Breeding Systems and Interspecific Hybridisation in the Genus *Eucalyptus* L'Hér.

By

Mark Fredric Ellis

Department of Horticulture, Viticulture and Oenology
Waite Agricultural Research Institute
The University of Adelaide
South Australia

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***Eucalyptus leucoxylon* F. Muell.**

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APPENDIX: Ellis, M. F., Sedgley, M., and Gardner, J. A. 1991. Interspecific pollen-pistil interaction in *Eucalyptus* L'Hér.(Myrtaceae): The effect of taxonomic distance. *Annals of Botany* **68**, 185-94.

SUMMARY

This study investigated the reproductive biology of four *Eucalyptus* species of the subgenus *Symphyomyrtus*; *E. spathulata*, *E. cladocalyx* and *E. leptophylla* of the section *Bisectaria*, and *E. leucoxylon* of the section *Adnataria*.

Aspects of the breeding system, floral morphology and pistil cytology were studied in three trees each of *E. spathulata*, *E. cladocalyx* and *E. leptophylla*. *E. spathulata* and *E. leptophylla* were found to be highly self incompatible, setting very low levels of seed from controlled self pollination. *E. cladocalyx* trees ranged from self compatible to self incompatible. Reductions were seen in both the number of capsules and the numbers of seed per capsule, from self pollination. The mechanism of self incompatibility was investigated in the pistil by following the success of cross and self pollinations with fluorescence microscopy. In *E. cladocalyx* and *E. leptophylla* no reduction in ovule penetration was seen from self pollination while in *E. spathulata* a significant reduction was seen in two trees but not the third, indicating post-zygotic mechanisms of self incompatibility operating in all three species, with some pre-zygotic control in *E. spathulata*. Floral architecture differed between the three species in the structure of the inflorescence units, flower morphology, and anther, pollen and ovule numbers per flower. Pistil cytology was similar for all three species but there were differences in the length of the stylar canal, the degree of sclerotinisation of the style, stigma morphology and volume of transmitting tissue.

The breeding system of *E. leucoxylon* was investigated with emphasis on the unusual features of gynodioecy and secondary pollen presentation. Fifty seven percent of trees in the study population were found to be male sterile, with pollen grains aborted during development between tetrad formation and anthesis. Hermaphrodite trees presented 93 percent of pollen grains on the upper style and stigma, with only seven percent of pollen grains remaining on the anthers. In the absence of pollinators hermaphrodite trees set low levels of autogamous seed compared with open pollinated pistils. Counts of pollen tubes in open pollinated pistils of each morph revealed that female trees were pollen limited in the study population. Multilocus estimates of outcrossing rates were determined for female

isozyme electrophoresis. Female trees showed values of ($\hat{t}$) approaching 1.0, indicating complete outcrossing, while hermaphrodite trees showed significant levels of selfed seed in open pollinated seed crops, but still maintained an outcrossing rate higher than that reported for most eucalypts.

The pollen-pistil interaction was investigated in three intraspecific, 57 interspecific and six intergeneric crosses using the three species *Eucalyptus spathulata*, *E. cladoclayx* and *E. leptophylla* as female parents. Interspecific prefertilisation isolation was found to occur in the pistil and manifested as a number of pollen tube abnormalities in the style and ovary associated with a lowered probability of ovule penetration. The major selection points in the pistil were the upper style and the ovary. The severity of abnormalities and the probability of pollen tube arrest in the pistil was proportional to the taxonomic distance between parent species. Ovule penetrations were seen mainly in crosses within the section *Bisectaria* or between the sections *Bisectaria* and *Adnataria*. Pollen storage, style length and mean maximum temperature during the flowering period of the male parent had no significant effect on pollen tube growth in the crosses used.

Intra and interspecific combinations which showed ovule penetration were repeated the following flowering season and monitored for seed set. Most combinations produced capsules although only combinations within the section *Bisectaria* and between the sections *Bisectaria* and *Adnataria* set viable seed. There was considerable variation between years in cross compatibility with some female trees which showed no ovule penetration in the first year setting viable seed in the same combinations the following year. Many close combinations set levels of seed approaching intraspecific cross pollinations, while interspecific crosses outperformed intraspecific self pollinations in both capsule set and the number of seeds per capsule. Some crosses between the sections *Bisectaria* and *Adnataria* showed hybrid breakdown at the early seedling stage. Hybrid parentage was confirmed through intermediate seedling morphology.

The findings of this study have implications for the ecology and conservation of *Eucalyptus* species, for breeding strategies in tree improvement programmes and for the taxonomic relationships of the species.

Declaration

I HEREBY DECLARE that the work presented in this thesis has been carried out by myself and does not incorporate any material previously submitted for another degree in any university. To the best of my knowledge and belief, it does not contain any material previously written or published by another person, except where due reference is made in the text. I am willing to make the thesis available for photocopy and loan if it is accepted for the award of the degree.

M. F. Ellis

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1. INTRODUCTION

1.1 THE GENUS *EUCALYPTUS*

The genus *Eucalyptus* L'Hér. consists of over 500 species most of which occur on the Australian continent with a few species occurring in New Guinea and nearby islands. The genus dominates much of the Australian flora, finding a niche to be exploited in all of the many climatic and ecological regimes across the continent. It occurs primarily as the dominant or co-dominant tree genus, and ranges from stunted coastal mallee (multiple branches from the basal lignotuber) only a meter high to the tallest flowering plant in the world, *E. regnans*, the Mountain Ash up to 100 meters tall. Variability within the genus and within species is high, a wide range of forms, with a variety of growth habits, morphological variations, environmental tolerances and characteristics are found. As such the genus represents a huge store of genetic variability, a potential only starting to be utilized by foresters and horticulturalists.

Until recently utilization of eucalypts in Australia was based mainly upon naturally occurring stands, the main uses being timber production (Boland *et al.*, 1984), as a source of nectar and pollen for honey production and to a much lesser extent eucalyptus oil production (Small, 1977), with some planting for amenity purposes. However soon after the introduction of eucalypts overseas into countries with similar climates their potential for commercial plantations was realised. Today eucalypts form a major part of tropical and subtropical-temperate forestry in Africa, Asia and the Americas and are the basis of industries of timber, pulp and eucalyptus oil production.

Other uses are developing for the versatile genus. Many species have been used in ornamental horticulture and amenity plantings and many more have potential, possessing the attributes of fast growth rate, attractive flowers, hardiness and being attractors of native birds. In agriculture overclearing has led to soil erosion and dryland salting. The establishment of windbreaks and salt resistant species helps alleviate these effects, eucalypts forming the majority of the plantings. Commercial woodlotting and agroforestry are also gaining popularity as farmers try to diversify farm incomes and

ameliorate adverse climatic conditions, with eucalypts playing a significant role (Gholtz, 1987). Eucalypts can also be used as a commercial crop on rehabilitated mine sites, salinized land and woodlots irrigated with waste water (Buckley, 1988; Allender, 1988), their characteristic of high rates of water usage being turned to advantage in this situation.

In Australia, as the need to protect remnant natural forest is realised, more emphasis is being placed on eucalypt plantations (Boutland and Byron, 1987). Growth rates in plantations are often faster than in regrowth natural forest and productivity is higher due to uniformity of age and form. Plantation productivity is partly determined by the genetic quality of the seed source. To increase growth rates, improve tree form and select for specific requirements for local environmental conditions or end products, breeding and genotype selection is needed. This has stimulated research into breeding systems and the development of breeding programmes with multiple objectives of increasing yields, form and environmental tolerances.

Effective conservation and management of native forest and woodlands depends upon a sound knowledge of the biology and genetics of the constituent species. Issues affecting the viability of populations include habitat fragmentation, land clearance, timber harvesting, altered fire and recruitment frequencies, disturbance, and introductions of non indigenous plants and animals. Further research into the processes governing reproduction in *Eucalyptus* will enable informed decision making and ensure viability of populations.

1.2 PROJECT AIMS

In an effort to extend our knowledge of the factors controlling reproduction and gene flow within and between eucalypt populations this project seeks to add information on the following areas in the genus *Eucalyptus*.

- To investigate breeding systems of species in the section *Bisectaria*, a group for which little breeding system information is available.

- To investigate aspects of floral morphology and reproductive behaviour in *E. leucoxylon*, including the features of pollen presentation and gynodioecy, a reproductive strategy not previously reported in *Eucalyptus* , and to assess their effect on the breeding system.

- To investigate the existence of physiological pre-fertilisation interspecific isolation mechanisms in the genus and their relationship with taxonomic distance.

- To monitor the process of hybridisation to the F1 seedling stage in combinations that lack physiological pre-fertilisation isolation mechanisms, measuring the variables of seed set and viability and their relation to taxonomic distances.

The results from this series of investigations can be used for the formulation of breeding programmes for the domestication and improvement of commercial eucalypt species, and will facilitate a better understanding of ecological and reproductive processes.

2. LITERATURE REVIEW

2.1 TAXONOMY

Eucalyptus was first described in 1788 by L'Héritier, the type specimen being *Eucalyptus obliqua* L'Hér. from Tasmania, although some other species were initially placed in other genera eg. *Symphyomyrtus lehmanii* Schauer (Lehman, 1844) and *Eudesmia tetragona* R.Br (Flinders, 1814). The system used to arrange species into groups by Bentham (1867) in *Flora Australiensis* was based on antherial characteristics and was used in modified form by several authors in further reviews of the genus (Mueller, 1879-84; Maiden, 1909-33; Blakely, 1934). Maiden (1909-33) in his revision of the eucalypts reviewed the characteristics available for arranging the known species into natural groups. He considered characteristics of habit, bark, timber, exudations, petiole, leaf, cotyledon and inflorescences including anthers, buds, pollen grains and fruit.

Blakely (1934), recognising 500 species and 150 varieties, used characteristics of the stamen for classification of sections and subsections and other vegetative and floral characteristics for series and subseries. At this level the classification is artificial (Chippendale, 1988). Pryor and Johnson (1971) proposed a system of classification not relying so heavily on antherial characteristics but based on opercular structures, ovule and seed coat structure as described by Grosse and Zimmer,(1958), and morphological features such as arrangement of stamens in the bud and other chemical, biotic and genetical systems.

They divided the genus into 7 informal subgenera (*Blakella*, *Corymbia*, *Gaubea*, *Idiogenes*, *Eudesmia*, *Monocalyptus* and *Symphyomyrtus*) with *Angophora* Cav. remaining as a separate genus. This did not rule out the possibility of raising the subgenera to generic status (as suggested by Johnson and Briggs, 1983), after clarification of the question of mono or polyphyletic origin of the subgenera (Pryor, 1976). Ladiges and Humphries (1983) however considered *Eucalyptus* to be a monophyletic group and *Angophora* to be a sister group not equivalent to the subgenera. Subgenera are characterised by distinct groups of characters without intermediates and gradations between groups. Features used for separation of subgenera are anatomy and development of the operculum, ovule orientation, cotyledon morphology, etc. (Carr and Carr, 1959,1968;

Pryor and Knox, 1971; Johnson, 1976). The subgenera also show some trends in preference for ecological regimes and habitats (Florence, 1981). However there is some controversy over the status of some subgenera. Johnson (1976) subdivided *Symphyomyrtus* by placing the sections *Equatoria* and *Howittaria* in a new subgenus *Telocalyptus*, but Ladiges and Humphries (1983) believe it does not warrant classification as a subgenus. Similarly they say the monotypic subgenus *Idiogenes* and the small subgenus *Gaubea* should not be recognised and together with *Monocalyptus* form a monophyletic group. The status of the subgenus *Eudesmia* is also uncertain, some members show a greater affinity with *Symphyomyrtus* than with other members of *Eudesmia*, indicating its polyphyletic origins (Ladiges and Humphries, 1983).

Within the subgenera the relationships in *Monocalyptus* have been most extensively researched. Ladiges *et.al.* (1983, 1986, 1987) have used cladistic and biogeographical methods to clarify the relationships within the subgenus, and have proposed changes at the level of section and below for some groups.

In *Symphyomyrtus* there is little literature pertaining to relationships. Pryor (1959) stated there was evidence of the groups *Adnata* and *Macranthera* (sections *Bisectaria* and *Transversaria*) from the fossil record in the Miocene indicating these groups had already diverged from each other. Pryor and Johnson (1971) allude to an affinity between the sections *Bisectaria* and *Transversaria*, and *Transversaria* and *Exsertaria* but a lack of affinity between *Exsertaria* and *Dumaria*. Cladistic analyses have yielded information on the possible evolutionary relationships within *Symphyomyrtus* (Chappill, 1988) which differ from the observations of Pryor and Johnson (1971).

In the classification proposed by Pryor and Johnson (1971) the categories of genus, subgenus, section, series, subseries, superspecies, species and subspecies were used, and were intended to reflect phylogeny. Further research has resulted in some changes to these groups and more are proposed (Ladiges *et al.*, 1984)

Johnson (1976) addressed the problem of grouping populations into categories of species. In most cases the constituent species of the superspecies show some intergradation or are very similar and could in some opinions be regarded as conspecific. Conversely some subspecies could be raised to specific level. Johnson (1976) stated that it

does not matter if populations are regarded as separate species or as subspecies as long as 1) there is a name in some rank for the focal character combination, 2) their ecological and geographic status is recognised, 3) their affinity is perceived and 4) situations resulting from interbreeding between them can be recognised. In *Quercus* L., a genus with much intergradation and gene exchange between species, Burger (1975) supported the concept of species being applied to natural populations easy of recognition and not genetically isolated populations.

2.2 BREEDING SYSTEMS

"The breeding system of an organism describes the probabilities of different kinds of gametes coming together to form zygotes" (Eldridge, 1976), and is controlled by many physiological, floral and genetical mechanisms.

2.2.1 The flower

The typical *Eucalyptus* flower is bisexual and has numerous free stamens in several rows inserted on a staminal ring surrounding the simple style. The ovary is adnate to the lower part of or the whole of the hypanthium and contains 2-7 locules, each cell containing numerous ovules. The developing bud is protected by an operculum derived from the sepaline and/or the petaline whorls, and is shed in one or two parts as the flowers open (Carr and Carr, 1959).

The flowers occur in inflorescences commonly termed axilliary umbels, axilliary panicles or terminal panicles although the umbel is actually a condensed dicasium with some apparently single infloresences being composed of multiple units. The number of flowers per inflorescence varies between one and fifteen or more but for the lower numbers is usually consistent within species and is taxonomically useful (Pryor and Johnson, 1971).

Anther morphology varies widely between species (Blakely, 1934) and may differ in terms of anther size and shape, mode of attachment to the filament, prominence and location of the anther gland and mode of dehiscence. Despite this there is much structural similarity, all anthers consisting of two bisporangiate lobes (Davis, 1968, 1969). Pollen

grains are numerous (Moncur and Boland, 1989) and are typical of myrtaceaous pollen, they are triangular and tri-colpate.

The style is simple but differs in length, width and rigidity between species indicating differences in cytological composition that may reflect different growth conditions for pollen tubes. Stigma morphology is characteristic of taxonomic groups and varies between the mop like stigmas of the subgenus *Corymbia* with long papillae to the blunt or pinhead stigmas of the subgenus *Symphyomyrtus*, all stigmas are papillate and styles posses a stylar canal of varying lengths (Boland and Sedgley, 1986). On the classification of Heslop-Harrison and Shivanna (1977) the eucalypt stigma is wet, producing a secretion that covers the stigmatic surface in which pollen grains adhere and germinate (Anderson, 1984; Griffin and Hand, 1979).

