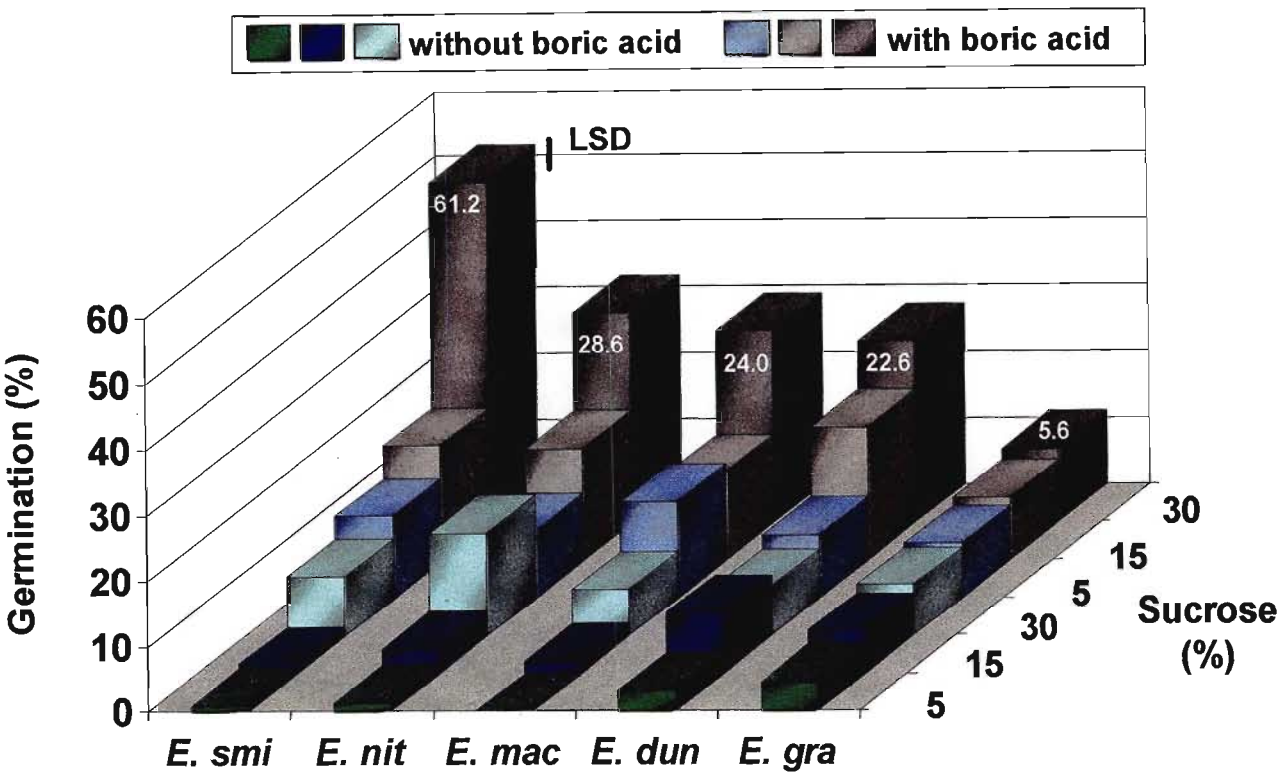


Figure 5.4: The germination % (transformed) of pollen, from five eucalypt species, on media containing different levels of sucrose with or without boric acid. Values on the bars represent actual germination % of the best treatments (LSD, $p<0.05$).

The presence of boric acid gave significantly better ($p<0.05$) germination in all species. However, some responded better than others. *Eucalyptus smithii* was significantly better ($p<0.05$) than all other species, at 26.7 % and *E. grandis* significantly worst at 4.6 %. There was no significant difference between *E. dunnii*, *E. nitens* and *E. macarthurii* overall. The best combination for germination of all species was 30 % sucrose with 150 mg l^{-1} boric acid, which gave a germination of 28.4 %. This was significantly better ($p<0.05$) than 15 % sucrose and boric acid which yielded 8.9 % germination overall. The worst boric

acid treatment was at the 5 % sucrose level, which yielded 5.4 % germination. For all clones of *E. smithii* tested, variations in sucrose concentrations in the presence or absence of boric acid had highly significant ($p<0.001$) results. Clone S7 gave the best germination followed by S334 and S72 respectively. The level of sucrose in the media had a significant effect on germination with 30 % sucrose and boric acid in the medium being best for all clones resulting in 61.2 % germination. The clone by sucrose interactions were not significant, however at 30 % sucrose, clones S334 and S72 gave significantly better ($p<0.05$) pollen germination than all other combinations. With the addition of boric acid to the medium a greater response in terms of germination was achieved particularly at 30 % sucrose. There were no significant differences between 5 and 15 % sucrose for all clones with or without boric acid. However, 5 and 15 % sucrose media containing boric acid was significantly better ($p<0.05$) than 5 and 15 % media without boric acid (Fig. 5.5).

Eucalyptus nitens responded to variations in sucrose concentrations in the presence or absence of boric acid and yielded highly significant ($p<0.001$) results. Clone N9 gave the best germination followed by N47 and N5 respectively. The level of sucrose in the media was important with 30 % sucrose being best and 150 mg l^{-1} boric acid in the medium giving significantly ($p<0.05$) better germination than no boric acid. The overall clone by sucrose interactions were not significant. However, clone N9 was significantly better ($p<0.05$) than clones N47 and N5 at each sucrose level. There were no significant differences between clones N47 and N5 at each sucrose level of 5 %, 15 % and 30 %. With the addition of boric acid to the medium a greater response in terms of germination was achieved at all levels of sucrose. There were no significant differences between clones N47 and N5 at all sucrose levels tested, but significant ($p<0.05$) improvements in germination of up to 20 % were recorded. Clone N9 showed the greatest response with the best germination of 46.3 % with 30 % sucrose and a boric acid-containing medium (Fig. 5.6).

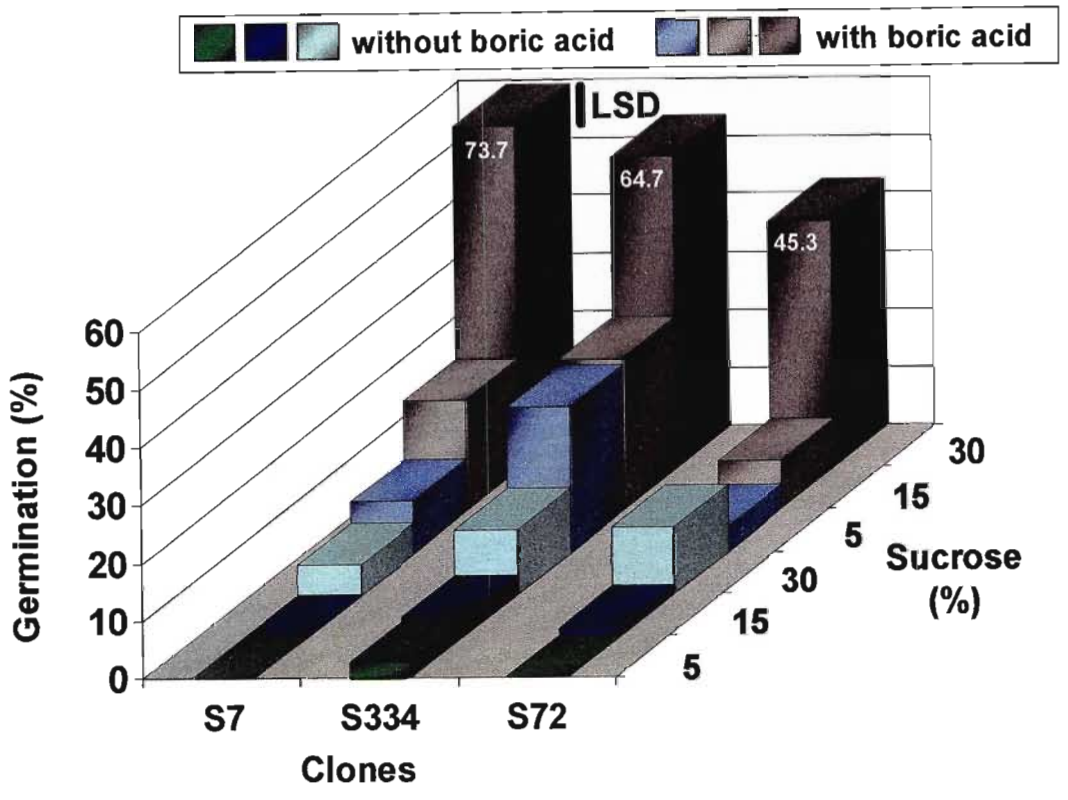


Figure 5.5: The pollen germination % (transformed) of three clones of *E. smithii* on media containing different levels of sucrose with and without boric acid. Values on the bars represent the actual germination % of the best treatments (LSD, $p<0.05$).

Eucalyptus dunnii responded to variations in sucrose concentrations in the presence or absence of boric acid and yielded highly significant ($p<0.001$) results. Clone D35 gave the best overall germination followed by D38 and D74 respectively. The level of sucrose in the media was important with 30 % sucrose being best and boric acid in the medium giving significantly ($p<0.05$) better germination than no boric acid. The overall clone by sucrose interactions were significant, with all clones improving germination percentage from 5-30 % sucrose, clone D 74 showed a drop in germination at 30 % sucrose from the 15 % sucrose medium, however this drop was not significant. There were no significant differences between the germination responses of clones D38 and D35. Although the sucrose concentration had a significant ($p<0.05$) impact on

pollen germination, with 30 % sucrose with boric acid giving the best response (Fig. 5.7). Clone D74 showed the best germination response (8.3 %) on 15 %

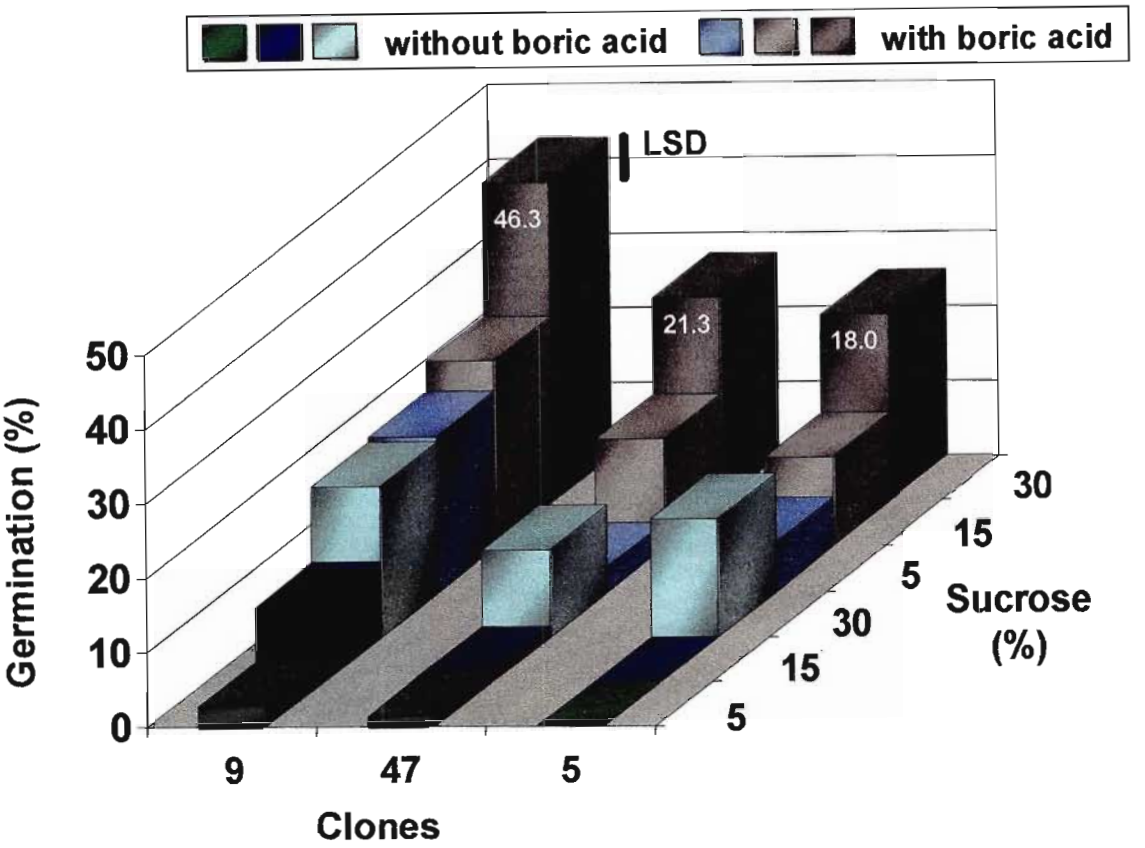


Figure 5.6: The pollen germination % (transformed) of three clones of *E. nitens* on media containing different levels of sucrose with and without boric acid. Values on the bars represent actual germination % of the best treatments (LSD, $p<0.05$).

sucrose with boric acid, and while not significant, it decreased to 8.0 % on the 30 % sucrose and boric acid medium. Similarly for all clones germination decreased with an increase in sucrose from 15-30 % in the absence of boric acid. Both clones D35 and D38 responded to 30 % sucrose and boric acid with germination figures of 30 % and 29 % respectively (Fig 5.7).

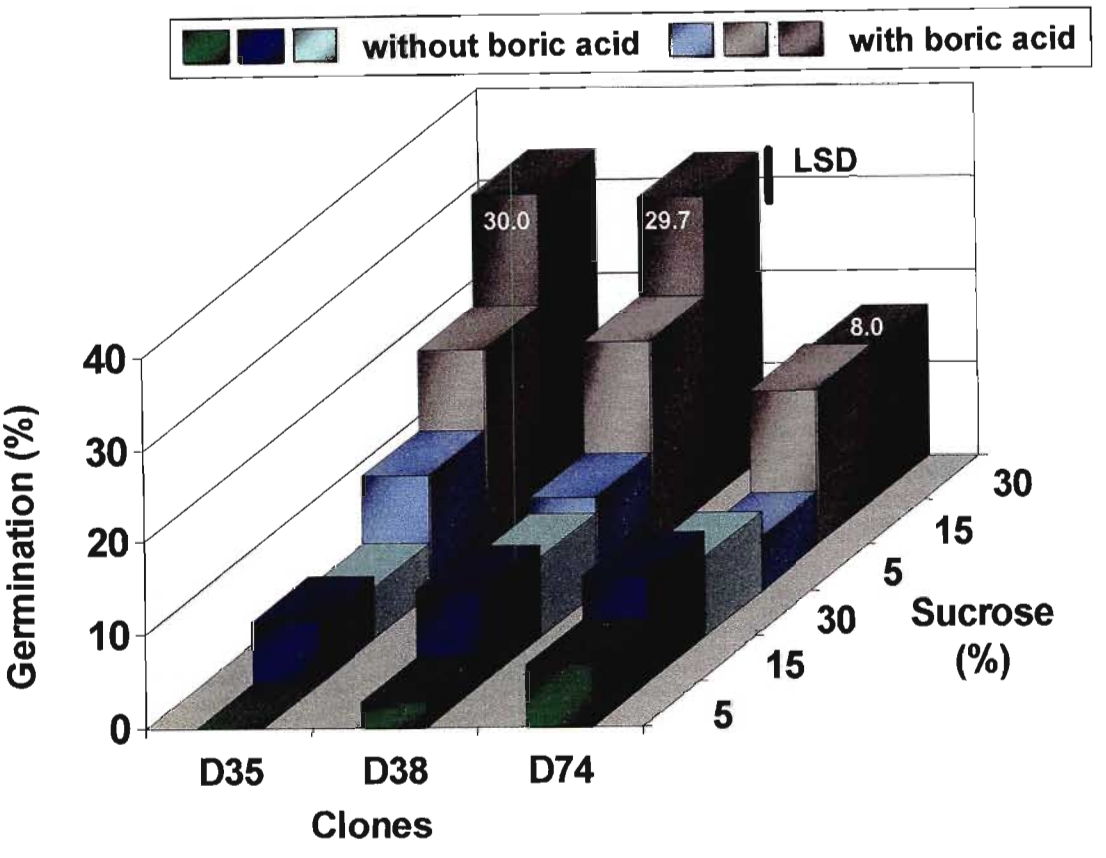


Figure 5.7: The pollen germination % (transformed) of three clones of *E. dunnii* on media containing different levels of sucrose with and without boric acid. Values on the bars represent the actual germination % of the best treatments (LSD, $p<0.05$).

Eucalyptus macarthurii responded to variations in sucrose concentrations in the presence or absence of boric acid and yielded highly significant ($p<0.001$) results. Clone M198 gave the best overall germination followed by M1 and M60 respectively. The level of sucrose in the media was important with 30 % sucrose being best and boric acid in the medium giving significantly ($p<0.05$) better germination than without boric acid. The overall clone by sucrose interactions were significant, with all clones showing improved germination with an increase of sucrose from 5-30 %. Clones M1 and M60 showed a significant decrease ($p<0.05$) in germination with 15 %, however this drop was not evident at 30 % sucrose. Clone M198 was the only clone to show a significant improvement at

each sucrose level. Overall all clones showed significant ($p<0.05$) improvements in pollen germination with 5 to 30 % sucrose. (Fig. 5.8).

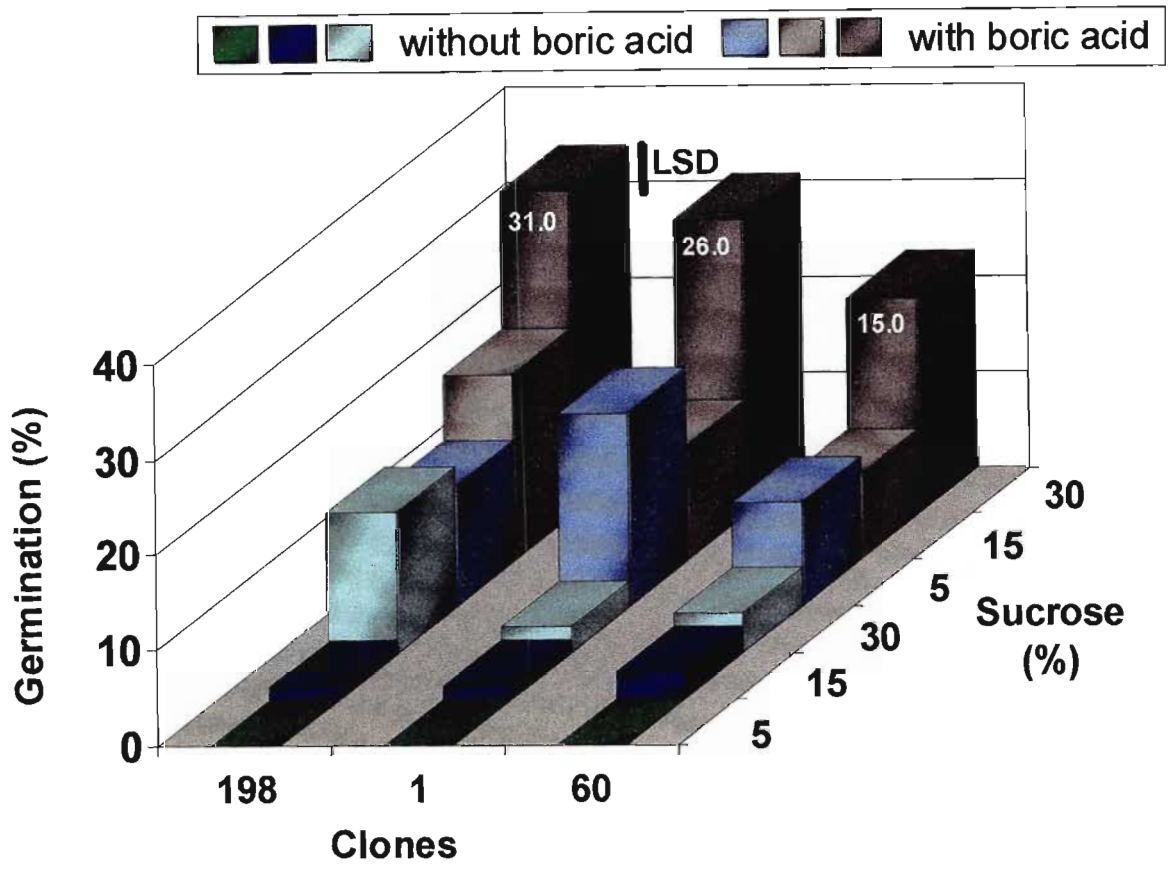


Figure 5.8: The pollen germination % (transformed) of three clones of *E. macarthurii* on media containing different levels of sucrose with and without boric acid. Values on the bars represent actual germination % of the best treatments (LSD, $p<0.05$).