The eucalypt ovary is multilocular (Cremer, 1965) with many ovules per locule (Davis, 1969) some of which are sterile and are termed ovulodes (Carr and Carr, 1962). The number of locules and ovules in each varies between species, the number and placement of ovules and ovulodes on the placenta being taxonomically valuable information (Carr and Carr, 1962). The eucalypt capsule is a false fruit developed from the inferior ovary which is adnate to and surrounded by the calyx tube (Cremer, 1965). Mature capsules contain fertile seed, aborted seed and chaff (congenitally sterile ovules) (Drake, 1975). In some species these seed types are readily distinguishable from each other morphologically, in others more detailed investigation of their contents must be made before identification. In *Eucalyptus camaldulensis* Dehnh. Zucconi (1959) found the chaff particles, which consisted of external tegument and funiculus only were situated in the apical cone of the ovary and were quite distinct in shape and colour from the fertile seeds. Sterile seeds resulting from the abortion of ovules at all stages from gametogenesis to embryogenesis were found below the chaff while the fertile seeds containing a complete embryo were found at the base of the ovary. However Davis (1969) found in *E. stellulata* Sieber ex DC., 50% of fertile ovules showed abnormalities which led to their eventual degeneration, the collapsed ovule consisting of integuments only. This was not restricted to position on the placenta.

In *E. regnans* the two vertical rows of ovules in each locule consist of two horizontal rows of ovulodes at the distal end of the placenta and three rows of ovules , with significant variation in the proportion of filled seed at each position (Griffin *et.al.* 1987). However no capsules were found with an ovule/seed ratio of greater than 50% and the mean population estimate was only 9% which could not be increased by controlled outcrossing.

So in eucalypts the number of seeds set is related to the number of fertile ovules, in some cases their position on the placenta and maternal resource allocation (Griffin *et al.*, 1987; Stephenson, 1981).

2.2.2 Floral initiation

In *Eucalyptus* the floral buds are at first concealed by an operculine involucre which is shed during development , after which the buds develop as exposed umbels (Ashton, 1975). There is wide variation in the duration of bud development , in *E. grandis* W.Hill ex Maiden the floral buds are first recognisable four months before anthesis (Davis 1968), whereas in *E. regnans* F. Muell., *E delegatensis* R. Baker and *E. fastigata* Deane and Maiden they are initiated 29 months before flowering (Fielding, 1956; Ashton, 1975), 12 months before anthesis in *E. macarthuri* Deane and Maiden and six and a half months in *E. cinerea* F. Muell. ex Maiden (Polunina, 1963).

The season of flowering may vary significantly in some species,. flowering occurring intermittently (Griffin, 1982) e.g. *E. nutans* F.Muell. (Waite Arboretum records) others having a well defined flowering period (Griffin, 1980). Other species e.g. *E. leucoxylon* F. Muell. and *E. sideroxylon* Cunn. ex Woolls have a very long flowering period spanning up to six months (Waite Arboretum records).

In fact Davis (1968) showed that *E. cinerea* and *E. melliodora* Cunn. ex Schauer flower at the same month in its natural habitat in Australia as when planted in the northern hemisphere, despite seasonal reversals. The same is true for sporogenesis in *E. camaldulensis* in Italy, although anthesis is delayed until summer in both hemispheres.

Flower abundance and subsequent fruiting varies between seasons, Cunningham (1957) and Ashton (1975) reported a two year cycle for *E. regnans* with heavier cropping every fourth year.

2.2.3 Pollination

The flowers of most eucalypts are generalised in structure and not adapted for specific pollen vectors, and as such are frequently visited by a range of potential animal pollinators (Eldridge, 1976; Ford *et al.*, 1979). The Australian biota is noted for its high percentage of bird pollinated plants, and the abundance and diversity of nectar feeding birds (Ford and Paton, 1986). Birds have been observed visiting flowers of about half of the species of eucalypts (Ford *et al.*, 1979). Most eucalypt flowers are visited by a range of potential pollinators including native insects, (bees, flies, moths beetles etc.) introduced honey bees (*Apis mellifera* L.) and small arboreal mammals. Some eucalypt species show a degree of adaptation to a particular pollinator. The large flowers of *E. cosmophylla* F. Muell. produce copious nectar (200-400 Joules nectar per day Ford *et al.*, 1979) and are particularly attractive to birds, which carry pollen on the beak and feathers. *E. stoatei* C.Gardner. seems to be exclusively bird pollinated, the stamens of the large pendulous flowers forming an impenetrable dome over the floral cup thus denying entry to insects (Hopper and Moran, 1981). Smaller flowers attract mainly insects (Ford *et al.*, 1979). The foraging habits of the pollinator will determine the amount of self/cross pollen delivered to the stigma. Hopper and Moran (1981) observed that 18% of honeyeaters movements on *E. stoatei* was between individuals, implying a high degree of cross pollination.

2.2.4 Self compatibility

This is an important factor in determining the genetic makeup of the seed crop, as almost all species tested seem to be capable of setting seed after self pollination (Eldridge, 1976). Hodgson (1976,b,c) found seed yields in *E. grandis* after selfing were 2-47% of those from cross pollination. Several studies have used allozyme analysis to determine outcrossing versus inbreeding rates in natural seed populations of eucalypts. Similar

results have been obtained for several species, with outcrossing rates ranging from 63% for *E. pauciflora* Sieber ex Sprengel (Phillips and Brown, 1977), 76% for *E. obliqua* (Brown *et al.*, 1975) and 65-85% for *E. delegatensis* (Moran and Brown, 1980). All species produce a significant proportion of self fertilised seed. *E. delegatensis* retains seed crops of several successive seasons in its canopy. Moran and Brown (1980) found the inbreeding rate decreases with age of the seed crop in *E.delegatensis* implying either differences in outcrossing rates with season or differential viability of inbred as opposed to outcrossed seed since fertilization. The latter explanation is supported by the findings of Phillips and Brown (1977) that when measured in seedlings the effective outcrossing rate was increased to 84% in *E. pauciflora* suggesting a higher mortality for inbred seeds.

Controlled self and cross pollination experiments in *E. regnans* showed preferential outcrossing. When a 1:1 self:cross pollen mix was used an average of 81% of resultant seeds were outcrossed (Griffin *et al.*, 1987). As no obvious differences between self and cross pollen tube growth in the style had been observed previously they suggested that the mechanisms for preferential outcrossing in *E. regnans* operate post fertilization and are dependant on both embryo genotype and maternal resource allocation. This conclusion was further supported by Sedgley and Smith (1989) who found no difference in ovule penetration between self and cross pollinations. Such mechanisms have been shown for other genera (Seavey and Bawa, 1986) and may function to increase fitness of the mother plant by directing resources to the fittest offspring and aborting the rest. In another species, *E. woodwardii* Maiden, Sedgley and Smith (1989) found that although pollen tube growth in the styles was comparable, fewer ovules were penetrated by self than cross pollen indicating there may be a pre-zygotic component to the relationship. Inbreeding depression resulting from self fertilization has been demonstrated in *E. regnans* (Eldridge and Griffin, 1983; Griffin and Cotterill, 1988) and in *E. gunnii* J. D. Hook. (Cauvin *et al.*, 1987; Potts and Cauvin, 1988) where in field trials self pollinated families showed low survival and growth relative to cross pollinated families.

The evidence so far points towards a successive weeding out of inbred genotypes from seed set (Griffin *et al.*, 1987), to germination (Phillips and Brown, 1977), to growth and competition in the wild (Phillips and Brown, 1977; Eldridge and Griffin, 1983), with

the end result that the percentage of mature dominants produced from outcrossing does not reflect the percentage of self versus cross pollen delivered to the stigma. So in the absence of a strong self incompatibility mechanism *Eucalyptus* achieves a high degree of outcrossing.

2.3 REPRODUCTIVE ECOLOGY

Flowering plants generally produce many more seeds than are needed for individual replacement. This is due to extreme temporal and spatial patchiness in the regeneration niche, ie. conditions necessary for germination and successful establishment. Eucalypts are no exception, most mature trees produce seed crops each year although cyclic variation in the size of seed crops have been noted (Cunningham, 1975). The long developmental phase from floral initiation to anthesis and subsequently to seed maturation causes reductions in reproductive units through herbivory, environmental damage and abscission through stress (Drake, 1981). Further reductions in seed and capsules occur due to abscisson from reproductive failure through pollen limitation or may occur due to incompatible matings (Sedgley, 1989) or selective fruit and seed abortion (Stephenson and Bertin, 1983)

Capsule dehiscence occurs after the fruit dries out, tissue shrinkage causing the valves at the top of the fruit to open (Cremer, 1965). Some species shed seed as soon as the capsules are ripe but most retain seed in the canopy for several years (Cremer, 1965; Cunningham, 1957, Moran and Brown, 1980). Seed is shed when branches die, are severed or in response to aging of the limb or environmental factors such as fire (O'Dowd and Gill, 1980). Factors causing seedfall are often linked to those creating a suitable niche for germination. *E. regnans* which grows in high rainfall areas of Victoria and Tasmania sheds its seed in response to fire, in which mature trees are killed. Fire reduces competition from established plants and creates the conditions necessary for seedling germination and establishment. *E. camaldulensis* sheds seeds soon after maturity in late summer, while seed dispersal and seedling recruitment follows seed shed during flooding events (Bren 1988).

Mallee species recruit infrequently needing a combination of fire and several subsequent years of good rainfall for successful recruitment (Wellington and Noble, 1985a). Competition from established plants for soil moisture usually prevents successful establishment. Such species are often long lived and are capable of surviving fire through regeneration from the basal lignotuber, so recruitment events are infrequent.

Eucalypt seeds are generally small and light (Grosse and Zimmer, 1958) and have short dispersal ranges, as seen through patterns of natural regeneration (Venning, 1988), although seeds of *E. calophylla* R. Br. ex Lindley are winged perhaps aiding wind dispersal (Langkamp, 1987).

Massive seed loses also occur after capsule dehiscence and seed scattering, through deposition in unfavourable habitats, herbivory and removal by ants (Wellington and Noble, 1985b). Hodgkinson *et al.*, (1980) noted a significant decrease in the number of *E. populnea* F. Muell. seeds in topsoil over a twelve month period.

Thus recruitment events are often infrequent in this long lived tree genus requiring a combination of events for successful establishment. Reproductive strategies are a trade off between providing a sufficient seed store to exploit regeneration opportunities and maintaining the genetic quality of the seed pool.

2.4 NATURAL HYBRIDISATION.

2.4.1 Occurrence of intermediates

Natural hybridisation of sympatric eucalypt species pairs has been reported many times. The first records of hybrids in the field were based mainly on intermediate morphology usually in mixed stands. However recent studies involve a more thorough investigation of morphological, ecological and phenological characteristics, chemical properties and progeny testing and may be compared with manipulative hybridisation of the two putative parents. Using these parameters a more accurate picture can be drawn upon the origin of the individuals in question, whether they be isolated individuals, a segregating swarm or a broad cline.

Difficulties often arise in the correct interpretation of stands of individuals showing characteristics intermediate between two species. The possibility exists that the

intermediates are formed by interspecific hybridisation, and represent the F1 or maybe more complex hybrids consisting of later generations and backcrosses, this being referred to as a hybrid swarm. Other possibilities include the remnants of cline forms between two closely related species which may indicate incomplete divergence and speciation, or lastly the development of intermediate phenotype through selection from a single species. There are many examples of striking convergent and parallel evolution among plant species especially in relation to similar environmental conditions.

Parsons and Kirkpatrick (1972) identified these three possible origins for stands of individuals intermediate in morphology between *E. cypellocarpa* L. Johnson and *E. goniocalyx* F. Muell. ex Miq. but where the pure species were absent and may have represented old swarms stabilised by selection from which the parents had disappeared. Such swarms are termed "phantom hybrids " indicating the absence of one or more putative parents. A similar occurrence was noted in an *E. cypellocarpa* population by Kirkpatrick *et al.*, (1973) in which individuals intermediate between *E. cypellocarpa* and *E. globulus* Labill. were identified and showed characteristics of hybrids, while the nearest *E. globulus* Labill.was 6.4 km away.

Clinal variations have been demonstrated within many eucalypt species in response to environmental conditions, e.g. with respect to altitude in *E. pauciflora* (Pryor, 1957), with respect to latitude in *E. viminalis* Labill. and *E. dalrympleana* Maiden (Phillips and Reid, 1980). A continuous cline was also demonstrated between *E. viminalis* and *E. dalrympleana* at the southern region of their distribution while on the mainland hybrid zones 50m wide between these two species had been reported (Phillips and Reid, 1980). Whereas complete clinal zones were found extensively in Tasmania, mainland stands of the pure species were more phenotypically distinct than those occurring further south. It is interesting to note that Pryor and Johnson (1971) placed these two species in the same series but different subseries perhaps indicating their degree of relationship. These types of patterns may be the result of incomplete speciation, with the extremes of the clines being recognised as separate species, or the results of past hybridisations introducing variability into populations providing a morphological link between the species. Past hybridisations

now stabilised have been suggested as a possible explanation for variation within *E. ovata* Labill. (Clucas and Ladiges, 1979) and *E. leucoxylon* (Boland, 1978)

2.4.2 Hybrid fitness

In many instances of putative natural hybridsation the individuals examined appear to be F1 progeny due to their low variability and the typical morphological segregation of their seedlings (Ashton and Sandiford, 1988; Clifford, 1954; Drake 1980, 1981a; Hopper *et. al.* 1978; Pryor, 1956b; 1981b). These initial hybrids may be the result of chance events such as fire enabling recruitment within the time the hybrid seed is retained in the canopy, or be regular occurrences at the interface of two potentially interfertile species. However their contribution to the gene pool and thus gene flow between the species will depend on their reproductive output and the adaptiveness of their progeny to local conditions. These correspond to the three phases of hybridisation described by Drake (1980), i) F1 plant establishment, ii) plant fertility, iii) evolutionary potential. He noted two species pairs which produce natural hybrids, *E. melanophloia* F. Muell. x *E. crebra* F. Muell. and *E. populnea* x *E. crebra*. *E. melanophloia* x *crebra* does not progress beyond phase one. In terms of canopy size, proportion of trees bearing fruit, number of fruit set and susceptibility to biotic damage, this hybrid had a very low reproductive output being 10% of the average of parental trees. This effect was also shown by Hopper *et al.*, (1978) in that seed set of *E. preissiana* Schauer x *buprestium* F. Muell. individuals was less than the average of parental species. This is consistent with the hypothesis that the hybrids were showing partial F2 breakdown. *E. populnea* x.*crebra* F1's however showed high fertility compared with the parents and there was evidence of expansion of the hybrid population into one or both parent species habitats. Other studies of hybrid seed production show intermediate production and viability compared with the parents e.g. *E. elaeophora* F. Muell. x *goniocalyx* F. Muell. ex Miq (Clifford, 1960).