Eucalyptus grandis did not respond as expected with a very low overall germination, however variations in sucrose concentrations in the presence or absence of boric acid had highly significant ($p<0.001$) results. Clone G 902 gave the best overall germination followed by G1361 and G45 respectively. The level of sucrose in the media was important with 30 % sucrose being best overall and boric acid in the medium giving significantly ($p<0.05$) better germination than no

boric acid. The overall clone by sucrose interactions were significant, clone G1361 showed a significant drop ($p<0.05$) in germination at 15 % sucrose from the 5 % sucrose medium, however this drop was not evident at 30 % sucrose. Clones G902 and G45 were best with 15 % sucrose and dropped at the 30 % sucrose level although not significant. Failure of the G45 pollen to germinate properly affected the coefficient of variation (CV %) in this trial, impacting negatively on the results (Fig. 5.9).

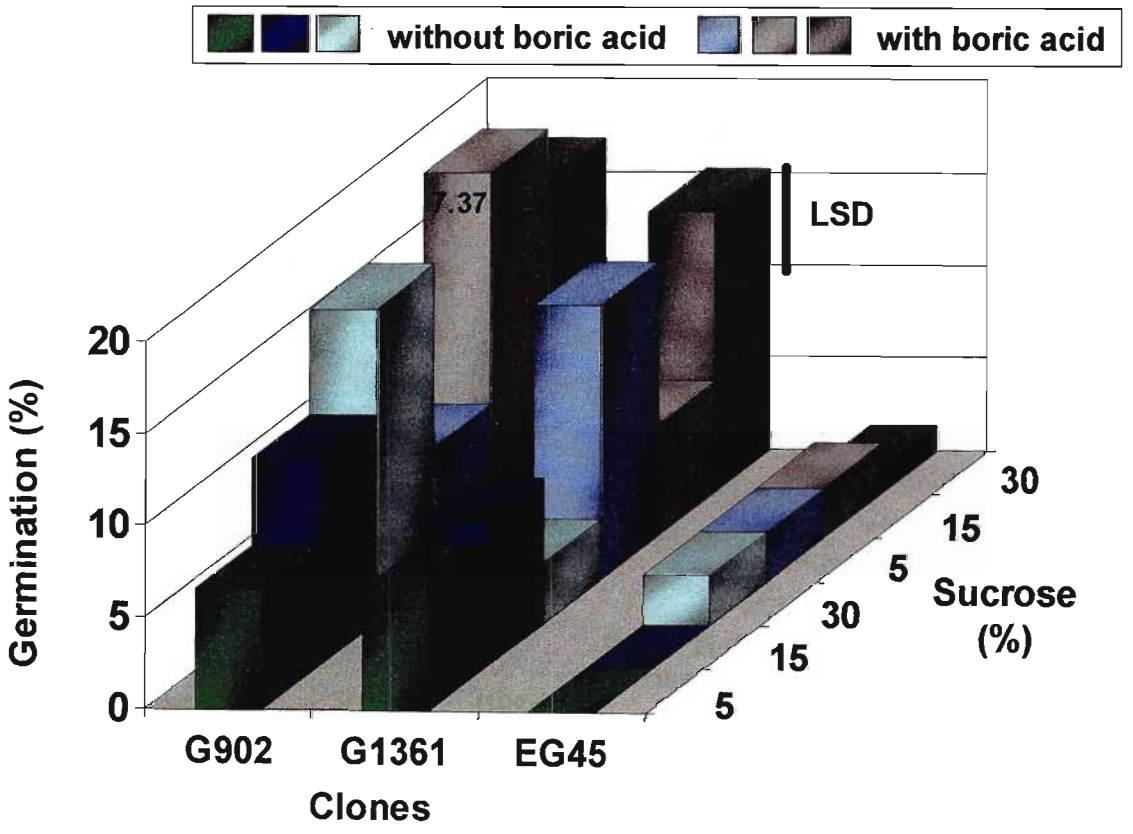


Figure 5.9: The pollen germination % (transformed) of three clones of *E. grandis* on media containing different levels of sucrose with and without boric acid. Values on the bars represent the actual germination % of the best treatment (LSD, $p<0.05$).

5.4 Discussion

There is a considerable variability in flower size within *Eucalyptus* and closely related taxa, which form morphological series varying markedly in flower and fruit size. According to PATEL *et al.* (1984) little variation exists throughout the family, when considering the morphological appearance of pollen. It is evident that pollen from the five species viewed that no single morphological character could be applied to separate them by species (Fig. 5.1). All the pollen grains are tricolporate and triangular in shape. Most of the variation that exists between species relates to pollen size differences.

The importance of pollen size difference relates to the ability to sucessfully produce interspecific hybrid progeny. The structural barriers arising from disparities in pistil and pollen tube length is thus a major post-mating barrier to interspecific hybridisation (GORE *et al.* 1990). The results indicate as much variation in the size of the pollen grains when comparing five species of eucalypt from the subgenus *Symphyomyrtus.* Significant differences in the area of pollen grains were found between and within species. The adjusted mean surface area of the pollen grains ranked *E. nitens* as having the smallest sized pollen grains (243.378 μm^2) and *E. dunnii* the largest pollen grains measuring 357.654 μm^2. There was no significant difference between *E. dunnii* and *E. macarthurii* at the species level. *Eucalyptus smithii*, *E. grandis* and *E. nitens* were significantly smaller than both *E. dunnii* and *E. macarthurii* (Table 5.6).

When comparing five species of *Eucalyptus,* GORE *et al.* (1990), found that *E. globulus* had the largest pollen grains followed by *E. unigera*, *E. ovata*, *E. gunnii* and *E. nitens* with the smallest. The *in vitro* pollen tube lengths followed a similar pattern with *E. globulus* having the longest pollen tubes and *E. nitens* the smallest with *E. unigera*, *E. gunnii* and *E. ovata* not significantly different from each other but significantly ($P<0.05$) larger than *E. nitens*. Furthermore GORE *et al.* (1990) was able to get a positive correlation ($r = 0.79$) between pollen grain

diameter and *in vitro* pollen tube length although in this case not significant. Similarly, PATEL *et al.* (1984) found little variation throughout the family, with pollen grains measuring 10-15 µm in the widest dimension in the unhydrated state. Pollen grains are generally tricolporate with a smooth surface and either acute or obtuse corners with distinct triangular shape.

Pollen was successfully stained and evaluated using Alexander's Stain. Highly significant differences ($p<0.001$) in clonal responses were observed reflecting a wide range of potential levels of viability. According to NEPI and FRANCHI (2000) pollen viability estimates will be higher as those with stained cytoplasm are not necessarily viable. When comparing Alexander's Stain with *in vitro* pollen germination using stored and fresh pollen, highly significant differences ($p<0.01$) were found between species, media and pollen type. *Eucalyptus nitens* pollen germinated significantly better ($p<0.01$) than *E. grandis* 53.2 % over 32.5 %. Fresh pollen gave a significantly better ($p<0.01$) response over stored pollen overall and Alexander's Stain was significantly better ($p<0.01$) than *in vitro* germination (Fig. 5.1). The most important result was that no significant difference could be found between fresh and stored pollen for both *E. nitens* and *E. grandis* using Alexander's Stain. In contrast germination of fresh pollen *in vitro* was significantly better ($p<0.01$) than stored pollen for the same *E. nitens* and *E. grandis* clones, a clear indication of the overestimation of viability when using Alexander's Stain. Similarly the FCR test gave similar results to that of Alexander's Stain. The FCR test overestimated the potential viability.

Results from the *in vitro* germination clearly indicated the importance of sucrose in the pollen germination medium. The most successful germination media for the *Eucalyptus* species ranged from 20–30 % sucrose with boric acid (Table 2.3). Variations in sucrose concentrations in the presence or absence of boric acid yielded highly significant ($p<0.001$) results for all species tested. *Eucalyptus smithii* gave the best germination followed by *E. nitens*, *E. dunnii*, *E. macarthurii* and *E. grandis*, which was worst. The marked differences in the optimum sucrose

for germination was important with 30% being best for all species and the presence of boric acid in the medium gave significantly better germination. According to POTTS and MARSDEN-SMEDLEY (1989) the stimulatory effect of boric acid in pollen germination media is widespread. The stimulatory effects direct and may increase absorption, translocation and metabolism of sugars. It may increase oxygen uptake and improve pectin synthesis. In the absence of boric acid germination is very poor.

This is consistent with the extremely poor growth achieved at all levels of sucrose for the species when tested without boric acid (Fig. 5.4). From the present results 150 mg l^{-1} as opposed to no boric acid was effective in improving germination significantly ($p<0.001$) although no intermediate level of boric acid was tested. Similarly, POTTS and MARSDEN-SMEDLEY (1989) found optimal sucrose levels to range between 20-30 %, over a range of boric acid levels between 0 and 100 mg l^{-1}. This is consistent with the results for the species tested with some clonal interactions with changes in sucrose level.

A successful system of viability testing is a prerequisite for not only pollen research but vital for successful controlled pollinations (POTTS and MARSDEN-SMEDLEY 1989). The use of various cytochemical methods, were shown to be mere indicators of potential viability and lack the reliability for adequate testing of stored pollen. While even the validity of *in vitro* testing may be questioned it has in this study identified differences and similarities in response between species and clones within species to varying levels of sucrose.

Chapter 6

POLLINATING AGENTS

6.1 Introduction

The Australian members of the Myrtaceae show a great deal of diversity in pollination mechanisms and pollinators (BEARDSELL *et al.* 1993). According to PRYOR (1976) eucalypts are pollinated by a diverse number of insects and birds. Most species appear to be pollinated by insects that are attracted to the nectar secreted into the bowl shaped flowers of many species (BEARDSELL *et al.* 1993). There is little evidence to indicate that wind plays any major role in the pollination process although some species such as *E. tereticornis* and *E. blakelyi* may be partly wind pollinated (PRYOR 1976).

Eucalypts with small flowers are predominantly entomophilous (insect pollinated) whereas species with large flowers are mostly ornithophilous (bird pollinated) (FORD *et al.* 1979). The most common pollinators of eucalypts are bees from the family Collectidae (ARMSTRONG 1979). Wasps are less likely visitors, however thynnid wasps have been observed on eucalypt flowers (ASHTON 1975). The major plantation species have small flowers and no apparent adaptation to particular vectors. Honeybees, *Apis mellifera*, are attracted to the flowers by the nectar and pollen, which adheres to all parts of their bodies. The honeybees are regarded as an effective substitute for natural pollinators in the exotic environment (ELDRIDGE *et al.* 1994). Pollen is virtually the only source of natural protein available to bees, influencing both breeding and longevity (MONCUR *et al.* 1995). Both the quality and quantity of pollen protein can independently, and in combination influence supply of essential amino acids, which ultimately become available to the bees. Often insufficient eucalypt pollen

is available to meet the protein requirements necessary to maintain body protein during honey flows (MONCUR *et al.*1991).

According to HORSKINS and TURNER (1999) honeybees were the most frequent visitors to the flowers of *E. cosata,* collecting pollen and nectar at all times of the day. Foraging commenced at temperatures of 11.9 °C (± 1.4) which are much cooler than the temperatures at which native bees begin to forage (21.0 °C ± 2.0). Honeybees also exhibit within and between canopy foraging behaviour, which is indicative of successful pollinators (HORSKINS and TURNER 1999). However, over a period of several days, observed restricted foraging without movement between plants. Environmental factors such as drought or prolonged rainfall may affect the normal rhythm of flowering, furthermore cold, wet and windy weather conditions, during flowering could prevent foraging (HINGSTON and POTTS 1998).

6.2 Materials and Methods

6.2.1 Plant materials

Two trials were conducted in the clonal (grafted) orchards of *E. nitens*, *E. macarthurii* and *E. grandis* planted at the Shaw Research Centre (SRC) in KwaZulu-Natal, South Africa. The orchards are situated at 29 ^{0} 29 ' South, 30 ^{0} 11 ' East at 1100 m above see level. The climate is cool (MAT 16.7 ^{0}C) with a January mean monthly maximum of 25.8 ^{0}C and July minimum of 4.4 ^{0}C. An estimated mean annual rainfall of 998 mm and median annual rainfall of 899 mm has been reported (PALLETT and MITCHELL 1993). The second survey of potential pollinators of *E. grandis* was conducted in the clonal hedges planted at a closer espacement of 1m between adjacent trees and not the 4x6 m espacement of the other orchards. At the time of the survey the *E. grandis* trees were in full flower, following treatment with paclobutrazol in the previous season.

6.2.2 *Impact of pollinators on capsule survival and seed yield*

In order to test the impact of pollinators on capsule survival, selected branches were completely isolated during peak flowering and compared to capsule survival without any isolation. The primary aim of this trial was to determine whether total exclusion of pollinators during flowering impacted on capsule abortion rate and seed set. Selected parents of *E. nitens*, *E. grandis* and *E. macarthurii* from various breeding populations were used and selected branches, bearing buds, were counted, bagged and labeled on grafted trees of various ages. For total isolation a bag (nappy liner) was used excluding all insects from the flowers (Fig. 6.1). All treatments and observations were conducted in the clonal grafted orchard described previously. The branches were allowed to flower naturally and capsules monitored (on two occasions) and harvested when ripe.

Figure 6.1: Typical isolation and labeling techniques used in Experiment one. (A) *E. macarthurii* branches labeled with no isolation and (B) *E. macarthurii* branches labeled and isolated (B).

6.2.3 Potential pollinators of Eucalyptus grandis

This study aimed to identify the potential pollinators of *E. grandis* in an exotic environment. To achieve this the identities of diurnal visitors were investigated at an order level. The hedge site was monitored over a period of five days, during different times of the day for intervals of one hour. Individual trees of *E. grandis* were observed with the visits recorded when insects arrived to forage in the canopy of particular trees. Tree canopies were short between 1 and 2 m making it possible to monitor the whole canopy at once. Temperatures were obtained from the weather station within 100m of the survey point.

6.2.4 Data analysis

The first experiment was analysed as a completely random design. The ANOVA was conducted on angular transformed data and the least significant difference (LSD) was calculated using the GENSTAT Statistical Programme. Experiment two was analysed using a Two-Way Contingency Chi –squared table tests to evaluate the statistical significance of independence.

6.3 Results

6.3.1 Impact of pollinators on capsule survival and seed yield

By excluding all potential pollinators from visiting the flowers an attempt was made to quantify the importance of pollinators and their effect on seed yield. A significant drop ($p<0.05$) in capsule survival between open and isolated flowers was observed in *E. grandis* and *E. macarthurii* with no significant difference in survival for *E. nitens*. Seed yield per capsule also dropped for all species following extraction of the seed from the remaining capsules, with highly significant differences ($p<0.001$) for seeds per capsule occurring between the two

treatments of *E. grandis*. While the seed yield per capsule was better in the open control treatment of *E. nitens* and *E. macarthurii* over that of the isolated treatment, unexplained capsule losses made it difficult to detect significant differences due to reduced degrees of freedom in the analysis (Table 6.1). However, seed set in all species was adversely affected by a lack of insect activity at the time of flowering (Table 6.1).

Table 6.1: A comparison of the mean (clones bulked) capsule survival and seed yield per capsule following open pollination and complete (closed) isolation.

Species	Clones Bulked	Open pollinated		Complete isolation	
		Capsule Survival (%)	No. Seed per Capsule	Capsule Survival (%)	No. Seed per Capsule
E. grandis	Mean	51.8	2.2	33.3 *	0.17 **
E. nitens	Mean	19.3	2.20	17.4 ns	1.06 ns
E. macarthurii	Mean	50.9	2.4	14.7 **	1.5 ns

ns = non significant; * = ($p<0.05$); ** = ($p<0.01$)

6.3.2 Potential pollinators of Eucalyptus grandis

Seven taxa were encountered, with a total of 20 species foraging on the flowers of *E. grandis* (Table 6.2). Honeybees and ants were the most dominant and active foragers in the flowers. The observed moths, while high in number relative to other insects only made fleeting visits to flowers. From the analysis it was evident that there is a highly significant ($p<0.001$) association between temperature and different species that can be found foraging in the flowers of *E. grandis*. In most case bees, moths and ants preferred the higher temperature classes from 20-29 °C and above. While flies and beetles were found in greater numbers from 15-22 °C. Butterflies and wasps showed no particular preference but had a tendency to be more active at the higher temperature classes.

Table 6.2: The diurnal insect taxa and species recorded visiting the flowers of *E. grandis* during April.

Taxa	Number of species per taxon	Ambient temperature at the time of the survey			
		< 15 °C	17-20 °C	20-22 °C	> 29 °C
		Number of visits by insects within the various temperature ranges			
Diptera (Flies)	5	12	4	9	5
Lepidoptera (Butterflies)	3	1	0	5	3
Hymenoptera (Bees)	4	0	2	15	22
Hymenoptera (Wasps)	5	1	2	3	5
Coleoptera (Beetles)	2	8	3	6	1
Hymenoptera (Ants)	1	0	11	36	12
Lepidoptera (Moths)	1	0	6	24	12

χ^2 89.02795 (*df*=18) (P<0.001)

6.4 Discussion

Failure of some *Eucalyptus* species to produce regular flowering crops and a lack of sufficient cross pollination between unrelated parents has been identified as the main reasons for irregular seed yields. In some instances with sufficient flowering but a lack of suitable pollinators, particularly in the exotic environment will lead to poor seed set (MONCUR *et al.* 1995). The diversity and abundance of each taxon is dependent on a wide range of different factors such as the plant species, time of flowering and climatic conditions at the time of flowering.

According to WILLIAMS and POTTS (1996) most flowering of *Eucalyptus* occurs from September to March (spring to summer) when temperatures are elevated, with very little flowering over winter. *Eucalyptus urnigera* is the only subalpine species in which flowering is centered over the winter period in Tasmania and appears to be bird pollinated. According to JONES *et al.* (2000) species such as *E. nitens* and to a lesser degree *E. smithii* have flowering times in South Africa which are centered in the winter months, a time not necessarily conducive to the presence of a large groups of potential pollinators. According to HORSKINS and

TURNER (1999) as many as 76 species of invertebrates were recorded visiting *E. cosata* in Victoria Australia during September and November. Similarly HINGSTON and POTTS (1998) identified seven species of bird and 71 insect species active in the canopies of *E. globulus* stands in Tasmania during November and December. From the results only twenty insect species could be identified on *E. grandis* during the survey period in April (Table 6.2). While this is well short of the insect communities identified in the natural ranges of *E.globulus* and *E. cosata*, honeybees were particularly dominant.

From the isolation experiment it is evident that restricted access to flowers by insects will impact on the overall survival of capsules and eventual seed yields. A significant drop ($p<0.05$) in capsule survival was observed across *E. grandis* clones from 51.8 % to 33.3 %. Similarly, a highly significant decrease ($p<0.001$) in capsule survival was observed across *E. macarthurii* clones from 50.9 % to 14.7 % between complete isolation and open pollinated treatments. It is important to note that according to JONES *et al.* (2000) both *E. grandis* and *E. macarthurii* flower during the warmer months of summer and spring. *Eucalyptus nitens* is largely a winter flowering species (JONES *et al.* 2000) and showed no significant difference ($p>0.05$) in capsule survival between complete isolation at 17.4 % survival and open pollinated flowers at 19.3% survival (Table 6.1). Similarly DU TOIT (1995) found that only 19 % of isolated Bengal litchi flowers set fruit as opposed to 84 % of the open pollinated flowers setting fruit representing an increase of over 300 %. The present results demonstrate the importance of sufficient pollinators during flowering. In the case *E. nitens* insufficient pollinators at the time of flowering and or nectar and pollen quantities and qualities may be inadequate. According to MONCUR *et al.* (1995) disappointing seed yields from established orchards outside of Australia have been related to the lack of pollen vectors, particularly in the Congo (eucalypts) and China (acacias).