If the F1 is fertile then the next most important factor in the success of hybrid forms is the interaction with the local habitat, which will determine to what extent progeny of the hybrids and their backcrosses will survive. The possibilities are a segregating swarm in which all combinations are viable and in which many parental characteristics

occur, a segregating swarm in which most intermediate forms are not viable (Pryor, 1950),or the intermediates themselves being most suited to exploiting a particular environmental niche with the elimination of parental types (Potts and Reid, 1983). Hartney (1965) suggested that where hybridisation is successful the lack of random recombination between characters, by partial linkage or genotype elimination at the gametic or zygotic stage prevents the total breakdown of distinctions between species. Such coherence results in the majority of characters remaining in their parental combinations. The possibility of long distance pollen migration and subsequent selection for parental types in the progeny of later generations led to the suggestion that hybridisation acts as a mechanism of species migration (Potts and Jackson, 1986; Potts and Reid, 1988). Rogers and Westman (1979) stated that the presence of niche complementarity amongst sympatric eucalypt species pairs places hybrid offspring of intermediate morphology at a disadvantage relative to the pure species. Davidson *et al.* (1987) found no evidence of introgression between *E. pulchella* Desf. and *E. delegatensis* in the field yet found putative hybrids in the progeny of two *E. pulchella* trees raised in seed beds. In some cases however the intermediates may colonise local microhabitats more efficiently than either parent or provide a foothold for genetic combinations with a nearby species via long distance pollen dispersal (Potts and Reid, 1983).

2.4.3 Patterns of hybridisation

In natural situations the frequency of hybridisation depends upon a number of conditions most importantly,the co-occurence of species pairs, synchronous flowering and the presence of pollinating agents. The problems of geographic isolation are overcome in mixed species plantations both in Australia and overseas and flowering times are often varied with the selection of particular genotypes for commercial use. Manipulative hybridisations performed by emasculation and controlled interspecific pollination can further increase the range of genotype combinations (van Wyk, 1977).

The other factor determining the success of hybridisation between taxa is the extent of reproductive isolation. Pryor and Johnson (1971) noted there was no evidence of interbreeding between the subgenera. Griffin *et al.*, (1988) in a review of the occurrence of

natural and manipulated hybrids went further to assess the records of hybridisation on the basis of relationship using Pryor and Johnson's (1971) taxonomic revision of the genus. Their findings agreed with the general observations made by previous authors, that the subgenera are effectively isolated from each other, but that hybrids within them are frequent and fertile. There are no reports of natural intersubgeneric hybrids and only reports of failed attempts at manipulative crossings resulting in no seed set or weak seedlings (Griffin *et al.*, 1988).

Within subgenera all possible species pairs were ranked according to 1) taxonomic position (intraseries, intrasection, intersection), and 2) the distance between the two species at the nearest part of their natural range. Each known hybrid was assigned a reliability rank, determined on whether it was known from a single herbarium record or if more extensive analysis was performed to establish its identity. All but five of the 520 recorded natural hybrids are between species pairs of the lowest geographical rank.

No natural hybrid combinations were recorded for *Idiogenes* (monotypic), *Gaubea* (two species) or *Telocalyptus* (four species). Within the other subgenera the occurrence of hybrid combinations reflects the hierachy of taxonomic affinities. In decreasing frequency hybrids occur within series, between series and between sections. However there are deviations from this rule as in the frequencies of hybridisation in certain groups. Griffin *et.al.* (1988) underline the fact that of all combinations geographically possible only 15% have been observed in nature, contrasting with the relative ease of obtaining manipulative hybrids. Of the 528 species reviewed 289 (55%) are recorded as occurring in at least one hybrid combination.

In conclusion it seems two major independent factors influence genetic integrity and gene flow between species, first the degree of reproductive isolation, which appears to be on the basis of degree of relationship of species pairs and secondly ecological adaptivness of any successful hybrid progeny.

2.5 CHROMOSOME STUDIES

Chromosome studies in *Eucalyptus* have revealed that most species have a constant 2N=22 with a few species 2N=24 (Federov, 1969; Rye, 1979). However up to

three differing counts for some species and the existence of fertile hybrids between species of different chromosome numbers leaves doubt as to whether numbers other than 2N=22 are accurate. This is made more plausible by the description of eucalypt chromosomes as small and easily subject to breakage (Ruggeri, 1960; Rye 1979). Related genera such as *Leptospermum* Forster and Forster f. and *Melaleuca* L. are also remarkably consistent in their chromosome number, most species being 2N=22 (Smith-White, 1948).

No natural polyploids have been identified in *Eucalyptus* but allotetrapoids and allotriploids have been synthesized experimentally and show promise in eucalypt improvement (Kapoor and Sharma, 1984b and 1985)

2.6 IMPROVEMENT

The increasing use of eucalypts for plantation forestry (both overseas and in Australia), for land reclamation or ornamental horticulture and amenity planting, requires the selection of the most suitable species and genotypes. As the genus contains more than 500 species with a wide range of growth habits and tolerances one or more species may be found suitable, but due to substantial variation within species selection of the best geographical form (or provenance) can lead to increases in yield or suitability. Introduction trials of eucalypt species outside their natural range have identified many species suited for production in local conditions. In a trial of 36 mainly eastern states species in the south east of South Australia Cotterill *et al.*, (1985) showed the potential of 13 species all of which out produced *Pinus radiata* D. Don (the major local plantation species) in the first four years of growth. Similar extensive testing has led to identification of species with potential in the U.S.A. (Hunt and Zobel, 1978), Mexico (Fierros and Musalem, 1978), South Africa (Anon., 1987) and the Northern Territory (Cracium, 1978).

Testing of physiological tolerances such as salinity tolerances (Blake, 1981; van der Moezel and Bell, 1987) and frost (Franclet and Boulay, 1982; Meskimen *et al.*, 1987) can also identify species with potential.

Field trials of different provenances of the same species have shown significant variation in survival and several growth parameters under local conditions (Burgess,

1975; Griffin *et al.*, 1982; McKimm and Ilic, 1987; Orme 1978; Sands, 1981; Siddiqui *et al.*, 1979). This is not surprising given the wide distributions of some eucalypt species, for example *E. tereticornis* Smith spans a latitudinal range of 7 degrees to 38 degrees (Matheson and Mullin, 1987), many other species with smaller distributions show a similar diversity of habitats and growth forms as in *E. obliqua* which ranges from a stunted small tree in coastal heaths to a forest tree up to 60m in height (Brown *et al.*, 1976; Chippendale and Wolf, 1981). In the plantation forestry situation selection of the provenance most suited to the climatic and soil conditions can increase yield and avoid losses due to environmental factors such as frost (Ades and Burgess, 1982; Rook *et al.*, 1980). Chemical analysis of tissue has also revealed differences between provenances which may be related to growth parameters and morphology (Abd-Alla *et al.*, 1980; Pedderick and Lennox, 1979)

Intensive eucalypt breeding programs now exist both in Australia and in several other countries, including France (Potts and Potts, 1986), India (Venkatesh and Sharma, 1980), South Africa (van Wyk, 1987), Brazil (Brune and Zobel, 1981), and the U.S.A. (Meskimen *et al.*, 1987). Breeding programs have different emphases depending on local conditions and climate, eventual uses of the crop and species used. A characteristic feature of the eucalypt plantations of Southern Africa is the narrow range of genotypes in the original introductions. Selection of superior forms and land races suited to local conditions from such a narrow genetic base leads to sub-optimal growth and other limitations. Comparisons of locally developed land races with native Australian provenances in field trials show that there are still significant gains to be made in the introduction of new material and through provenance testing (Matheson and Mullin, 1987). A re-introduction of improved strains from overseas seed orchards to Australia and comparison with local material has also shown the superiority of certain natural provenances (Orme, 1978). Ades and Burgess (1982) showed superiority of natural provenances of *E. grandis* over African improved seed lots in N.S.W., a result confirmed in field trials in South Africa. Field trials have shown that significant gains in productivity can be made by family selection within provenances (Ades and Burgess, 1982; Brown *et al.*, 1976; Wilcox *et al.*, 1980)

In the course of breeding and in mixed species plantations hybrid seed has been produced, in some cases the resultant hybrids show much potential in their growth characteristics and their progeny have been included in breeding programs (Marien and Thibout, 1978; Potts *et al.*, 1987, van Wyk, 1987; Venkatesh and Sharma, 1980)

Genetically superior material may be propagated by seed or clonally. Seed for plantations is collected from natural stands or from plantations and seed orchards in the case of land races. Seed orchards provide the most reliable source of superior cross pollinated seed due to the establishment of a wide variety of genotypes of superior quality. But potential is limited by the source of parent genotypes and the degree of outcrossing. Carson (1988) stressed the need for regional seed orchards with *Pinus radiata* , after finding significant genotype-location interaction. Practices ensuring a high frequency of out crossed seed include, gathering seed from stands after heavy flowering when the frequency of outcrossing is high, and increasing diversity of origin of stock in seed orchards. Too often improved seed is tested against degenerate, possibly inbred controls (Griffin, 1988).

Clonal forestry of eucalypts is used to perpetuate particularly good phenotypes or genetic combinations and is widely practised in France, Brazil and in the U.S.A.(Meskimen *et al.*, 1987; Potts and Potts, 1986).

2.7 PROPAGATION

In natural stands eucalypts reproduce almost exclusively by sexual reproduction, apomixis is unknown (Pryor and Johnson, 1971) and only a few northern species are rhizomatous (Boland, 1986). Although many species are able to coppice and regenerate from lignotuberous buds, this does not lead to the formation of new individuals (McComb and Bennet, 1986). The majority of commercially used eucalypts are propagated from seed. This is a simple procedure, seeds of most species germinate readily, with only a few species needing a period of cool moist stratification to break dormancy (Larsen, 1965; Turnbull and Doran, 1987). Seed remains viable for many years. When selecting for particular growth forms, physiological characteristics or morphologies, propagation from seed can lead to an undesirable variation in the progeny. Unless controlled pollinations

have been performed parentage of the seed is unknown and a significant proportion may be inbred leading to inbreeding depression (Griffin, 1988). For these reasons some breeding programs have employed vegetative propagation of superior clones.

Many tree crops can be propagated by hardwood cuttings, however adult eucalypt tissue contains a rooting inhibitor (Paton *et.al.* 1970) limiting rooting capacity to seedling material and rejuvenated shoots from epicormic buds. Epicormic shoots develop in most eucalypts with the exceptions of several important commercial species such as *E. regnans*, *E. nitens* (Deane and Maiden) and *E. delegatensis* etc (Hartney, 1980). Many species have been successfully propagated by this method which is used for clone multiplication in commercial plantations (Meskimen *et.al.* 1987, Potts and Potts, 1986; van Wyk, 1987). Igboanugo (1987) had limited success in rooting excised tissue from three year old lignotubers from three species.

The grafting of superior clones onto seedling rootstock is another method used although problems with graft incompatibility and low success rates render it rather expensive. It is used mainly for the establishment of seed orchards or the propagation of horticultural cultivars (Hodgson 1977; McCombe and Bennet 1986; Ryan, 1966; van Wyk 1977).

Tissue and organ culture is the other major method of vegetative propagation of eucalypts. In vitro propagation has the advantages of producing clones of large numbers of individuals in a relatively short space of time, using only small amounts of starting material. The limitations of in vitro propagation are the difficulty of sterilization of material from field grown trees and the rooting of shoots from mature plants (McComb and Bennet, 1986). Sterilization presents a problem as tissue is often killed in sterilizing solutions although this can be overcome in most species. Adult material is difficult to root in vitro but seedling explants give good shoot multiplication and rooting ability. Lakshmi-Sita and Vaidyanathan (1979) used five day old material of *E. citriodora* Hook. to produce 100 fold shoot multiplication in four months. The use of seedlings has the disadvantage of using untested material. Alternatively adult material can be rejuvenated by coppicing or maintenance in culture for long periods (Greenwood 1987; McCombe 1984). The breeding of frost resistant eucalypts in France depends on field testing of seedlings

followed by cutting back of superior plants to yield rejuvenated shoots for micropropagation. This technique is used to produce 25,000 plants a month from selected clones of *E. gunnii*, *E. dalrympleana* and their hybrids (Franclet and Boulay, 1982)

Adult material has been used successfully in several species eg. *E. citriodora* (Gupta *et.al.* 1981) but as shown by Burger (1987) the shoot multiplication and rooting ability of mature explants was inferior to explants from coppice.

Callus can be produced and cultured from both juvenile and mature material but shoots regenerated from mature callus cultures show the same low rooting ability of mature shoot explants (McCombe and Bennet, 1986). Although the cost of production of plants from tissue culture can be higher than seedling production the perpetuation of known genotypes can lead to significant gains.

2.8 METHODS OF HYBRIDISATION

The technique used for artificial hybridisation is very similar to that used in manipulative intraspecific pollination. As the eucalypt flower is bisexual it must be emasculated before the application of experimental pollen, stamens are removed by making a cut just below the staminal ring, above the ovary (Griffin and Hand, 1979). If done just prior to operculum shed and thus anther dehiscence, a pollen free pistil is achieved. It has been shown that most eucalypts are protandrous, so pollination cannot proceed until the stigma is receptive. Experiments investigating seed set, pistil age and condition have shown maximum receptivity is correlated with stigmatic secretion and usually occurs at about 6-12 days after operculum shed, when the stigma becomes swollen and exudes a sticky secretion, (Griffin and Hand, 1979; Hodgson, 1976a; Sedgley and Smith, 1989). The exact timing depends on the species and longevity of the flower and the stigma may remain receptive for up to ten days (Griffin and Hand, 1979). Pollinations before the stigma becomes receptive are mostly unsuccessful (Griffin and Hand, 1979; Sedgley and Smith, 1989). Fresh pollen may be applied by "anther" (from a fresh flower) or by brush, flowers used as a pollen source are bagged prior to anthesis to ensure an uncontaminated pollen source.

The use of interspecific pollen presents an additional problem in the form of availability of fresh pollen. If non-synchronously flowering species are used pollen must be stored. *Eucalyptus* pollen has been shown to be remarkably robust in terms of mechanical sturdiness, capacity to withstand heat stress and immersion in hypotonic solutions (Heslop-Harrison and Heslop-Harrison, 1985). Griffin *et al.*, (1982) described a method for pollen storage which after purification involved desiccation for 48 hours then storage at room temperature or at -16° C. Results of *in vitro* pollen viability tests showed that high viability was maintained for one year at -16°C but room temperature storage was only adequate for much shorter periods. Similar techniques have been reported for other woody plant genera (Akihama and Omura, 1986; Williams and Rouse, 1988)

Techniques of controlled pollination without emasculation have been used. Su and Wu (1984) treated *E. saligna* Smith flowers with 400ppm ethephon to kill stamens and recorded no damage to pistils. Chemical emasculation techniques are not widely used .

2.9 MANIPULATIVE HYBRIDISATION

Hybridisation between dissimilar genotypes or species is a method often used in plant improvement and breeding. The advantages of combining two genotypes are many fold. The transfer of one or a few genes from one species to another can be beneficial in the development of disease resistance, plant or fruit quality, etc. New characters may be expressed by the F1 not seen in either parent, the increase in heterozygosity often leading to superior growth and yields via heterosis (Kriebel, 1973). Alloploid plants can be produced if parents of different ploidy levels are used. The success of hybridisation can also give information on the relationship of one species to another.

Hybridisation can be effective between different strains of the same species between species and sometimes between genera, the success of hybridisation usually being dependant on the degree of relatedness (Carr *et.al.* 1988) although groups differ widely in this respect. It is used routinely for increasing vigour, adaptability and introducing disease resistance of crop plants such as forage crops and legumes (Fehr and Hadley, 1980). Hybridisation in tree crops is still in its developmental stages. Natural hybrids were

recognised early in *Eucalyptus* (see Maiden, 1909-33), *Betula* L. (see Clausen 1970) and *Quercus* (Wiegand 1935), but due to long generation times and the resources needed for cultivar testing progress has been slow in research into the potential for hybridisation in the improvement of tree crops. Most of the work to date has been done on northern hemisphere forest crops and fruit trees.

Hybridisation is useful in transferring disease resistance from one species or cultivar to another. Dutch elm disease severely affects American elms while some Eurasian elms are largely resistant (Karnosky and Mickler 1986). Considerable research effort is being placed into developing resistant hybrids, but this is complicated by differences in ploidy level (Ager and Guries, 1982). Resistance to root rot was identified in avocado rootstocks of G755 variety, this variety was recently identified as a natural hybrid between avocado (*Persea americana* Miller) and coyu (*P.sheideana* Nees) (Ellstrand *et al.*, 1986). The hybrid *Picea glauca* (Moench.) Voss x *sitchensis* (Bong.) Carr. was recognised as being potentially cold resistant and fast growing, combining the two parental attributes (Fowler, 1987). *Eucalyptus* hybrids are used in selection trials in France for frost resistance (Potts *et al.*, 1987).

The transfer of particular attributes between varieties is not the only reason for hybridisation. Wide crosses within species or between related species often leads to an increase in heterozygosity resulting in hybrid vigour or heterosis. The hybrids show better growth and yields than either parents. This is common in crop plants (Forsberg and Smith, 1980), but is not the rule in tree crops. Some studies have found the usefulness of hybrids, Sijde and van der Roelofsen (1986) predicted a 50% increase of yield from pine hybrids over pure species from early growth results in South Africa. Venkatesh (1987) also reported higher yields from *Eucalyptus* hybrids than pure species in field trials. However other studies have shown hybrids to be intermediate between the parents (Brissette and Barnes, 1984). Wide hybridisation may be unsuccessful due to either the failure to produce F1 progeny or in terms of hybrid weakness or hybrid breakdown. However, hybrid breakdown and sterility is absent in several major tree crops eg. *Prunus* L. (Bell and Hough, 1986)

2.10 REPRODUCTIVE ISOLATION

Griffin *et al.*, (1988) calculated the frequencies of hybrid combinations between co-occurring eucalypt species with respect to various taxonomic ranks. For most groups only a small proportion of geographically possible combinations had been reported. This may be due to the incompleteness of records but it most probably reflects the absence of many combinations in the wild, indicating that some species are reproductively isolated from each other (Brooker, 1979). The mechanisms of isolation may be temporal, ecological or physiological and may exert their effect pre or post -zygotically. Differences in flowering time can be an effective barrier to interbreeding and although years may occur when the two flowering seasons overlap to some extent the probability of any hybrid seed formed in such chance events contributing to the next generation are low. Ecological isolating mechanisms such as absence of common pollinating vectors can effect pre-zygotic isolation, while niche complementarity can effect post-zygotic isolation by selecting against intermediate hybrid seedlings (Rogers and Westman, 1979).

Physiological isolating mechanisms can act at a number of stages of the reproductive cycle. They result in either no seed set or reduced viability of any resultant seedlings. Manipulative hybridisations have been performed on a number of species pairs (Griffin *et al.*, 1988), while many have been successful, a number of others have not yielded viable offspring. The majority of unsuccessful combinations involve intersubgeneric crosses, while some combinations within *Symphyomyrtus* have also been unsuccessful (Beardsell *et al.*, 1979; Pilipenko 1969). The reason for failure of these manipulated hybridisations must be characteristics inherent in the reproductive system of the species involved.

While the concept of increasing genetic disharmony with increasing taxonomic distance is widely accepted the amount this factor determines the extent of hybridisation and the amount determined by physiological isolating mechanisms such as incompatibility or incongruity within *Eucalyptus* is not known.

Incompatibility is known to operate at the intraspecific level in many genera (de Nettancourt, 1977). Self incompatibility may be defined as a genetically controlled mechanism which prevents the normal functioning of the pollen pistil relationship, and

restricts inbreeding. Some eucalypt species have been shown to exhibit weak self incompatibility (Potts and Savva, 1989; Sedgley and Smith, 1989). Interspecific incompatibility prevents gene flow between species, and is suggested to be under similar genetical control (de Nettancourt, 1977) and maintains an upper limit of outcrossing. However interspecific incompatibility has not been proven to be controlled by the same loci as self incompatibility (Ascher, 1986; Sedgley, 1989). Hogenboom (1975, 1984) suggested incongruity as the major limiting factor to interspecific hybridisation. The reproductive system of each species having diverged through evolution so co-ordination between pollen and pistil of different species is incomplete, leading to a breakdown of the pollination pathway or later after fusion of the nuclei.

Breakdowns may occur at any stage of the pollen pistil interaction from pollen deposition on the stigmatic surface to seed formation. In the *Eucalyptus* flower, at peak receptivity the stigmatic surface is covered by an extracellular secretion (Griffin and Hand, 1979). Analysis of the constituents of stigmatic secretion in other species reveals carbohydrates, proteins, lipids, enzymes, phenolics and amino acids (Heslop-Harrison and Shivana,1977; Knox, 1984). It is in this medium that pollen adhesion, hydration and germination occur. A failure to respond to the chemical signals of the stigma or inappropriateness of the stigmatic secretion can result in a failure of pollen pistil interaction at this stage. In poplar (Guries and Stettler, 1976) and *Ulmus* L. (Bob *et al.*, 1986) both of which possess dry stigmas, the major barrier to wide hybridisation within the genus is the failure of pollen germination on the foreign stigma. However Williams *et al.*, (1982a) also noted this as a characteristic point of arrest in some interspecific *Rhododendron* L. crosses, the *Rhododendron* stigma is covered in secretion at receptivity, as in *Eucalyptus* .

After germination pollen tubes must penetrate the stigmatic surface. The failure to penetrate has been observed in some incompatible crosses in poplar (Gaget *et al.*, 1984), *Rhododendron* (Williams *et al.*, 1982b), and *Ulmus* (Ager and Guries, 1982). Within the stylar transmitting tissue pollen tubes commence growth towards the ovary. Cytological studies of style composition show both the spherical, glandular cells of the stigmatic zone and the thick walled more elongate cells of the transmitting tissue may

secrete an extracellular mucilage into the intercellular spaces of the wall. It is through this intercellular matrix the pollen tube grows (Knox, 1984).

Most dicotyledons have solid styles but *Eucalyptus* possesses a semi-solid style with a stylar canal of varying lengths (Boland and Sedgley, 1986). In open styles characteristic of many monocotyledons, pollen tubes grow through the mucilage filled canal. Observations of pollen tube growth in *Eucalyptus* have shown pollen tubes in compatible crosses grow intercellularly through the transmitting tissue, after germinating on the stigma surface adjacent to the papillae and to only a limited extent in the stylar canal (Sedgley and Smith, 1989).

The style is an important place for pollen tube rejection by the pistil (Villar and Gaget, 1988). It is the major site of expression of the gametophytic self incompatibility response (de Nettancourt, 1977). Glycoproteins involved in the S allele incompatibility response have been detected in the stylar matrix (Knox and Williams, 1986). The stylar cells may also communicate with the ovary by way of plasmodesmata, stimulating a post pollination response (Knox and Williams, 1986). Pollen tubes of incompatible interspecific crosses may also be arrested in the stylar region (Williams *et al.*, 1982a). This could be due to the exhaustion of reserves contained in the pollen grain, the stylar tissue withholding nutrients required for its heteromorphic nutrition, or to active inhibition of pollen tube growth (Sedgley, 1989). The growth of the pollen tubes may be directed towards the ovary chemotrophically by the transmitting tissue (Knox, 1984).

Another factor influencing the growth of interspecific pollen in the style is style length. Disparate style lengths have been shown to be effective barriers to interspecific fertilization in *Rhododendron* (Williams and Rouse, 1988) and *Darwinia* Rudge (Briggs, 1964), the pollen of short styled species either failing to reach the ovary of the long styled species or failing to effect fertilization after reaching the ovary. Some abnormalities of tube growth have also been noted in crosses involving pollen of long styled species in the pistils of short styled *Rhododendron* species (Williams *et al.*, 1986; Williams and Rouse, 1988).

As the pollen tube elongates callose plugs are formed to partition the actively growing tip containing the vegetative nucleus, generative cell and other organelles from the older parts of the pollen tube consisting of vacuolated cytoplasm (Sedgley *et al.*, 1985).

Entry into the ovary, micropyle and embryo sac are all possible points of arrest of incompatible pollen tubes as seen in *Rhododendron* (Williams *et al.*, 1982b) and other species (Seavey and Bawa, 1986). The arrest of pollen tubes at any point from stigma to ovary is usually associated with a number of syndromes, both errors in tip growth and anomalies in callose deposition in external walls and plugs (Williams *et al.*, 1982b). In interspecific crosses the same pollen may fail at different points in the pistils of different species (Williams *et al.*, 1985)

The most commonly used technique for observing the growth of pollen tubes in the pistil is fluorescence microscopy. Sections or squashes are stained with aniline blue and callose in the pollen tube walls fluoresces yellow-green contrasting with the dull stylar tissue (Ager and Gueries 1982; Martin, 1959, Sedgley and Blesing, 1982; Sedgley and Smith 1989). Scanning electron microscopy is also used for examination of pollen stigma interactions and the pathway of pollen tube growth in the ovary (Vilar *et al.*, 1987).

Entry of pollen tubes into the embryo sac and subsequent release of gametes is harder to observe and requires serial sectioning of embryo sacs (Williams *et al.*, 1982b). After fertilization by interspecific pollen, zygote development may be arrested for several reasons such as disharmonies between the two genomes, between one genome and the other cytoplasm or disharmony between the zygote and the endosperm (Ahmad *et al.*, 1988; Fehr and Hadley, 1980). Maternal resource allocation may play a part in determining which zygotes are allowed to complete development. Preferential seed or fruit abortion may select the fittest zygote genotypes thus eliminating weak combinations (Stephenson and Bertin, 1983).

2.11 ALTERNATIVE METHODS OF HYBRIDISATION

Barriers to effective fertilization occur between many potentially interfertile species, and to overcome these barriers a number of techniques of by-passing or reducing the effect of the barrier have been developed. As the site of foreign pollen rejection in most species is the stigma or style most methods involve facilitating the entry of pollen tubes into the ovary.

Compatible pollen has often been used to elicit the post pollination response necessary for satisfactory tube growth. The "mentor pollen " can be applied as a mixture with foreign pollen (Payan and Martin, 1975), as killed mentor pollen (Gaude *et al.*, 1985) or as pollen extract (Knox *et al.*, 1972). The function of the mentor pollen is thought to be to provide recognition substances (cell wall proteins), missing from the pollen of foreign species. Hybrid seed set has been stimulated in Poplar (Knox *et al* 1972b) by this method.

Another method of inducing germination and tube growth on foreign stigmas is the use of solvents applied to the stigma surface. Whitecross and Willing (1975) suggested that if pollen wall proteins are involved in the recognition process, then a maternally derived substance on the stigma surface must also be involved. The treatment of poplar stigmas with lipid solvents increased the yield of hybrid seed following interspecific pollination, higher yields were achieved than with mentor pollen. Pryor and Willing (1974) used hexane applied to stigmas to obtain hybrids between incompatible pairs of eucalypts. Temperature can also affect the degree of inhibition of pollen tube growth in foreign styles as found in *Cucumis* L. (Franken *et al.*, 1988)

Some techniques used to reduce stylar inhibition of pollen tube growth involve reducing the distance the pollen tubes have to grow. In *Prunus* this is accomplished by using the shortest styled species as the seed parent (Layne and Sherman, 1986; Weinberger, 1975). Other techniques include style amputation and pollination of the cut stump (Raff, 1983), or intraovarian pollination (Heslop-Harrison *et al.*, 1985; Zentkeler, 1980).

In some interspecific hybridisations viable embryos are formed but are later aborted due to non-functioning of the endosperm or interaction with the maternal genotype. With the development of in vitro cell culture methods for propagation of tree crops comes the possibility of embryo rescue (Raghavan, 1977). Vamaguchi *et al.*, (1987) performed embryo rescue successfully on *Camellia* L. interspecific hybrids, the embryos were excised just prior to degeneration of the endosperm. Embryo culture may also be used to accelerate germination and multiply clones without waiting for seed maturation (Raghavan, 1980).

Another approach to hybridisation of incompatible species is the by-passing of sexual reproduction via somatic hybridisation. This technique has been used widely on herbaceous genera and with some success in woody plants (Grosser *et al.*, 1988; Schieder and Vasil, 1980; Williams *et al.*, 1987,). Somatic hybridisation involves the fusion of somatic protoplasts of two species, effectively producing allotetraploids. Fusion products are screened for hybrid cells and plantlets regenerated in vitro (Vasil and Vasil, 1980).

Embryo rescue and somatic hybridisation have not been performed on *Eucalyptus* but advances in cell culture techniques (McCombe and Bennet, 1986) and protoplast separation from *Eucalyptus* show some potential for these methods (Rhaghavan, 1980).

Another method for combining genotypes of incompatible species is to use a bridge species to transfer genes from one species to another. This method has the disadvantages of long generation times and the introduction of unwanted characteristics from the bridge species. Viable tri-hybrid eucalypts have been produced successfully by Venkatesh and Sharma (1980).

2.12 IDENTIFICATION OF HYBRIDS

The method usually used for the verification of hybrid *Eucalyptus* seed is the screening of seedlings for morphological characteristics intermediate between putative parents. Several groups of eucalypts show characteristic cotyledon shape (Johnson, 1976). Hybrids between such groups can be identified by intermediate cotyledon shape (Beardsell *et al.*, 1979; Kapoor and Sharma, 1984a; Pryor 1954a, 1956a, 1958). Other seedling characteristics such as phyllotaxy (Brooker, 1970), glaucousness and leaf shape can also be indicative of hybrid origin (Ruggeri, 1959). Seedling morphology has been used in verification of hybrid origin in other genera eg. *Rhododendron* (Rouse *et al.*, 1985).

This technique works well when morphological markers are present at the seedling stage and allows large numbers of seeds, potentially from open pollinated sources to be screened.

A more conclusive technique applied to other tree crops is isozyme analysis. The genotypes of species with respect to particular loci can be established by starch gel

electrophoresis and species and cultivars can be characterised by the occurrence or frequency of particular isozymes (Arulsekar and Parfitt, 1986; Weeden and Lamb, 1985). F1 hybrids can be identified by their combination of parental isozymes (Chaparro *et al.*, 1987; Parfitt *et al.*, 1985). This method allows determination of the origin of cultivars by comparing isozyme composition with other members of the group (Ellstrand *et al.*, 1986; Hakoda, 1987; Hancock and Iezzoni, 1988).

Isozyme analysis has been used in *Eucalyptus* mainly for determination of outcrossing rates, as within species variation at some loci is sufficient to establish parentage of intraspecific crosses (Fripp, 1982; Fripp *et al.*, 1986; Griffin *et al* . 1987; Hopper and Moran, 1981). Isozymes have also been used to characterize species and populations. For example *E. saligna* and *E. grandis* are morphologically similar and are often confused and Burgess and Bell (1983) identified isozyme characteristics strongly diagnostic for each species. Moran and Hopper (1983) found wide differentiation of populations of *E. caesia* Benth. at 11 loci and Moran and Bell (1983) grouped polymorphic loci in *Eucalyptus* into three categories according to the level of genetic variability. From the range of eucalypt species tested it appears that similarity of isozyme patterns depends on the degree of relationship. Thus isozyme analysis has potential for the identification of *Eucalyptus* hybrids, especially from controlled crosses or seed batches of known parentage.