Apis mellifera (honeybee) has long been associated with the pollination of

Eucalyptus species world wide, chiefly for the production of honey. According to MONCUR *et al.* (1991) in order to achieve high honey production rates, pollen must have adequate protein levels and flowers with good nectar flow. Good nectar flow is reduced by low morning and evening temperatures and thus less populous colonies of honeybees are found at high altitude. Furthermore flow does not start below certain temperatures, the threshold varying from one species to the next, thus effectively reducing foraging activity. According to DU TOIT (1995) honeybee foraging in Bengal litchi increased from 08h00 to 12h00 for pollen with nectar collection at the highest rate early in the morning. HORSKINS and TURNER (1999) found that nectar availability in *E. cosata* was greatest in the morning with honeybees collecting both pollen and nectar throughout the day. Honeybees favoured pollen collections in the morning commencing at temperatures as low as 11.9 °C (± 1.4). From the present survey bees favoured the warmer temperatures between 20-29 °C, with little or no visits at temperature less than 15 °C. However, this could be affected by other factors such as clouds, wind and interactions with environment at the time of flowering. Ants were regular visitors to the flowers of *E. grandis*, specifically for nectar (visual assessment) and not pollen. Similarly HORSKINS and TURNER (1999) found that ants almost exclusively utilized nectar. Due to the nature of their feeding habits at the base of the stigma and within single canopies at any one time, ants contribute little to the pollination in *E. grandis* and in fact could reduce visits by honeybees by utilising available nectar. Beetles had a tendency to forage fairly aggressively carrying visible quantities pollen on their bodies. Wasps, butterflies and moths visited flowers at random moving between canopies rather than between flowers within single canopies. Flies were present feeding mainly on nectar and moving all over individual flowers. Of all the insects observed in this preliminary survey, honeybees were the most dominant and active pollinators of *E. grandis*.

According to MONCUR *et al.* (1995) there are a few reports of increased seed yields from open pollinated seed orchards following the introduction of *Apis*

mellifera (Table 2. 5). The ability of honeybees to pollinate eucalypt flowers and increase production largely depends on factors such as the eucalypt species, flowering season, climatic conditions during flowering, site conditions, pollen and nectar quantity and quality. Little is known about the insect communities responsible for the pollination of eucalypts in South Africa and further research is required to model the impact of site and climatic conditions on the presence of pollinators at the time of flowering. Furthermore management strategies need to be developed to utilize honeybees in hostile environments (cold and dry) where certain eucalypts such as *E. nitens* are grown.

Chapter 7

BREEDING AND POLLINATION IN EUCALYPTS

7.1 Introduction

In the past traditional eucalypt breeding focussed on the improvement of the pure species. The greatest advance in industrial plantation forestry of the past 20 years has undoubtedly been the development of clones from hybrid genotypes (GRIFFIN *et al.* 2000). To ensure continuous genetic improvement of the population, controlled crosses can be made to concentrate the best alleles from a range of selected parent trees. Controlled pollination, involves the transfer of pollen from anthers of a selected male parent to the stigmatic surface of a female parent. The stigma is isolated from all other pollen sources. Pollen germinates on the stigma, grows down the style to the ovary and fertilizes the ovules (MONCUR 1995). Most of the commercially important eucalypts have mixed but predominantly outcrossed breeding systems (GRIFFIN 1989). Self-fertilization results in inbreeding depression and must be minimized and avoided (SEDGELY and GRIFFIN 1989).

In eucalypts, the male phase generally begins approximately two days after anthesis, with pollen-shed occurring in response to temperature and other environmental factors. The female stage begins with the appearance of a sticky secretion on the stigmatic surface. Hybridization between species from the major eucalypt subgenera does not occur either naturally or artificially (PRYOR and JOHNSON 1971; GRIFFIN *et al.* 1988). The main reasons for this, are pre-zygotic barriers, the first of which is a unilateral structural barrier. The amount of pollen tube inhibition has been shown to increase with increasing taxonomic distance between parents (POTTS *et al.* 2000). This broad trend for expression of the F_1 hybrid inviability to increase with increasing taxonomic distance between

parents has been demonstrated by comparing the percentage of normal progeny as a percentage of the seed sown.

In the temperate, summer rainfall regions of South Africa *E. dunnii*, *E. smithii*, *E. macarthurii*, *E. nitens* and *E. grandis* are the main eucalypt species of commercial importance. For all species, there has been a clear focus on genetic improvement through breeding programmes over the past few years (CLARKE and JONES 1998). In addition to the development of each of the separate species, there has also been an interest in the production of hybrid combinations with other species to improve characteristics important for plantation forestry. The hybridization could improve the frost tolerance of *E. dunnii* and *E. grandis* when crossed with *E. macarthurii*. Resistance to attack by *Phytophthora* may possibly be reduce in *E. smithii* when hybridized with *E. grandis*. The pulping properties of *E. dunnii* and *E. grandis* could improve if hybrid combinations with *E. smithii* are successful.

7.2 Materials and Methods

7.2.1 Plant materials

The breeding populations for all species are made up of open-pollinated families from selections made in the land-race in South Africa and in progeny trials which include families from a number of provenances collected in the natural range in Australia. The orchards have been established on level terrain at a wide espacement of 4x6 m. The ramets of each clone are randomized throughout each orchard and vary in age due to annual additions and replacements. For the purposes of generating sufficient flowers all orchards were treated with paclobutrazol, applied as a soil drench.

The controlled pollinations were conducted in the clonal orchards of *E. nitens*, *E. dunnii*, *E. macarthurii*, *E. smithii* and *E. grandis* planted at the Shaw Research Centre (SRC) in KwaZulu-Natal, South Africa. The orchards are situated at 29 ^{0} 29 ' South, 30 ^{0} 11 ' East at 1100 m above sea level. The climate is cool (MAT 16.7 ^{0}C) with a January mean monthly maximum of 25.8 ^{0}C and July minimum of 4.4 ^{0}C. An estimated mean annual rainfall of 998 mm and median annual rainfall of 899 mm has been reported (PALLETT and MITCHELL 1993).

7.2.2 Controlled pollination

A range of controlled crosses were conducted over the years following different mating designs depending on the objectives. The controlled pollination data is a compilation of a series of attempts to produce viable progeny of both pure and hybrid combinations. The number of mother trees varied for species from one season to the next largely due to insufficient and or sporadic flowering. The combination of graft incompatibility, disease and paclobutrazol resulted in losses of some mother trees before capsule collection. The number of flowers pollinated varied widely due to factors such as quantities of pollen and flowers available. While this does make interpretation more difficult, the results do provide insight into what seed yields per capsule can be expected from full-sib crosses and what hybrid combinations are possible.

Pollen was collected by placing excised branches into buckets of water. All opened flowers were removed leaving unopened flowers to open at room temperature under controlled conditions. On a daily basis, opened flowers were collected, anthers cut and placed into desiccators until dry. Pollen was extracted by shaking the pollen through a sieve (of < 50 μm) and, collecting and placing the clean pollen into plastic straws. Each clone was catalogued and stored separately (multiple straws) in airtight bottles with silica gel at 2-4 ^{0}C from 0 -24 months. Pollen was also brought from external sources such as Shell Forestry

Technology Unit, in the UK and used in a collaborative project to produce hybrids, particularly with *E. globulus* and *E. maidenii.*

The focus of the controlled crossing programme was to produce full-sib progeny for each of the pure species, to advance the generations, and to attempt some hybrid combinations. All crosses were conducted using the traditional approach to controlled pollination. Close to anthesis, buds were emasculated by carefully removing the stamens and anthers using a scalpel. Once all flower buds were emasculated, the section of branch was isolated with a bag (nappy liner) to exclude all insects from the flowers (Fig. 6.1). Depending on the species, pollen was applied to receptive stigmas with a fine toothpick and following pollination, the flowers were again isolated. When the stigma had blackened, the isolation bags were removed to allow full development of the capsules. From approximately 8-12 months later, remaining capsules were collected and seed extracted and counted.

7.2.3 Data analysis

Due to the nature of the collections with some parent trees eliminated because of low or no capsule production, most of the data was analysed as a completely random design to obtain means for various treatments and combinations. The ANOVA was conducted using the GENSTAT Statistical Programme.

7.3 Results

7.3.1 Intra-specific crosses

It was evident from the analysis of a random set of clones within each species that it is possible to improve the seed yield per capsule by way of controlled pollination (Table 7.1). *Eucalyptus macarthurii* produced on average 3.6 seeds per capsule in the clonal orchards following open pollination during peak flowering in August to September. With controlled pollination 7.6 seeds per capsule was achieved. Similarly, with *E. nitens*, which flowers from March to October open pollinated seed yields per capsule were low at 2.4 seeds. In controlled pollinations, 3.7 seeds per capsule were extracted.

Table 7.1: Mean seed yields per capsule harvested following intra-specific controlled pollination of some cold tolerant eucalypts at the Shaw Research Centre (SRC).

Intra-specific Crosses	Pollination Type	Number of Capsules Harvested	Total Number of Extracted Seeds	Number of Seeds per Capsule
E. macarthurii x E. macarthurii	Open pollinated	1165	4203	3.6
E. macarthuriii x E. macarthurii	Control pollinated	682	5193	7.6
E. nitens x E. nitens	Open pollinated	2943	7151	2.4
E. nitens x E. nitens	Control pollinated	1872	6942	3.7

7.3.2 Inter-specific crosses

After a series of attempts to produce hybrid combinations, it was evident from the capsules collected that *E. dunnii* combines well with a number of related species (Table 7.2). The best seed yield per capsule was achieved with *E. globulus* and *E. maidenii* as the hybrid partner, producing 7.8 and 11.4 seeds per capsule respectively. Combinations with *E. smithii* and *E. camaldulensis* were also good

but the small sample size may have biased this result. Combinations with *E. macarthurii* and *E. nitens* were the least productive, with both only generating 3.4 seeds per capsule.

Eucalyptus nitens produces flowers during the dry winter months of the year and controlled pollinations proved difficult due to losses of mother trees, capsule abortion and cold conditions. Again, combinations with *E. globulus* proved to be the most successful with 4.1 seeds per capsule. This may be due to the pollen tube and style length relationship. *Eucalyptus globulus* pollen has sufficient reserves to produce adequate pollen tubes (GORE *et al.* 1990). The poorest performance was the combination of *E. nitens* with *E. dunnii* with the production of only 1.9 seed per capsule (Table 7.3).