2.13 CHARACTERISTICS OF HYBRIDS AND FIELD TRIALS

Studies on the morphology of F1 *Eucalyptus* hybrids generally show characteristics intermediate between parental types. This attribute is an aid to detection of natural hybrids in the field. The juvenile foliage is often a distinctive part of a species' morphology, hybrids between species with different juvenile foliage usually display intermediate qualities as in *E. viminalis* x *bridgesiana* R. Baker (Pryor, 1950) which is intermediate in degree of glaucousness, phyllotaxy and leaf shape between parents, with generations later than F2 segregating for these characteristics.

Adult foliage of hybrids also shows intermediate characteristics as shown by morphometric analysis of hybrids of *E. risdonii* J.D.Hook and *E. amygdalina* Labill.

(Potts and Reid, 1985) and *E. regnans* x *macrorhynda* F. Muell. ex Benth. (Ashton and Sandiford, 1988). Venkatesh and Sharma (1979) compared *E.tereticornis* x *grandis* controlled hybrids with putative natural hybrids which were shown to be intermediate between parental types in characteristics such as leaf disposition, shape, length, colour and stomatal frequency. In more complex hybrids, later than F1 or backcrosses the inheritance of characteristics such as leaf shape have been shown to be additive (Potts and Reid, 1985). Flowers and fruit which are important taxonomic features in *Eucalyptus* have been observed in a number of hybrids. The number of flowers per inflorescence and other features such as exerted or inserted valves are relatively constant within species with other characteristics such as disk width, locule number and fruit size being slightly more variable but nevertheless useful.

Pryor (1954b) found that in crosses between three and seven flowered species the seven flowered condition was partially dominant with three flowered umbels often found in the basal pair of axils of the F1 flowering shoot. While departures from the three or seven flowered condition occurs in some F1s, fruit size is usually intermediate between parental types, although according to Pryor (1976) F1 fruit size is usually the logarithmic mean rather than the arithmetic mean of the parents. Other features such as bark characteristics, tree form and site preferences have been shown as intermediate (Ashton and Sandiford, 1988; Potts and Reid, 1983). van Wyk (1987) showed an increase in wood density of *E. grandis* hybrids compared with pure *E. grandis* , however did not compare it with the other parent species. Venkatesh and Sharma (1979) investigated inheritance of characteristics such as lignotuber development and flowering periodicity in *E. tereticornis* and found inheritance to be dominant for those traits.

The main aim of most eucalypt breeding programs is to produce faster growing, high quality strains that will give increased yields of timber and shorter rotation times. Trials of hybrid eucalypts have been performed in India, South Africa, France and the U.S.A. and many studies indicate that there is a significant gain to be made in the use of hybrids exhibiting heterosis.

Field trials of open pollinated F1 progeny of *E. grandis* (second parent either *E. camaldulensis* , *E. robusta* Smith, *E. tereticornis* or *E. rudis* Endl.) compared with

their pure *E. grandis* half siblings in the southern U.S.A.showed an 80% increase in volume over the pure species and a similar increase of 19% in height (Meskimen and Franklin, 1984). Likewise heterosis has been reported in *E. grandis* x *tereticornis* (van Wyk 1987; Venkatesh and Sharma, 1979), *E. tereticornis* x *camaldulensis* (Venkatesh, 1978; Venkatesh and Sharma, 1977, 1978) and *E. grandis* x *urophylla* S. T. Blake (van Wyk, 1987).

These studies and their conclusions on heterosis are limited in the use of controls of pure species. In most instances hybrids were compared with only one of the parents (the other one was sometimes unknown). Often open pollinated families of the pure species were used. As shown by seed production data open pollinated seed crops contain a significant proportion of inbred seed which may exhibit inbreeding depression, especially when the gene pool of the original population was restricted in its parentage (Griffin, 1988).

Other studies have found no evidence of heterosis, with hybrid growth rates being equal to or intermediate between parental rates (Potts *et al.*, 1987; West, 1981). Moreover some crosses give rise to inferior offspring, or outbreeding depression as seen in, *E. gunnii* x *ovata* (Potts *et al.*, 1987).

The potential to breed eucalypt hybrids that are better suited to particular environmental conditions or soil types is an area that has hardly been touched. Franclet and Boulay (1982) included hybrids of *E. gunnii* and *E. dalrympleana* in the frost tolerance breeding program. These hybrids have shown promise as a source of superior material. There is no literature pertaining to the inheritance of physiological characteristics such as salinity tolerance of eucalypt hybrids.

Natural hybrids are often detected in ecological niches slightly different to their parents. *E. obliqua* x *pulchella* was observed growing in gullies not colonised by the surrounding *E. pulchella* (Potts and Reid, 1983).

The breeding of superior lines utilizing hybridisation often depends upon the production of advanced generation hybrids and the success of this procedure will be determined by the fertility of the interspecific hybrids and the fitness of their segregates and backcrosses. Seed production data of naturally occurring hybrids has shown variation in

fertility in various hybrids. Clifford (1960) first reported hybrid seed production data in *E. elaeophora* x *goniocalyx* hybrids, in which production was intermediate between parents and seed viability not significantly different, indicating full fertility. However Drake (1981a) showed differences between two hybrids of *E. crebra*. *E. melanophloia* x *crebra* showed lower viable seed output than the parent species but differences were due to variables such as canopy size, proportion of canopy bearing fruit and susceptibility to biotic damage, but a significant number of seeds were produced per tree (Drake, 1981b) indicating the overall viability. Other hybrids have shown lower than parental seed production, *E. obliqua* x *pulchella* (Potts and Reid, 1983), *E. preissiana* x *buprestium* (Hopper and Coates, 1978). *E. populnea* x *crebra* on the other hand showed no difference in fertility between taxa (Drake, 1981a). Advanced generation hybrids and backcrosses are regularly found in natural stands showing the lack of hybrid breakdown in many hybrid combinations (Potts and Reid 1985; Pryor 1950). Hybrid breakdown is sometimes manifested at the seedling stage by lower growth rates. Pryor (1950) showed the lack of vigour in types of intermediate morphology in segregates of *E. viminalis* x *cinerea* F.Muell. ex Benth., perhaps due to inharmonious gene combinations.

However the selection of fully fertile strains or vegetative propagation of superior clones allows the utilization of advanced generation hybrids. Although improvement is still warranted (Brune and Zobel, 1981) much of the stock used for plantation forestry in Brazil is of complex hybrid origin. Combining more than two species by a series of hybridisations is also possible, Venkatesh and Sharma (1980) reported a trispecific hybrid *E.* (*camaldulensis* x *tereticornis*) x *grandis*. Although this combination was inferior to the dispecific hybrid it shows the potential for the transfer of characteristics between species, and perhaps the potential to use "bridge species" between inviable combinations.

3. PLANT MATERIAL

Twenty five species were used in this project (Table 3.1). Four species were used extensively in controlled pollination experiments as female parents and for breeding system estimation, the rest were used as pollen donors. Trees used in controlled pollinations and for phenological observations were located in parkland plantations in Adelaide (latitude 35° 08'S, longitude 139° 08'E), (*E. spathulata* Hook. and *E. leptophylla* F. Muell. ex Miq.(formerly included in *E. foecunda* Schauer, see Brooker 1988)) and Callington 50 km southeast of Adelaide, (latitude 35° 07'S, longitude 139° 02'E),(*E. cladocalyx* F. Muell. and *E. leucoxylon* F. Muell.). Pollen was collected from trees in the same plantations, and natural stands in Adelaide, Callington and Hobart, Tasmania (latitude 42° 53'S, longitude 147° 19'E). Herbarium specimens of representatives of each species have been lodged with the South Australian Herbarium.

Table 3.1: List of species used and locations

Angophora costata (Gaertn.) Britten,	p	Waite Institute grounds
Eucalyptus albida Maiden and Blakely,	p	Waite Arboretum
E. botryoides Smith,	p	Waite Institute grounds
E. camaldulensis Dehnh.,	p	Waite Institute grounds
E. citriodora Hook.,	p	Waite Institute grounds
E. cladocalyx F. Muell.,	p	Monarto plantation, Callington
E. diversifolia Bonpl.,	p	Waite Arboretum
E. ficifolia F.Muell.,	p	Waite Institute grounds
E. flindersii Boomsma,	p	Waite Arboretum
E. grandis W.Hill ex Maiden,	p	Black Hill Flora Centre
E. lansdowneana F.Muell. and J.Brown,	p	Waite Arboretum
E. leptophylla F. Muell.	p	Black Hill Flora Centre
E. leucoxylon F. Muell.	p	Monarto plantation, Callington
E. maculata Hook.,	p	Black Hill Flora Centre
E. melliodora Cunn. ex Schauer,	p	Black Hill Flora Centre
E. obliqua L'Hér.,	n	Cleland Conservation Park
E. occidentalis Endl.	p	Waite Arboretum
E. platypus var. *heterophylla* Blakely,	p	Monarto plantation, Callington
E. pulchella Desf.,	n	Mt Nelson Hobart, Tasmania
E. sargentii Maiden	p	Monarto plantation, Callington
E. spathulata Hook.,	p	Waite Institute grounds
E. tereticornis Smith,	p	Waite Arboretum
E. viridis R.Baker,	p	Black Hill Flora Centre
E. yalatensis Boomsma,	p	Monarto plantation, Callington
Melaleuca nesophila F.Muell.	p	Waite Institute grounds

p = plantation, n = natural stand.

4. BREEDING SYSTEMS OF THREE SPECIES FROM THE SECTION *BISECTARIA*.

4.1 INTRODUCTION

There have been several determinations of breeding systems of *Eucalyptus* species using a variety of methods. Relative contributions of outcrossed versus selfed seed to the gene pool have been estimated using isozyme analysis for many species (Brown *et al.*, 1975; Fripp *et al.*, 1986; Moran and Brown, 1980; Phillips and Brown, 1977; Yeh *et al.*, 1983), and self compatibility has also been assessed through controlled pollinations (Potts and Savva, 1988). However the mechanisms of self compatibility have been studied in only two species, *Eucalyptus regnans* (Sedgley *et al.*, 1989) and *E. woodwardii* (Sedgley, 1989, Sedgley and Smith, 1989) through observation of the pollen pistil interaction.

The genus contains species with distinct habitat preferences and ecological niches. It would therefore be expected to show a variety of adaptations in the breeding system suited to particular habitats, pollinators and life strategies. Gross floral morphology is very consistent across the taxonomic groups, but there are differences in flower size, ovule and seed number per flower, seed weight (Langkamp, 1987) and the inflorescence unit, ie. resource allocation in reproductive output. There are also more subtle differences in floral structure, and some of these have taxonomic and thus evolutionary significance. Those that have already been documented are stigma morphology (Boland and Sedgley, 1986), anther morphology (Bentham, 1867; Blakely, 1934; Pryor, 1953) and ovule arrangement on the placenta (Carr and Carr, 1962).

Many of the species so far investigated for breeding system variables come from high rainfall forest habitats and are commercially important timber crops, although a few rare or isolated species have also been studied (Fripp, 1982; Peters *et al.*, 1990; Sampson *et al.*, 1989). The subgenus *Symphyomyrtus* section *Bisectaria* contains more than 100 species, most of which are mallees and inhabit dryland regions. Many have potential as wood crops and shelter belts in dryland, saline or degraded areas. They are also the dominant overstorey in much of the Mediterranean climate region in Australia.

In this section aspects of floral morphology and breeding systems of three species from the Section *Bisectaria* were investigated. *E. cladocalyx* and *E. leptophylla* are indigenous to South Australia and *E. spathulata* to Western Australia. *E. cladocalyx* occurs in three disjunct locations in South Australia in rainfall areas of approximately 550 mm and ranges from a small crooked tree to a tall forest tree 30 m high (Boomsma, 1972). The variety used in this study is from the Eyre Peninsular and is often referred to in cultivation as *E. cladocalyx* var. *nana* due to its small bushy habit. *E. leptophylla* occurs in South Australia, New South Wales and Victoria as a mallee three to eight metres tall in areas of rainfall 250-500 mm (Boomsma, 1972; Brooker, 1988). *E. spathulata* occurs in south west Western Australia in the 342-500 mm rainfall belt and can be a mallee or a tree up to 12 m (Chippendale, 1973).

4.2 MATERIALS AND METHODS

4.2.1 Plant material

Eucalyptus spathulata ssp *spathulata*, *E. cladocalyx* var *nana* and *E. leptophylla* . trees used in this investigation were described in chapter 3. All three species are currently placed in the subgenus *Symphyomyrtus* section *Bisectaria* (Pryor and Johnson, 1971), and represent the series *Elongatae*, *Microcorythae* and *Porantherae* respectively (Chippendale, 1988). Trees were observed over a three year period for bud, flower and capsule development.

4.2.2 Floral morphology and post anthesis development

Ten buds on each of three trees per species were tagged at anthesis and monitored daily for changes to perianth, pistil and androecium. Statistics on bud, flower and fruit morphology and retention were gathered from ten floral units from each tree. Three tagged shoots per tree were examined macroscopically each month for evidence of floral bud initiation. To obtain pollen counts five buds from each tree were allowed to open in the laboratory and pollen was suspended in a mixture of water and wetting agent. Four replicate subsamples of pollen from each flower were counted using a haemocytometer.

4.2.3 Controlled pollinations

The controlled pollination technique is shown in Figure 4.1A-D. Buds at anthesis (operculum lift) were emasculated by cutting into the stamenophore just below the point of attachment of the filaments, and bagged with glassine paper bags to exclude pollinators (Hodgson, 1976a). Any buds on the branch not at this stage were removed. Prior to pollination, bags were opened and pistils pollinated either by using a pollen laden gelatine capsule or by brushing freshly dehisced anthers across the stigma, until the surface was covered with a heavy dusting of pollen. All buds within a bag received the same treatment. Branches were then rebagged until pistils were harvested for microscopical examination or bags removed two weeks later after the pistils were no longer receptive and left for seed set. Controls were treated similarly but without pollination. Pollen was collected from flowers bagged before anthesis to ensure no contamination from stray pollen.

4.2.4 Time of stigmatic receptivity

Two methods of assessing pistil receptivity were used. Controlled pollinations were carried out at two day intervals using two sources of cross intraspecific pollen, pistils were harvested 24 hours after pollination, fixed immediately in Carnoy's fixative and stored at 4°C. Pistils were hydrated through an alcohol series, styles were then separated from the pistils and scored longitudinally to facilitate squashing. Tissue was softened in 0·8 N NaOH at 60°C for up to one hour, stained in decolourised aniline blue overnight then squashed in 80% glycerol and viewed under fluorescence microscopy (Martin, 1959). The number of germinated pollen grains on the stigma in the squash preparations were counted in ten replicate pistils per treatment.

A sequence of both open pollinated and emasculated, bagged, unpollinated pistils were also collected at two day intervals and fixed in FPA 50 (5 parts formalin, 5 parts propionic acid, 90 parts 50% ethanol, 0.5% caffeine). After dehydration through an alcohol series, critical point drying and sputter coating with gold, stigma surfaces were observed using a Phillips 505 Scanning Electron Microscope operated at 20 kV for morphological changes related to attainment of receptivity.