Table 7.2: Mean seed yields per capsule harvested following inter-specific controlled pollination of *E. dunnii* mother trees at the Shaw Research Centre (SRC).

Inter-specific Crosses	Number of Capsules Harvested	Total Number of Extracted Seeds	Number of Seeds per Capsule
E. dunnii x E. nitens	102	343	3.4
E. dunnii x E. macarthurii	2677	9143	3.4
E. dunnii x E. grandis	1421	4552	4.6
E. dunnii x E. urophylla	113	514	4.6
E. dunnii x E. smithii	11	71	6.5
E. dunnii x E. globulus	482	3751	7.8 *
E. dunnii x E. maidenii	145	1652	11.4 *
Mean	**4951**	**20026**	**4.0**

* Pollen from external source

Table 7.3: Mean seed yields per capsule harvested following inter-specific controlled pollination of *E. nitens* mother trees at the Shaw Research Centre (SRC).

Inter-specific Crosses	Number of Capsules Harvested	Total Number of Extracted Seed	Number of Seeds per Capsule
E. nitens x E. dunnii	146	277	1.9
E. nitens x E. globulus	389	1585	4.1*
E. nitens x E. grandis	447	1183	2.6
E. nitens x E. macarthurii	40	107	2.7
Mean	**1022**	**3152**	**3.1**

* Pollen from external source

Intra-specific crosses with *E. macarthurii* have been successful. Those in combination with *E. dunnii* were the best with a yield of 6.2 seeds per capsule. Controlled crosses with *E. grandis* produced 5.8 seeds per capsule (Table 7.4). Combinations with both *E. camaldulensis* and *E. globulus* were equally successful. However, the sample size was small.

Table 7.4: Mean seed yields per capsule harvested following inter-specific controlled pollination of *E. macarthurii* mother trees at the Shaw Research Centre (SRC).

Inter-specific Crosses	Number of Capsules Harvested	Total Number of Extracted Seeds	Number of Seeds per Capsule
E. macarthurii x E. dunnii	352	2187	6.21
E. macarthurii x E. camaldulensis	15	91	6.07 *
E. macarthurii x E. globulus	72	426	5.91 *
E. macarthurii x E. grandis	134	772	5.76
Mean	**573**	**3476**	**6.1**

* Pollen from external source

7.4 Discussion

The use of controlled pollination for the production of full-sib progeny to advance the generations of pure species and for the development of new hybrid genotypes is an important component of any tree breeding programme. The techniques applied demonstrate the inherent potential of each species, in that controlled pollinations produce much higher seed yields per capsule as opposed to open pollination with species such as *E. macarthurii* and *E. nitens*. Similarly, TIBBITS (1989) demonstrated an increase from 3.8 seeds per capsule, following open pollination of *E. nitens*, compared to an increase of 7.9 seeds per capsule after controlled pollination in orchards in Tasmania.

Many intersectional F_1 hybrids within *Symphyomyrtus* were unsuccessful or exhibit high levels of inviability. According to TIBBITS (2000), there is a trend for increasing success of controlled pollination with increasing taxonomic affinity between maternal and paternal species. Abnormal phenotypes are more often encountered when crossing species from the section *Maidenaria,* with crosses between species from the sections *Exsertaria* and *Transversaria* being atypically successful (POTTS *et al.* 2000; DE ASSIS 2000). This is evident from the crosses between *E. nitens* and *E. globulus* which produced much higher seed yields per capsule (4.1) as opposed to those of *E. nitens* with *E. grandis*, which only yielded an average of 2.6 seeds per capsule. TIBBITS (2000) had similar results with *E. nitens* x *E. globulus* yielding 6.8 seeds per capsule and *E. nitens* x *E. grandis*, 4.4 seeds per capsule. A similar trend could be observed with crosses between *E. dunnii* and other species. Although *E. dunnii, E. nitens and E. macarthurii* are closely related, the hybrid combinations produce on average lower yields than combinations with E. *grandis* and *E. urophylla* the more distant relatives. The crosses between *E. macarthurii* and other species showed very little difference for seed yield per capsule between the combinations irrespective of genetic distance.

While seed yield per capsule is a good measure of the pollination techniques applied it often has no bearing on the viability and field performance of the progeny produced. According to POTTS *et al.* (2000), vigorous individuals can be identified in the progeny, however the frequency of F_1 inviability is higher than that encountered in taxonomically closer species. A high level of seedling abnormalities and dwarfs are common with an increase in genetic distance crosses.

Chapter 8

SEED PRODUCTION

8.1 Introduction

In open pollinated seed orchards the ratio of viable seed produced relative to available ovules is low. Compared with open pollination, controlled pollination increases the of number seeds formed in each capsule. This is a clear indication that insufficient pollen is reaching the stigma (MONCUR *et al.* 1995). Seed orchards based on a number of provenances and families may comprise trees exhibiting a range of flowering periodicity thus resulting in poor synchronisation of pollination amongst trees.

Extracted seed usually consists of a mixture of fertile seed and chaff (unfertilized ovules). In *Eucalyptus alba*, a member of the *Symphyomyrtus,* fertile seed is produced towards the bottom of the placenta with two types of chaff, the elongated and cubic forms originating from different positions in the placenta region. By contrast members of the *Monocalyptus* produce fewer and less variable structures, with viable seed developing from the lowermost ovules (BOLAND *et al.* 1980).

The proportion by weight of viable seed relative to the overall dirty seed in a seed lot may vary greatly from species to species, orchard location and from season to season. According to GROSE and ZIMMER (1958) most eucalypts should comprise 3-20 % of the total dirty seed collected. It is vital to get an idea of what the expected recovery rates per species should be on an annual basis in order to gain insight into which factors, may require manipulation to influence the outcome. These may include environmental factors, orchard design, canopy

management or genetics. Given the potential of flowers to produce far in excess of what is currently realised, further investments are warranted to improve the genetic worth and reduce production costs of seed.

8.2 Materials and Methods

8.2.1 Plant materials

All seed harvesting operations were conducted across a range of orchards in the eastern half of the country, in the south at the Shaw Research Centre (SRC) in KwaZulu-Natal, situated at 29 ^{0} 29 ' South, 30 ^{0} 11 ' East at 1100 m above sea level (Fig. 8.1). The northern most orchards extreme extend as far north as Rooihoogte near Carolina in Mpumalanga situated at 26 ^{0} 10 ' South, 30 ^{0} 25 ' East at 1650 m above sea level. These summer rainfall areas have an estimated mean annual rainfall of between 850–1000 mm with peak temperatures in January and minima in July. Frost is common across all sites during the winter but less severe in KwaZulu-Natal (Table 8.1) (PALLETT and MITCHELL 1993).

Table 8.1: A summary of the seed orchards used in this study.

Orchard Number	Species	Type	Location	Year of Establishment	Breeding Programme
EB001T	*E. dunnii*	BSO	SRC KwaZulu Natal	1987	Sappi
EC015T	*E. smithii*	BSO	SRC KwaZulu Natal	1991	Sappi
EX002T	*E. smithii*	BSO	Shafton KwaZulu Natal	1987	Sappi
RP004T	*E. smithii*	LR	Hlelo Mpumalanga	1982	Sappi
EB004T	*E. macarthurii*	BSO	Rooihoogte Mpumalanga	1991	Sappi
EB005T	*E. macarthurii*	BSO	Hlelo Mpumalanga	1991	Sappi
EB008T	*E. macarthurii*	BSO	Comrie KwaZulu Natal	1991	Sappi
C1/82/NIT.AMS	*E. nitens*	BSO	Amsterdam Mpumalanga	1982	ICFR
C5/88/NIT.JAG	*E. nitens*	BSO	Jagtlust Mpumalanga	1988	ICFR

Eucalyptus capsules were collected by selective pruning of branches with the use of tree climbers or by felling whole trees. All capsules were kept separate by

tree and delivered to the seed processing facility at the Shaw Research Centre. Following drying of the capsules under normal ambient temperatures, the dirty seed (seed and chaff) was separated by hand and the mass recorded. With the help of the seed processing unit each batch of seed was cleaned and separated into five grades, the first four of which ranged between 600 μm and 1000 μm. All seed below 600 μm was considered as chaff or uncleanable.

Figure 8.1: A typical mature *E. dunnii* breeding seedling seed orchard.

8.2.2 Pure seed recovery rates of Eucalyptus dunnii, E. macarthurii, E. nitens and E. smithii

The by weight proportion of viable seed in a seed lot may vary greatly from species to species, orchard location and from season to season (Table 2.10). Very little information is available regarding the expected pure seed yields for a

range of *Eucalyptus* species grown in managed orchards (Fig. 8.1). This information is important to the understanding of the efficiency of the breeding system of the various species and essential to predict future seed yields.

Since 1998 seed collection operations have been conducted across Sappi forests landholdings and accurate records of the collections and processing have been kept. In most cases capsules were collected (Fig. 8.2) and kept separate by tree, however in some land race orchards, trees were bulked. The data from these various seed collection operations have been collated firstly to determine the pure seed recovery rates by species and secondly, to identify the possible impacts of location from season to season.

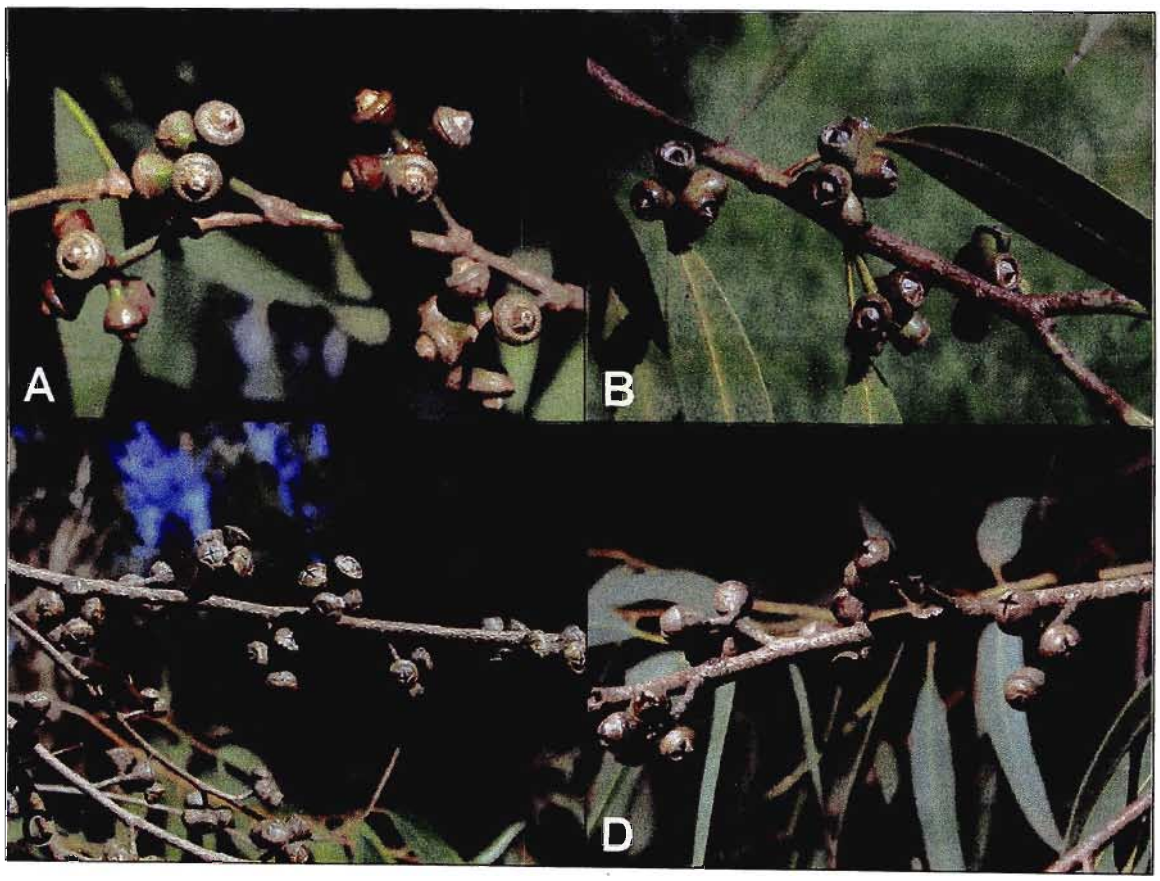


Figure 8.2: Ripening capsules of *Eucalyptus species* (A) *E. dunnii*; (B) *E. nitens*; (C) *E. macarthurii* and (D) *E. smithii*.

8.2.3 Data analysis

Due to the nature of the collections with some parent trees eliminated because of low or no capsule production, most of the data was analysed as a completely random design. We are unable to assume the equality of variance. The t-test is only an approximate test with the degrees of freedom calculated as a weighted average. The ANOVA and t-tests were conducted on angular transformed data using the GENSTAT Statistical Programme.

8.3 Results

8.3.1 Pure seed recovery rates of Eucalyptus dunnii, E. macarthurii, E. nitens and E. smithii

During the period 1999 to 2001 five separate harvests of *E. dunnii* were conducted in seed orchard EB001T at the Shaw Research Centre. Each harvest was restricted to one replication in the orchard that had an abundance of ripe capsules. From the analysis, when ignoring family, it was evident that both the year of harvest and replication showed highly significant differences ($p<0.001$) with regard to pure seed yields (Table 8.2). The harvest conducted in 1999 was significantly better than both the 2000 and 2001 harvests, while no differences could be detected between the seed harvests of 2000 and 2001.

The differences were largely due to the contribution of replication ten with an impressive yield of 34.2 % in 1999, however replication twelve only yielded 26.3 % in the same year. The overall pure seed yield that can be expected for *E. dunnii* from this site is 26.0 %, with the highest recorded yield of 47.6 % and the lowest of 4.2 % (Table 8.3).

Table 8.2: Matrix comparing differences between means of pure seed yields per replication following harvesting in the *E. dunnii* orchard EB001T, at the Shaw Research Centre over the period 1999 to 2001.

Replications						Pure seed recovery	
5		7	9	10	12	Angular Transformed Data	%
5		*(t37=0.58) ns*	*(t49=1.25.) ns*	*(t42=3.96) ****	*(t31=0.70.) ns*	29.06	23.9
7	*(t37=0.58) ns*		*(t52=0.37) ns*	*(t45=4.25) ****	*(t34=1.12) ns*	28.40	23.2
9	*(t49=1.25.) ns*	*(t52=0.37) ns*		*(t57=6.27) ****	*(t46=1.97) ns*	27.90	22.1
10	*(t42=3.96) ****	*(t45=4.25) ****	*t57=6.27) ****		*(t39=2.83) **	35.57	34.17
12	*(t31=0.70) ns*	*(t34=1.12) ns*	*(t46=1.97) ns*	*(t39=2.83) **		30.58	26.3
***E. dunnii* mean**						**30.31**	**26.0**

Ns = non signficant; * = (p<0.05); *** = (p<0.001)

Table 8.3. The overall pure seed recovered from collections of *E. dunnii* over the period 1999 to 2001 at the Shaw Research Centre.

Collection Year	Replication	No Trees	Pure Seed	
			Angular transformed data	Percentage
Differences between years				
1999	10 and 12	41	33.75 ***	31.3
2000	9	33	27.90	22.1
Mean difference in seed recovery between years is 5.843 ± 1.192 (t_{72} = 4.90) ***				
1999	10 and 12	41	33.75 ***	31.3
2001	5 and 7	38	28.69	23.5
Mean difference in seed recovery between years is 5.052 ± 1.263 (t_{77} = 4.00) ***				
2000	9	33	27.90	22.1
2001	5 and 7	38	28.69	23.5
Mean difference in seed recovery between years is 0.791 ± 1.116 (t_{69} = 0.71) ns				
***E. dunnii* mean**			30.31	**26.0**

ns = non significant; *** = (p<0.001)

A comparison between the two orchards of *E. macarthurii* in Mpumalanga has shown no significant difference between the two sites for pure seed recovery nor any significant difference between common families across the sites. The total seed yield at both sites was generally low due to light capsule crops with the overall recovery recorded at 25 %. At Hlelo the pure seed recovery was 22.5 % as opposed to Rooihoogte where the overall recovery was 26.9 % (Table 8.4). The recorded yield for single parent trees was 50.0 % at Hlelo and the lowest of 0 % at Rooihoogte. This large variation is typical of orchards that are just starting to produce seed. At Comrie in KwaZulu-Natal the best pure seed recovery for a single tree was 44.4 % and the lowest 16.1%. The capsule crop in this orchard was heavier reducing variation between trees. The mean pure seed yield that can be expected for *E. macarthurii* is 26.1 %.

Table 8.4: Pure seed recovered from three separate collections of *E. macarthurii* during 1999 in Mpumalanga and KwaZulu-Natal.

Site	Number of Trees	Pure Seed Recovery	
		Angular Transformed Data	Percentage
Differences between sites			
Hlelo	14	28.00 ns	22.5
Rooihoogte	19	30.4	26.9
Mean difference between sites is 2.4 ± 2.636 (t_{31}=0.96) ns			
Hlelo	14	28.00 ns	22.5
Comrie	29	31.26	27.2
Mean difference between sites is 3.26 ± 1.72 (t_{41}=1.92) ns			
Rooihoogte	19	30.4 ns	26.9
Comrie	29	31.26	27.2
Mean difference between sites is 0.86 ± 1.87 (t_{46}=0.46) ns			
Common families across both sites in Mpumalanga			
Hlelo	12	28.40	23.2
Rooihoogte	12	29.45	26.1
Mean difference between sites is 1.05 ± 3.508 (t_{22}=0.30) ns			
***E. macarthurii* mean**		30.5	**26.1**

ns = non significant

Harvesting of *E. nitens* capsules was also conducted during 2001 in orchards managed by the ICFR as part of the Co-operative Tree Breeding Programme. The collections and processing of the seed was conducted by Sappi Forests Research during 2001. From the results of this harvest the overall mean seed yield for *E. nitens* was 13.8 % with no significant difference between the two sites for seed yield (Table 8.5). The lowest recorded pure seed recovery was 3.5 % and the best was 31.7 % both from the Amsterdam site. At the Jagtlust site seed yields were lower in terms of overall pure seed recovery estimated at 11.1 % but the range between trees was less than that of the Amsterdam site.

Table 8.5: The percentage pure seed recovered from collections of *E. nitens* during 2001 in ICFR orchards in Mpumalanga.

Site	Trees Collected	Pure Seed Recovery	
		Angular Transformed Data	Percentage
Jagtlust	13	18.96	11.11
Amsterdam	33	22.11	14.84
Mean difference between sites is 3.158 ± 1.802 (t_{44}=1.75) ns			
***E. nitens* mean**		21.22	**13.8**

ns = non significant

Eucalyptus smithii was subjected to a similar investigation in which comparisons were made between three separate orchards in which seeds were harvested between 1999 and 2001. From the results it is evident that *E. smithii* has a low recovery rate of 18.0 %. The Shafton orchard site was the most productive with an overall pure seed recovery of 23.8 % followed by Tweedie at 15.4 % with the worst being at Hlelo in Mpumalanga at 12.7 % (Table 8.6). Both the Hlelo and Shafton orchards recorded the lowest recovery with some individual trees producing of no pure seed. The Shafton site had the greatest range of recovery rates followed by Hlelo and the SRC respectively.

A final analysis, was conducted to evaluate differences between species. The data was grouped ignoring sites and collection year for the purposes of ranking species according to pure seed recovery rates. The results clearly indicate that *E. nitens* produces significantly ($p<0.001$) less pure seed than the other three species. No significant difference could be found between *E. dunnii* and *E. macarthurii* in the analysis of this data set. *Eucalyptus smithii* was significantly better than *E. nitens* ($p<0.05$) but significantly ($p<0.001$) poorer than both *E. dunnii* and *E. macarthurii* respectively (Table 8.7).

Table 8.6: The percentage pure seed recovered from three separate harvests of *E. smithii* during 1999 in Mpumalanga and KwaZulu-Natal.