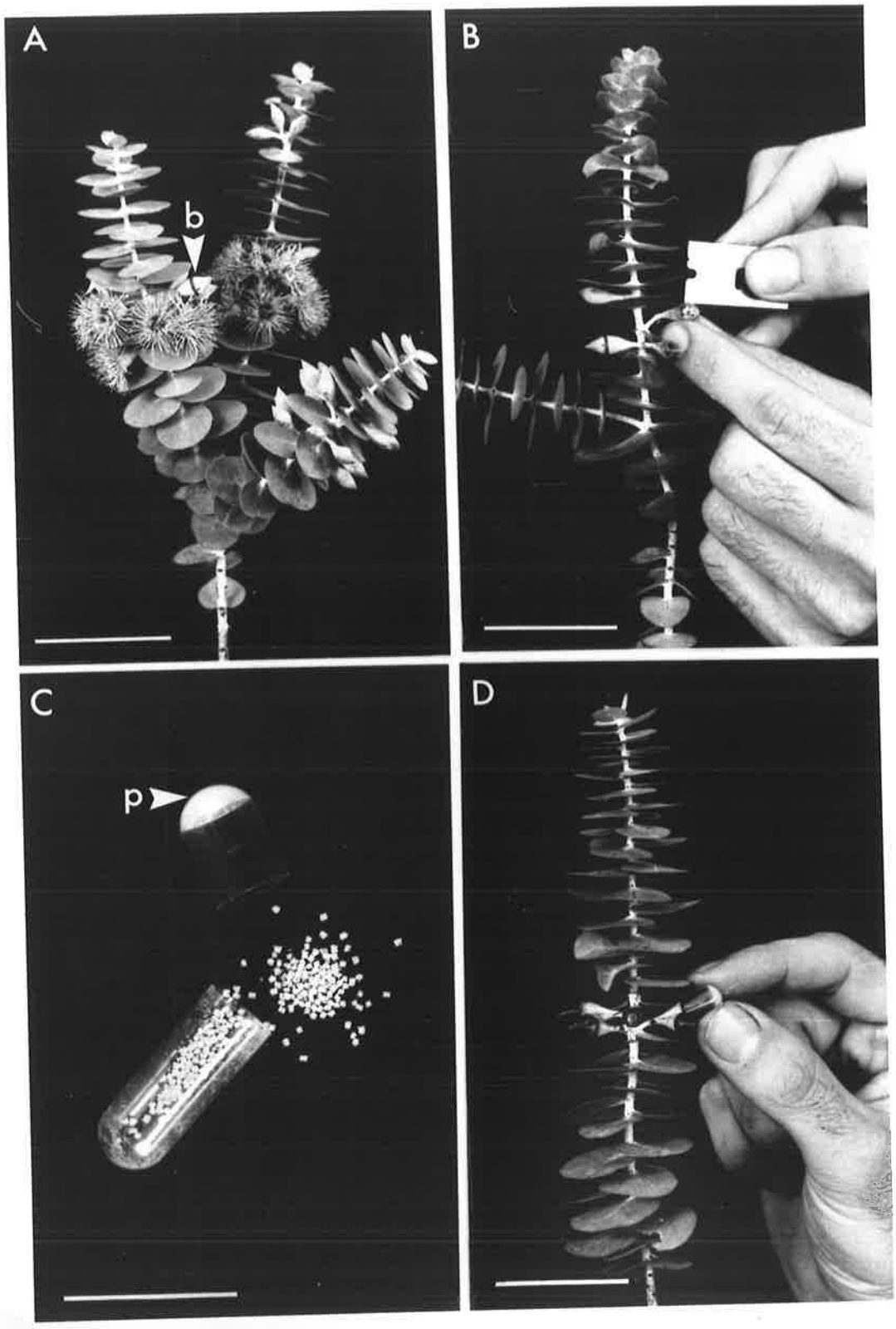
Figure 4.1. Emasculation and controlled pollination technique for *Eucalyptus*.

4.1A. Flowering branch of *E. kruseana*, showing buds at anthesis (b). Bar represents 5cm.

4.1B. Emasculation technique, buds are emasculated by cutting into the stamenophore just below the point of attachment of the filaments. Bar represents 5cm.

4.1C. Gelatine capsule containing desicated anther and pollen mixture, (p) pollen adheres electrostatically to the capsule. Bar represents 2cm.

4.1D. Pollination of emasculated flowers at receptivity direct from gelatine capsules. Bar represents 5cm.


A
b
B
C
p
D

4.2.5 Self compatibility

Controlled self and intraspecific cross pollinations were performed on three trees of each species. Cross treatments received pollen from three different conspecific sources and self treatments received geitonogamous pollen. Pistils were harvested ten days after pollination and processed for fluorescence microscopy. Counts were made of the number of pollen tubes in the upper style, the base of the style and the number of ovules penetrated by pollen tubes from twenty pistils for the three trees per species. Other flowers were pollinated with the same treatments and left for seed set. Resultant capsules were harvested one year after pollination and seed extracted after capsule dehiscence.

4.2.6 Comparative pistil cytology

To compare the pistil cytology of the three species, sections were prepared and stained histochemically. Both pollinated and unpollinated flowers were used. Unpollinated, bagged, emasculated flowers were harvested at day 0, at the time of receptivity and ten days after receptivity. Pollinations with cross intraspecific pollen were performed on emasculated flowers at peak receptivity and branches rebagged. Pollinated flowers were harvested two and ten days after pollination. Pistils were dissected and fixed in 3% glutaraldehyde in phosphate buffer under vacuum for 24 hours. Pistils were processed through a dehydration series of alcohols, embedded in glycol methacrylate and semi thin longitudinal (LS) and transverse (TS) sections ranging from 2.5 - 9 µm were prepared. Proteins were localised with Coomassie brilliant blue, carbohydrates with the Periodic Acid Schiffs reaction (PAS), phenolics with Toluidine blue O (TBO), and lipids with Sudan black B. Structure was further investigated with fluorescence microscopy using aniline blue for callose and Auramine O for cutins.

4.2.7 Statistical analysis

To test for differences in self and cross pollen tube growth in the pistil, the numbers of pollen tubes in the upper style and the lower style and the percent of pollen tubes reaching the base were compared using analysis of variance. Percent ovule penetration was calculated and an analysis of variance performed on the arc sin

transformed data. *E. cladocalyx* and *E. leptophylla* data fitted poorly to the normal model so data were ranked and tested by the Mann Whitney U test for non-parametric data (α = 0.05). Differences in capsule set and seeds per capsule from self and cross pollinations were analysed by two way ANOVA using the three trees per species as replicates, seeds per capsule data was log transformed, and additionally analysed on an individual tree basis. The self compatibility index (Kenrick, 1986) was calculated for each tree.

4.3 RESULTS

4.3.1 Floral morphology and post anthesis development

Floral buds appeared 26 months prior to anthesis in *E. spathulata,* and 14 months prior to anthesis in *E. cladocalyx* and *E. leptophylla.* Consequently three years crops of floral buds were present on a tree of *E. spathulata* at any one time and two of *E. cladocalyx* and *E. leptophylla* (Fig. 4.2A-C). Peak anthesis occurred in December for *E. spathulata,* December-January for *E. cladocalyx* and January-February for *E. leptophylla.* The inflorescence unit is the umbel and there was variation between the three species in the number of flowers per umbel, ovules per flower, anthers and pollen grains per flower and in floral dimensions (Table 4.1, Figs. 4.3-4.5). *E. leptophylla* was the only species with staminodes, the inner whorls of anthers were club shaped on short filaments and appeared to have been arrested at an early stage of development (Fig. 4.5D). Some pollen bearing, abnormal anthers were also present in the inner whorls. Pollen morphology was similar in the three species and was typical of tricolpate myrtaceaeous pollen.

The three species showed the same sequence of post-anthesis events and showed similarity in the duration of the floral stages (Table 4.2). In all species pollen removal occurred the same day as pollen dehiscence. Insect pollinator activity was high in the early morning and free pollen was rarely observed on flowers. Stigma secretion was first observed several days after pollen removal in *E. spathulata* and *E. cladocalyx* but shortly afterwards in *E. leptophylla.* The floral display consisting of stamens, was maintained until four days, eight days and seven days after peak receptivity (Table 4.3) in *E. spathulata*, *E. cladocalyx* and *E. leptophylla* respectively. There was no increase in

Figure 4.2. Floral development in *E. spathulata*, *E. cladocalyx* and *E. leptophylla*.

4.2A. Flowering branch of *E. spathulata* showing (a) floral buds two months after macrocsopic appearance, (b) floral buds 14 months after macroscopic appearance, (c) flowers and mature buds at anthesis, (d) mature fruit, set 12 months previously, and (e) fruit set 24 months previously. Bar represents 10cm.

4.2B. Flowering branch of *E. cladocalyx* showing (a) floral buds two months after macrocsopic appearance, and (b) flowers and mature buds at anthesis. Bar represents 10cm.

4.2C. Flowering branch of *E. leptophylla* showing (a) floral buds two months after macrocsopic appearance, (b) flowers and mature buds at anthesis, and (c) mature fruit set 12 months previously. Bar represents 10 cm.


A
a
b
c
d
e
B
a
b
C
a
b
c

Table 4.1: Floral characteristics of *Eucalyptus spathulata*, *E. cladocalyx* and *E. leptophylla*, (section *Bisectaria*) (means ± standard error).

	E. spathulata	*E. cladocalyx*	*E. leptophylla*
Maximum buds initiated per umbel	7	16	13
Flowers per umbel at anthesis	5·7 ± 0·2	8·1 ± 0·4	7·2 ± 0·4
Open pollinated fruit per umbel	2·9 ± 0·3	3·5 ± 0·3	4·8 ± 0·5
No. locules per flower	3·5 ± 0·1	3·2 ± 0·1	3·4 ± 0·1
No. ovules per flower	39·9 ± 0·6	56·9 ± 1·3	33·2 ± 0·6
No. anthers per flower	74·1 ± 2·1	192·3 ± 4·3	192·8 ± 2·7
No. staminodes per flower	0	0	73.9 ± 2·7
Diameter of pollen grains (μm)	25·4 ± 0·4	22·1 ± 0·3	14·1 ± 0·1
No. pollen grains per flower	$3{\cdot}6 \times 10^5$ $\pm 2{\cdot}6 \times 10^4$	$2{\cdot}2 \times 10^5$ $\pm 1{\cdot}5 \times 10^4$	$7{\cdot}3 \times 10^5$ $\pm 3{\cdot}1 \times 10^4$
Pollen / ovule ratio	$9{\cdot}1 \times 10^3$	$3{\cdot}9 \times 10^3$	$2{\cdot}2 \times 10^4$
Mature style length (mm)	6·0 ± 0·7	4·9 ± 0·4	4·6 ± 0·5

Figure 4.3. Floral morphology of *E. spathulata*.

4.3A. Floral cluster showing mature flowers, and (b) buds at operculum lift (anthesis). Bar represents 2cm.

4.3B. Diagramatic representation of *E. spathulata* flower

(i) dissected flower showing simple stigma (s), anthers (a) and ovary (o),

(ii) transverse section through hypanthium showing three locules (l),

(iii) arrangement of ovules (shaded) and ovulodes on the placenta.

4.3C. Scanning electon micrograph of *E. spathulata* anthers. Bar represents 1mm.

4.3D. Scanning electron micrograph of *E. spathulata* pollen, showing germination pores (g) and sporopollenin orbicules (o). Bar represents 50μm.

4.3E. Mature fruit, showing three valves at the top of the hypanthium. Bar represents 1cm.

4.3F. Contents of mature capsule, showing full seed (f) and chaff particles (c). Bar represents 1cm.

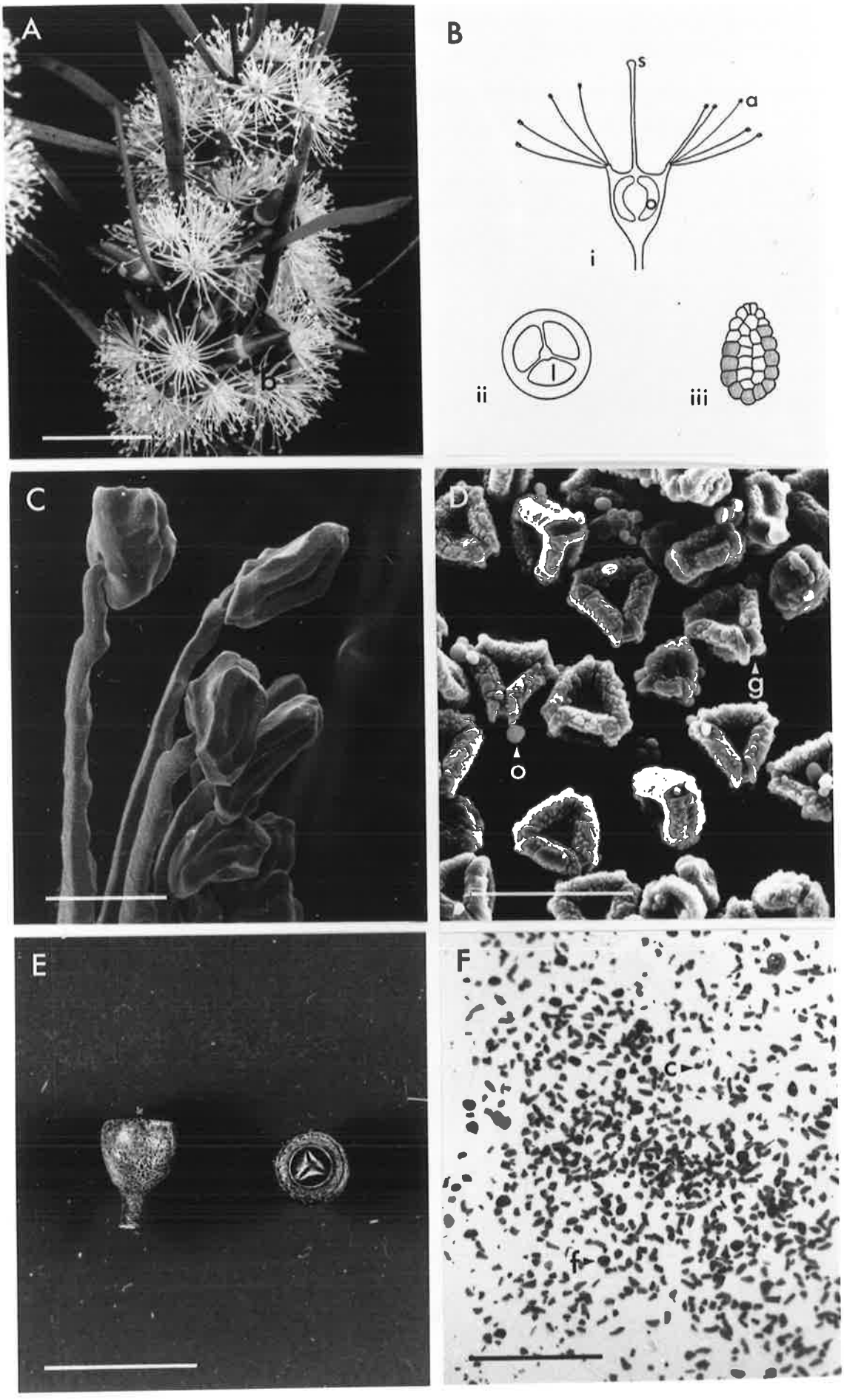

A
b
B
s
a
o
i
ii
iii
C
D
g
o
E
F
c
fr

Figure 4.4. Floral morphology of *E. cladocalyx*.

4.4A. Floral cluster showing mature flowers, and (b) buds at operculum lift (anthesis). Bar represents 5cm.