Site	Trees Collected	Pure Seed Recovery	
		Angular Transformed Data	Percentage
Differences between sites			
Shafton	38	28.16	23.8
Tweedie	25	22.61	15.4
Mean difference between sites is 5.55 ± 2.0 (t_{61}=2.78) *			
Shafton	38	28.16	23.8
Hlelo	30	19.91	12.74
Mean difference between sites is 8.25 ± 2.03 (t_{66}=4.07) ***			
Tweedie	25	22.61	15.4
Hlelo	30	19.91	12.74
Mean difference between sites is 2.7 ± 1.8 (t_{53}=1.53) ns			
***E. smithii* mean**		24.01	**18.0**

ns = non significant; * = ($p<0.05$); *** = ($p<0.001$)

Table 8.7: Matrix comparing differences between means of pure seed yields per species from various orchards for the period 1999-2001.

Species					Pure Seed Recovery	
	E. dunnii	*E. nitens*	*E. macarthurii*	*E. smithii*	Angular Transformed Data	%
E. dunnii		*(t156=9.07)* ***	*(t172=0.06)* ns	*(t203=6.36)* ***	30.31	26.0
E. nitens	*(t156=9.07)* ***		*(t106=7.66)* ***	*(t137=2.04)* *	21.22	13.8
E. macarthurii	*(t172=0.06)* ns	*(t106=7.66)* ***		*(t153=4.99)* ***	30.25	26.1
E. smithii	*(t203=6.36)* ***	*(t137=2.04)* *	*(t153=4.99)* ***		24.01	18.0
Mean					27.09	21.81

ns = non significant; * = ($p<0.05$); *** = ($p<0.001$)

8.4 Discussion

According to GROSE and ZIMMER (1958) most eucalypts should yield 3-20 % clean seed of the total dirty seed collected. Separating the seed from the chaff is often difficult, resulting in some seed losses. On a per species basis this is true for *E. nitens* having a mean recovery of 13.8 % and *E. smithii* as much as 18.0 %. Both *E. dunnii* and *E. macarthurii* produced a mean recovery well over 20 %. Individual trees within species demonstrate a great deal of variation ranging from 0 % to as much as 50 % recovery per tree. From the results *E. smithii* had the greatest range between trees followed by *E. macarthurii*, *E. nitens* and *E. dunnii*. There were differences between the harvesting year and location for the various species, however these are only indicators of possible trends, which require more detailed investigation in the future.

The complex interaction between environmental factors such as altitude, rainfall, and site together with species, flowering time and flower loading contribute to the ultimate recovery rates of pure seed. The potential of any species to produce seed has been clearly demonstrated through the use of controlled pollinations and in particular the one stop pollination technique. According to HARBARD *et*

al. (1999) pure seed recovery rates per capsule in *E. globulus* can be improved from 11.6 seed to 25.7 seeds per capsule using the one stop pollination technique. Similarly TIBBITS (1989) demonstrated with controlled pollination in *E. nitens* that seed yield per capsule could be improved from 3.8 seeds for open pollinated flowers to 7.9 seeds when manipulated. The wide range between recovery rates that were found for all species demonstrates the potential of each species. This variation is a clear indication that not only is insufficient pollen reaching the stigma, due to a possible lack of pollinating agents, but it is also possible that selfing is causing low seed set. The full potential of each species is often not realised due to a lack of synchronised flowering between adjacent trees as was the case in *E. globulus* growing in northwest Tasmania. Flowering occurred over five months with no pollen transfer between early and late flowering trees (MONCUR and BOLAND 2000). According to GROSSER *et al.* (2001) in a study of *E. nitens* progeny it was found that the parental contribution to the progeny varied amongst clones suggesting that panmictic pollinations are not occurring. This may be due to differences in flowering times subjecting each clone to a different pollen pool. The lack of panmictic pollination in *Eucalyptus* orchards highlights the need for information regarding flowering patterns, orchard design and pollinators to improve the genetic outcome and seed yields

Chapter 9

CONCLUSIONS

Flowering patterns of individual *Eucalyptus* species such as *E. nitens E. dunnii*, *E. macarthurii* and *E. smithii* are similar from one year to the next with same mean flowering peaks. TIBBITS (1989) found a good correlation in flowering from one year to the next and for trees within provenances of *E. nitens*, suggesting strong genetic control of flowering at least at the population level. It was found that very few species had similar flowering peaks to those documented for the natural distribution of each species. This was particularly apparent with *E. nitens* that flowered in mid-winter at the survey site in South Africa but is largely a summer flowering species in its natural range in Australia (GOODMAN 1973; POYNTON 1979; BOLAND *et al.* 1980; BROOKER and KLEINIG 1983). For the purposes of breeding and tree improvement it is apparent that some of the Cold Tolerant species have long reproductive sequences which delays the time from buds to seed, this is particularly apparent with species such as *E. smithii* and *E. dunnii.* The breeding cycles for these species are two to three times that of *E. grandis* due to an extended stage 2 bud phase. Both *E. nitens* and *E. macarthurii* have relatively short breeding cycles but are at least twice that of *E. grandis.* It may be possible to manipulate the environmental conditions to induce a flowering response and speed up the reproductive sequences of various species through cultural practices, such as nutrition , watering regimes and controlled environments thereby advancing or delaying the breeding cycle to best suite breeding and seed production objectives.

The use of paclobutrazol in breeding programmes can reduce the generation time significantly and increase seed production in orchards (MONCUR and BOLAND 2000). Paclobutrazol applied as soil drench resulted in an increase in the flower bud production in *E. nitens*, *E. smithii* and *E. grandis* in relation to little

or no flower production on young 1 to 2-year-old grafts at this site, particularly for *E. nitens* and *E. smithii*. The onset of early flowering not only allows for the production of seed but also facilitates the study of flowering patterns and clonal differences. The best time to apply paclobutrazol as a soil drench is early summer with the full impact of the application realised in the second season following application, approximately fourteen months later. This time delay could possibly be shortened with foliar spray applications or irrigation in clonal orchards to facilitate the uptake of the paclobutrazol during the winter months. The combination of reduced net biosynthesis of endogenous GA and exposure to cold temperatures with sufficient moisture will produced floral buds.

Successful seed set in eucalypts is dependant on many factors such as sufficient flowers, effective pollinators and viable pollen. With the production of full-sib seed through the use of controlled pollinations, viable pollen is an important component to successful seed set. The various cytochemical methods used were mere indicators of potential viability and lacked the reliability for adequate testing of stored pollen. While even the validity of *in vitro* testing may be questioned it has identified differences and similarities in response between species and clones within species. From the range of *in vitro* pollen viability studies the most successful media for all species tested was 30% sucrose with 150 mg l^{-1} boric acid. Without boric acid in the media the response after 24 hours was significantly poorer ($p<0.001$). POTTS and MARSDEN-SMEDLEY (1989) found optimal sucrose levels to range between 20-30 %, over a range of boric acid levels between 0 and 100 mg l^{-1}. GORE *et al.* (1990) was able to get a positive correlation ($r= 0.79$) between pollen grain diameter and *in vitro* pollen tube length although in this case not significant but highlight the importance of the direction of inter-specific hybrid combinations. Significant differences ($p<0.05$) in the area of pollen grains were found between and within species. There was no significant difference between *E. dunnii* and *E. macarthurii* at the species level. Pollen of *E. smithii*, *E. grandis* and *E. nitens* was significantly smaller than that of both *E. dunnii* and *E. macarthurii*. Overall *E. nitens* had the smallest pollen grains and

thus careful consideration needs to be given to how inter-specific crosses are made to ensure that pollen tubes can fertilize the ovules.

Sufficient flowering in orchards established in the exotic range does not necessarily lead to high seed yields and is often poor due to a lack of suitable pollinators (MONCUR *et al.* 1995). It is apparent that a reduction of potential pollinators not only leads to poor capsule survival but also poor seed set. The time of flowering could lead to reduce pollinator activity as there are indications that an association does exist between the presence of active pollinators and temperature. Little is known about the complex interaction between the environmental conditions, species flowering patterns, canopy structure, flower abundance and pollinators. The outcome of which, is often disappointing with reduced seed yields common across a number of orchards relative to the potential. The use of honeybees (*Apis*) does offer some hope but requires careful management to ensure that orchard designs and flower loadings facilitate good outcrossing and which impact the genetic worth and seed set.

The potential of flowers to set seed is clearly demonstrated by the difference between open pollinated flowers and controlled pollinated flowers following intra-specific crosses where differences in seed yield per capsule are very often more than double with *E. nitens* and *E. macarthurii*. TIBBITS (1989) demonstrated an increase from 3.8 seeds per capsule following open pollination of *E. nitens* with an increase to 7.9 seeds per capsule after controlled pollination in orchards in Tasmania. Similarly with inter-specific crosses higher seed yields are extracted from crosses between closely related species, but the emphasis is more focussed on number viable progeny from extracted seed. Abnormal phenotypes are more often encountered when crossing species from the section *Maidenaria,* with crosses between species from the sections *Exsertaria* and *Transversaria* (POTTS *et al.* 2000; DE ASSIS 2000).

Most eucalypts produce clean seed which ranges from 3-20 % of the total dirty seed collected (GROSE and ZIMMER 1958). In managed orchards this varies considerably from one year to the next with site, and from species to species. An extensive survey of orchards clearly demonstrated that *E. nitens* has the lowest clean seed recovery (13.8 %) significantly less than that of *E. smithii* (18.0 %) and both *E. macarthurii* and *E. dunnii* (26.1 % and 26.0 % respectively). Given the potential of each flower to produce far in excess of that which has been recorded emphasizes a possible lack of pollinators, or a high degree of selfing. It is also interesting to note that both *E. nitens* and *E. smithii* have some flowering which occurs during the cooler winter months of the year as opposed to *E. dunnii* and *E. macarthurii* which generally flower in the summer which could impact on the abundance of effective pollinators. From the comparison between seed yields of controlled pollinated flowers of *E. nitens* and *E. macarthurii* it is evident that *E. nitens* produces 50 % less seeds per capsule. A similar ratio is found when comparing the clean seed recovery rate from open pollinated orchards, suggesting that the different eucalypt species are predisposed to produce seed to certain threshold levels.

The seed yield potential in open pollinated orchards can be increased by including clones with overlapping flowering, enhancing the flower load through the use of growth retardants such as paclobutrazol and increasing the number of pollinators at the time of flowering.

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