4.4B. Diagramatic representation of a *E. cladocalyx* flower

(i) dissected flower showing simple stigma (s), anthers (a) and ovary (o),

(ii) transverse section through hypanthium showing three locules (l),

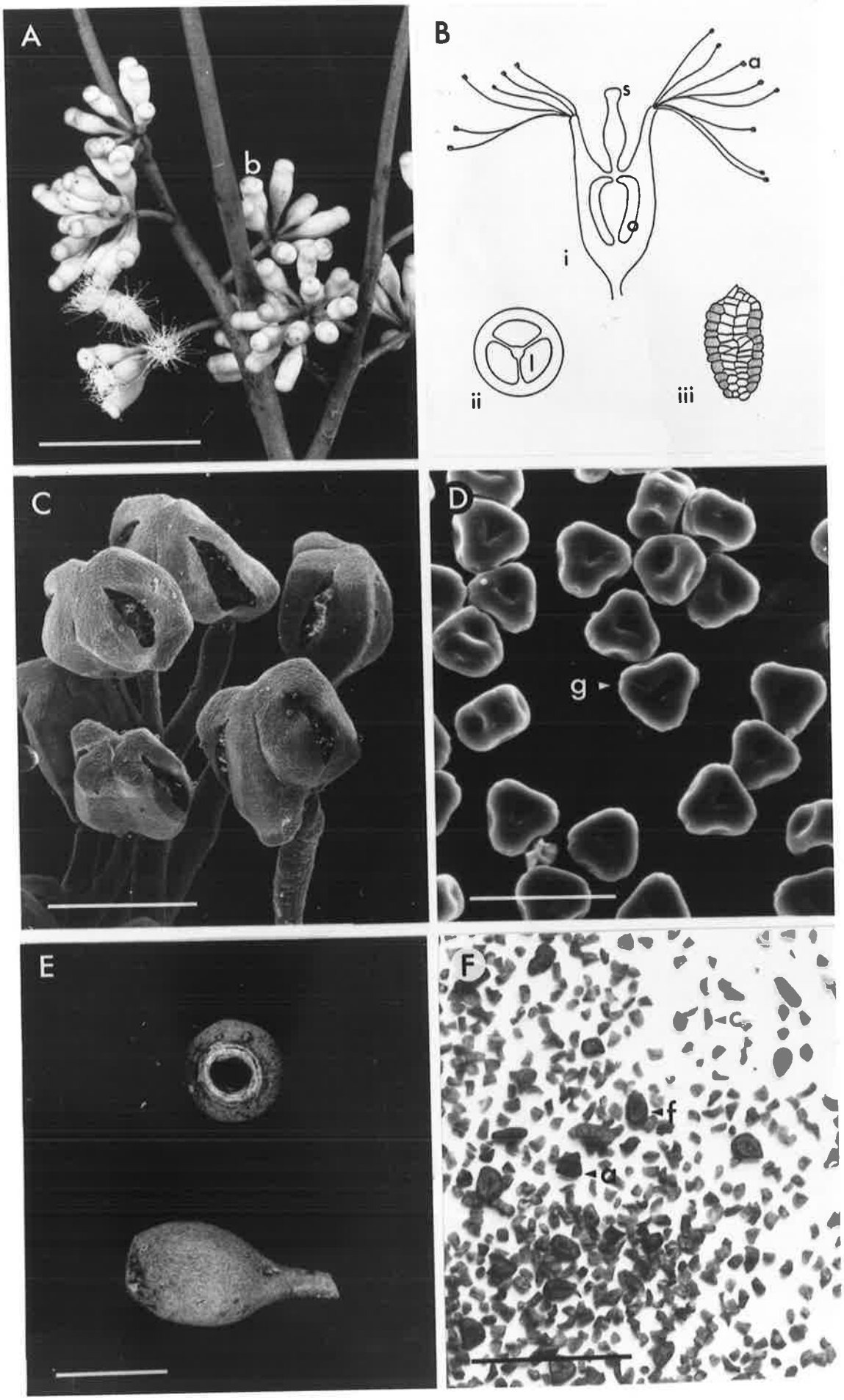
(iii) arrangement of ovules (shaded) and ovulodes on the placenta.

4.4C. Scanning electon micrograph of *E. cladocalyx* anthers showing dehiscence through elongate slits. Bar represents 0.5mm.

4.4D. Scanning electron micrograph of *E. cladocalyx* pollen, showing germination pores (g). Bar represents 50μm.

4.4E. Mature fruit. Bar represents 1mm.

4.4F. Contents of mature capsule, showing full seed (f) aborted seed (a) and chaff particles (c). Bar represents 10mm.


A
b
B
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s
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a

Figure 4.5. Floral morphology of *E. leptophylla*.

4.5A. Floral cluster showing mature flowers, and (b) buds at anthesis. Bar represents 2cm.

4.5B. Diagramatic representation of a *E. leptophylla* flower

(i) dissected flower showing simple stigma (s), anthers (a) and ovary (o),

(ii) transverse section through hypanthium showing three locules (l),

(iii) arrangement of ovules (shaded) and ovulodes on the placenta.

4.5C. Scanning electon micrograph of fertile *E. leptophylla* anthers from the outer whorls. Bar represents 0.5mm.

4.5D. Scanning electon micrograph of sterile *E. leptophylla* anthers or staminodes (st) from the inner whorls. Bar represents 0.2mm.

4.5E. Scanning electron micrograph of *E. leptophylla* pollen, showing germination pores (g). Bar represents 20μm.

4.5F. Mature fruit, showing three valves at the top of the hypanthium. Bar represents 1mm.

4.5G. Contents of mature capsule, showing full seed (f) and chaff particles (c). Bar represents 1cm.

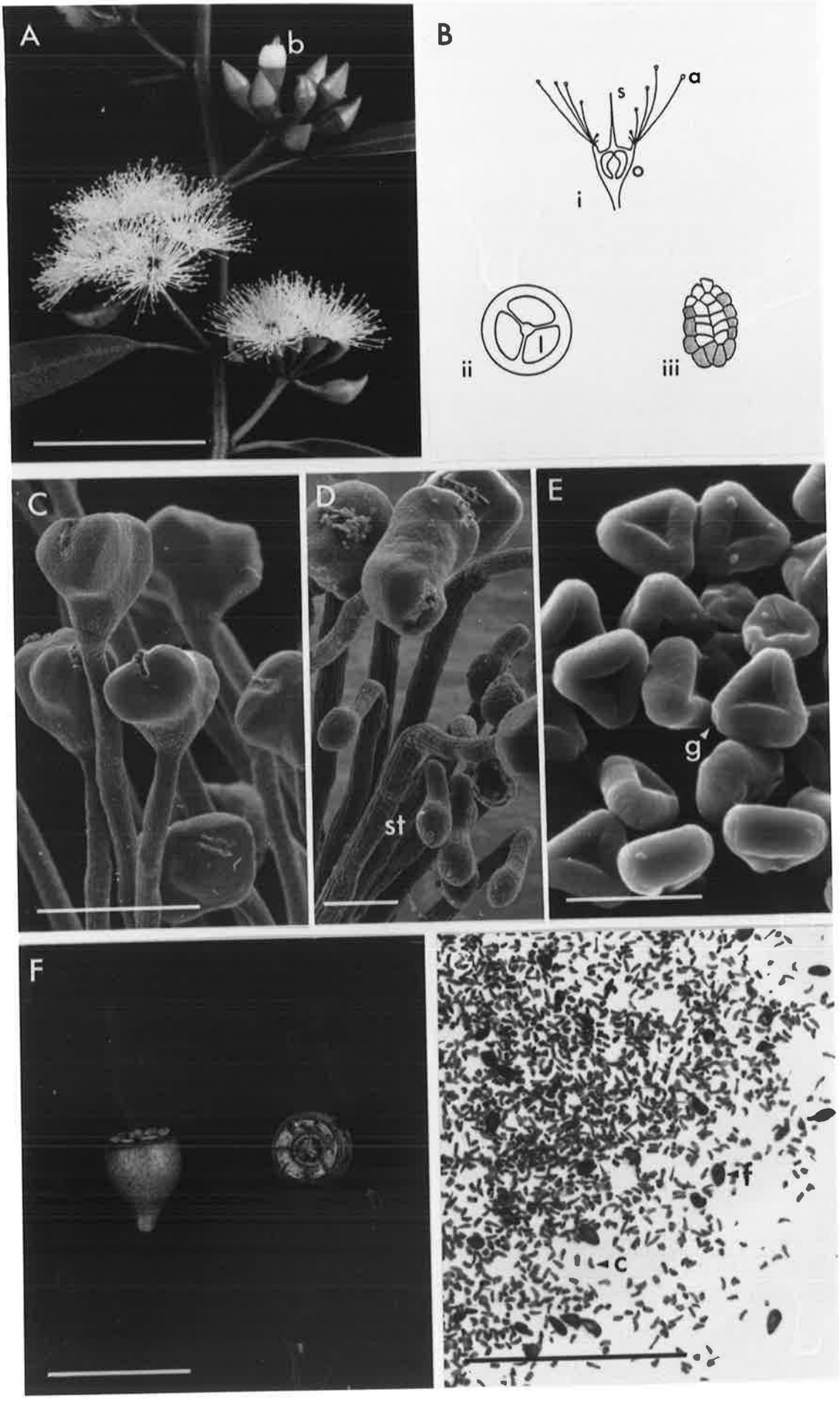
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Table 4.2: Timing of post anthesis development in *Eucalyptus spathulata*, *E. cladocalyx* and *E. leptophylla* . (* as assessed by electron microscopy, ** style does not abscise but withers gradually and may be retained on fruit).

	Mean days from operculum lift		
Stage	*E. spathulata*	*E. cladocalyx*	*E. leptophylla*
Operculum shed	2·2 ± 0·2	2·2 ± 0·1	1·5 ± 0·2
First stamens unfolded	2·6 ± 0·2	2·6 ± 0·1	2·8 ± 0·3
Last stamens unfolded	2·6 ± 0·2	2·8 ± 0·1	3·8 ± 0·3
Anther dehiscence	2·6 ± 0·2	2·8 ± 0·1	3·7 ± 0·3
Pollen removal	2·6 ± 0·2	2·9 ± 0·1	3·8 ± 0·3
Stigma secretion	5·1 ± 0·1	5·1 ± 0·1	4·0 *
First stamen abscission	12·0 ± 0·4	18·5 ± 0·2	15·1 ± 0·4
Last stamen abscission	15·7 ± 0·9	19·0 ± 0·0	17·3 ± 0·3
Style abscission	24·2 ± 0·4	20·2 ± 0·1	**

style length after anthesis. Style abscission did not occur until pollen tubes had reached the ovary. In *E. leptophylla* the style often did not abscise but withered and remained in place even until fruit maturation one year later.

4.3.2 Time of stigmatic receptivity

Pollen did not adhere to the stigmatic surface until several days after anthesis, after which pollen germination increased with time (Table 4.3). Peak receptivity was considered to be when pollen germination was in excess of the amount needed for fertilisation but not so late as to not allow time for pollen tubes to reach the ovary before style abscission. In all three species numbers of germinated pollen grains increased with time after anthesis. The flowers were bagged, and stigmatic secretion built up over time, not being removed by pollinators, thus allowing increased germination. Peak receptivity was estimated to be at day eight for *E. spathulata* and *E. leptophylla* and day ten for *E. cladocalyx*.

Morphological changes associated with attainment of receptivity were similar for all three species (Fig. 4.6A-F). At anthesis the stigma surface is covered with a cuticle that overlies the papilla cells (Fig. 4.6A-C, E). After anthesis expansion of the papilla cells ruptures the cuticle and allows extra-cellular secretion to accumulate on the stigma surface (Fig. 4.6D, F) At receptivity the stigma surface is fully expanded becoming broader and higher, with secretion covering and between the papillae. In pollinated pistils pollen grains can be seen germinating in the secretion and pollen tubes penetrating the stigma between the papilla cells (Fig. 4.6F).

4.3.3 Self compatibility

Healthy pollen tube growth was observed in both self and cross pollinations for all three species. When the success of pollen tube growth in the style was analysed (Table 4.4) it was found for *E. spathulata* that in trees 1 and 2 cross pollen tubes were more successful ($P < 0.05$) than self pollen tubes in reaching the base of the style, while tree 3 showed no difference between the treatments. *E. cladocalyx* showed much variation between trees, in tree 1 cross

Table 4.3: Time of stigmatic receptivity of *Eucalyptus spathulata*, *E. cladocalyx* and *E. leptophylla*, measured by pollen germination on pollinated stigmas. (Mean germinated pollen grains ± standard error).

Day	*E. spathulata*	*E. cladocalyx*	*E. leptophylla*
0	0	0	0
2	4·7 ± 4·7	0	0
4	6·1 ± 3·6	0	8·6 ± 2·8
6	25·2 ± 9·1	0	3·3 ± 1·2
8	378·7 ± 43·5	13·8 ± 4·8	16·6 ± 4·4
10	367·8 ± 47·6	256·0 ± 58·7	8·1 ± 2·2
12	640·3 ± 50·5	50·6 ± 21·3	28·8 ± 9·6
14	- -	182·1 ± 53·2	30·7 ± 9·1

Figure 4.6. Changes in eucalypt stigma mophology associated with attainment of receptivity.

4.6A. Scanning electron micrograph of *E. cladocalyx* stigma at anthesis. Bar represents 250μm.

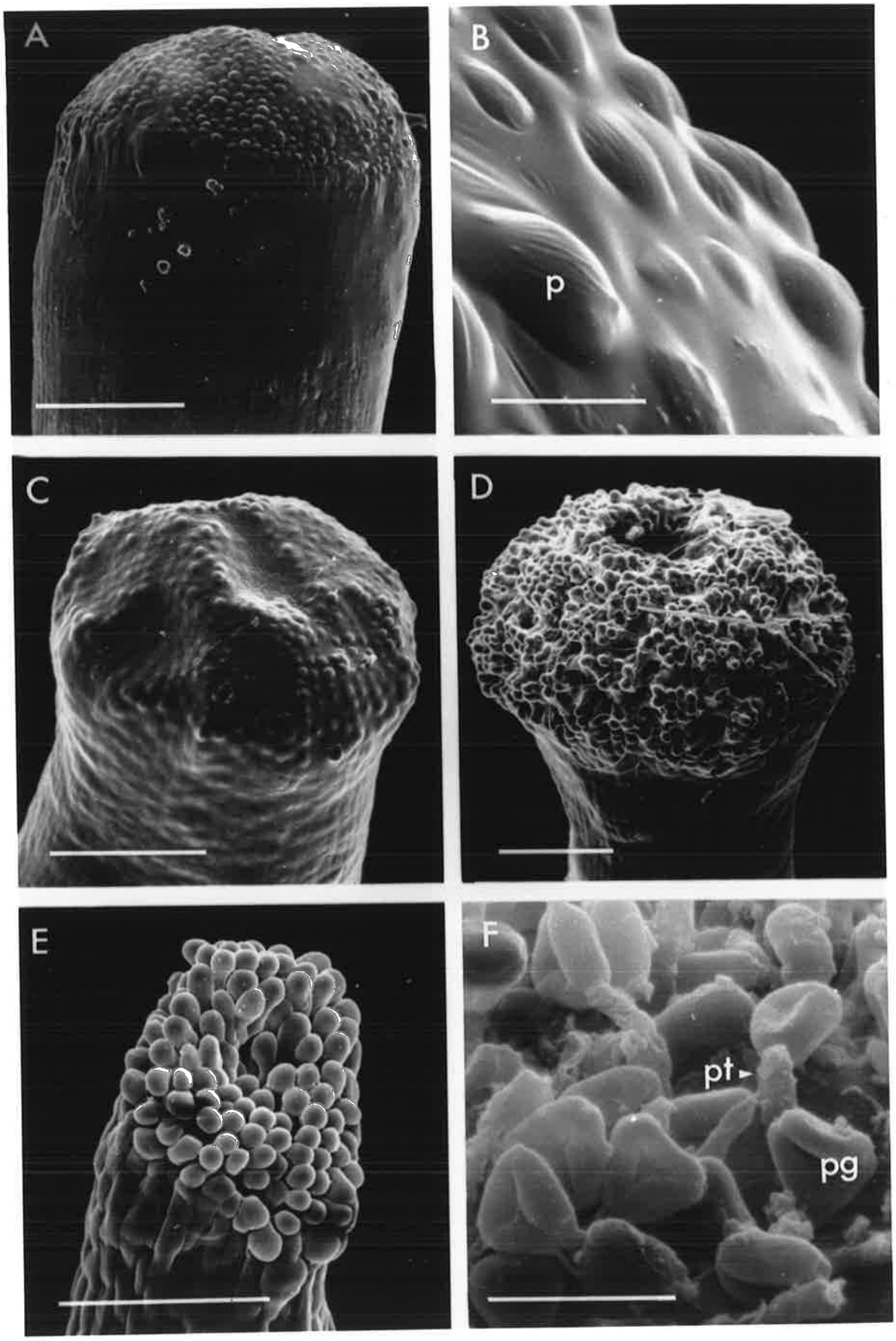
4.6B. Scanning electron micrograph of *E. cladocalyx* stigma at anthesis, papillae (p) are covered by a thick layer of cuticle. Bar represents 20μm.

4.6C. Scanning electron micrograph of *E. spathulata* stigma at anthesis. Bar represents 200μm.

4.6D. Scanning electron micrograph of *E. spathulata* stigma ten days after anthesis, showing an expansion of the stigmatic surface and presence of stigmatic secretion over and between the papillae. Bar represents 200μm.

4.6E. Scanning electron micrograph of *E. leptophylla* stigma at anthesis, showing assymetrical stigma. Bar represents 100μm.

4.6F. Scanning electron micrograph of *E. leptophylla* stigma surface ten days after anthesis and two days after pollination, showing germinated pollen grains (pg) and pollen tubes (pt) penetrating between the papillae cells. Bar represents 20μm.


A
B
p
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pt
pg

Table 4.4: Pollen tube growth in styles of *Eucalyptus spathulata*, *E. cladocalyx* and *E. leptophylla* following controlled pollination. (Mean number of pollen tubes ± standard error).

	Tree 1		Tree 2		Tree 3	
	Cross	Self	Cross	Self	Cross	Self
E. spathulata						
Upper style	234·1 ± 15.8	305·7 ± 17.6	243·8 ± 14.5	256·5 ±15.3	155·9 ± 15.8	192·8 ± 13.8
Lower style	145·7 ± 9.2	110·2 ± 6.3	127·1 ± 8.9	96·6 ± 5.8	90·0 ± 9.3	107·4 ± 7.6
Ovules penetrated	17·7 ± 1.5	2·7 ± 0.5	12·3 ± 2.6	4·2 ± 1.0	9·5 ± 1.7	8·7 ± 1.4
E. cladocalyx						
Upper style	170·1 ± 26.6	220·6 ± 20.8	290·6 ± 19.8	238·6 ± 19.9	111·6 ± 13.7	191·8 ± 16.3
Lower style	47·7 ± 9.9	33·1 ± 6.5	223·3 ± 20.3	201·8 ± 20.2	31·6 ± 4.9	96·7 ± 10.7
Ovules penetrated	1.0 ± 0.3	0.5 ± 0.3	4.7 ± 1.2	1.8 ± 0.7	1.8 ± 0.6	1.1 ± 0.3
E. leptophylla						
Upper style	24·8 ± 4.7	22·3 ± 4.8	43·6 ± 7.9	54·4 ± 10.1	26·7 ± 2.9	44·9 ± 37.8
Lower style	23·6 ± 4.2	16·7 ± 2.4	30·2 ± 6.0	34·0 ± 4.8	15·5 ± 4.5	37·8 ± 7.0
Ovules penetrated	0·9 ± 0.3	0·2 ± 0.1	2·6 ± 0.8	6·6 ± 1.3	0·4 ± 0.2	0·7 ± 0.3

pollen tubes performed significantly better than self, there was no significant difference in tree 2, while in tree 3 self tubes outperformed cross tubes. In *E. leptophylla,* trees 1 and 2 showed no significant difference but tree 3 showed some differences (P= 0.05) with self outperforming cross.

E. spathulata trees 1 and 2 showed a significant reduction in ovule penetration by self pollen tubes compared with cross pollen tubes (P<0.001, P<0.05 respectively). In *E. cladocalyx* and *E. leptophylla* there was no significant differences in the number of ovules penetrated by self and cross pollen tubes. The numbers of pollen tubes found in the ovary in all treatments of *E. cladocalyx* and *E. leptophylla* were lower than expected, which could have been due to inefficient pollination technique or adverse climatic or biotic influences.

It should be noted in most cases, even though some differences in pollen tube growth was detected between treatments, the number of pollen tubes that reached the base of the style was in excess of that needed for full fertilisation of ovules and the variation was probably the result of different numbers of pollen grains deposited on the stigma. Reductions in pollen tube numbers in the style is precipitated partly by competition for space as the lower style has a lower carrying capacity of pollen tubes than the upper style due to a narrower transmitting tissue. The reduction in ovule penetration in *E. spathulata* tree 1 and 2 is considered to be a real effect as ample pollen tubes were observed in the lower style.

The self compatibility index (Kenrick, 1986) was calculated as the ratio of seed produced by self pollination to seeds produced by cross pollination, taking into account the number of flowers pollinated (Table 4.5). *E. spathulata* and *E. leptophylla* trees showed low levels of self compatibility while *E. cladocalyx* trees ranged from self compatible to completely self incompatible. Analysis of capsule set data shows there was a significant reduction in capsule set following self pollination for *E. spathulata* (P<0.01) and *E. leptophylla* (P<0.05) but no significant difference for *E. cladocalyx* , probably due to the large variation between trees. The difference in the number of full seeds per capsule between self and cross pollinations was highly significant in *E. spathulata* (P<0.001) with significant differences between trees in the number of seeds per capsule (P<0.001).

Table 4.5: Capsule and seed set following control pollinations of *Eucalyptus spathulata*, *E. cladocalyx* and *E. leptophylla*. (Means ± standard error, n = number of flowers pollinated. SC index = seeds produced by self pollination/seeds produced by cross pollination).

	Tree 1		Tree 2		Tree 3	
	Cross	Self	Cross	Self	Cross	Self
E. spathulata						
% capsule set	91·3	7·8	70·6	12·0	64·6	0
seeds per capsule	10·9 ± 0·5	2·3 ± 0·5	5·3 ± 0·3	1·2 ± 0·1	11·4 ± 0·7	- -
n	81	77	102	83	116	100
SC index	0·02		0·04		0·00	
E. cladocalyx						
% capsule set	7·4	10·3	56·7	63.9	5·0	7·8
seeds per capsule	1·8 ± 0·3	1·5 ± 0·5	2·1 ± 0·3	0·04 ± 0·3	1·0 ± 0·0	0
n	81	97	120	72	20	102
SC index	1·16		0·02		0·00	
E. leptophylla						
% capsule set	36·2	2·8	63·4	26·1	66·7	1·3
seeds per capsule	2·2 ± 0·3	1·0 ± 0·0	3·1 ± 0·2	0·9 ± 0·2	2·7 ± 0·2	0·5 ± 0·5
n	47	72	134	65	102	74
SC index	0·04		0·12		0·01	

E. leptophylla also showed differences in the resultant seeds per capsule in trees 2 and 3 ($P<0.05$) but not tree 1. *E. cladocalyx* showed variation between trees ranging from significant differences in trees 2 ($P< 0.001$) and 3 (zero self seed set) to no significant difference in tree 1.

4.3.4 Comparative pistil cytology

The three species share many features of pistil cytology, but differ in the length of the stylar canal, degree of sclerotinisation, stigma morphology and transmitting tissue (Fig. 4.7A-F). A thick cuticle envelopes the style and stains positive with PAS, Auramine O and Sudan black B, indicating both carbohydrates and lipids (probably cutins) and may act as a barrier to water loss (Fig. 4.7A). Below the epidermal layer is a zone of stylar cortex containing vascular bundles, groups of sclerenchyma cells and in *E. spathulata* and *E. cladocalyx* , glands occur just below the epidermis (Fig. 4.7B). The glands are more prevalent towards the base of the style and consist of a lumen surrounded by seven or eight secretory cells which stain weakly with TBO and Sudan black B and are similar to the oil glands described by Carr and Carr (1969). *E. leptophylla* shows a dense continuous ring of short sclerenchyma cells, inside the cortex, which makes the style rigid and very brittle.

The stigmas of *E. spathulata* and *E. cladocalyx* conform to the pinhead type, whereas *E. leptophylla* is blunt (Fig. 4.7C) (Boland and Sedgley, 1986). The stigma surface is covered by unicellular papillae. At anthesis the papillae are densely packed, and are covered by a thick cuticle that stains PAS and Sudan Black positive, forming an impenetrable barrier to pollen adhesion, hydration and tube growth. At anthesis the stigmatic papillae of *E. leptophylla* are less obscured by the cuticle than the other two species. Papilla cells are spherical or tapered below which are files of transmitting tissue cells which become more elongate towards the base of the style (Fig. 4.7C). At the top of the style, the transmitting tissue fills the space within the epidermis, becoming narrower lower down where it is surrounded by cortex tissue. In *E. cladocalyx* the transmitting tissue is widest in the mid style, tapering rapidly towards the base. A three or four lobed stylar canal (depending on the number of locules) is evident from the stigma surface to approximately one third of the way down the style in *E. spathulata* and *E. cladocalyx*, in

Figure 4.7. Pistil cytology of *E. spathulata* and *E. leptophylla.*

4.7A. Light micrograph of longitudinal section through a *E. spathulata* stigma ten days after anthesis, stained with aurimine O, showing stylar canal (sc) lined with cuticle. Bar represents 500μm.

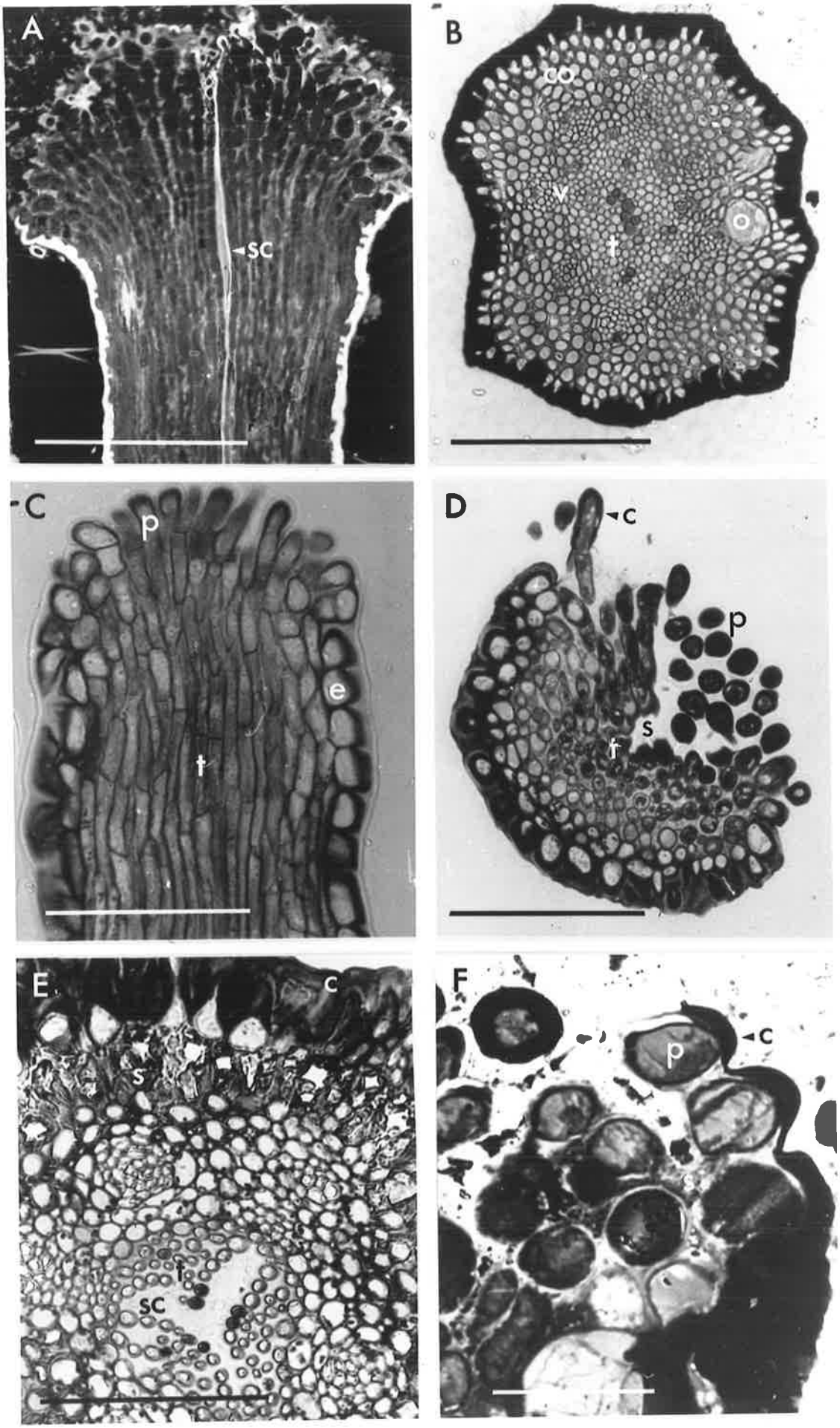
4.7B. Light micrograph of transverse section through the lower style of *E. spathulata* stained with PAS, showing transmitting tissue (t), vascular bundles (v) stylar cortex (co) and oil duct (o). Bar represents 200μm.

4.7C. Light micrograph of longitudinal section through a *E. leptophylla* stigma at anthesis stained with PAS, showing thick cuticle overlying the epidermis (e), papilla cells on stigma surface (p) and transmitting tissue cells (t). Bar represents 100μm.

4.7D. Light micrograph of a transverse section through an assymetrical *E. leptophylla* stigma at anthesis stained with PAS and TBO, showing thick cuticle (c) overlying the papilla cells (p), the start of the stylar canal (s) and transmitting tissue cells (t). Bar represents 100μm.

4.7E. Light micrograph of a transverse section through the lower style of *E. leptophylla* stained with PAS, showing the stylar canal (sc), transmitting tissue (t), a ring of sclerenchyma cells (s) and the thick cuticle (c) covering the epidermis. Bar represents 100μm.

4.7F Light micrograph of a longitudinal section through the stigma of *E. spathulata* ten days after anthesis stained with PAS and TBO, showing rupture of the continuous stigmatic cuticle by papilla cell (p) expansion, some remnants of the cuticle remain intact (c). Bar represents 50μm.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E. leptophylla the canal continued to the base of the style (Fig. 4.7E). The transmitting tissue cells adjacent to the stylar canal are covered by a layer of cuticle similar in composition to the cuticle covering the epidermis, although the cuticle is not present in the lower parts of the *E. leptophylla* stylar canal (Fig. 4.7E).

By the time of stigmatic receptivity, secretion is evident on the stigma surface, between the transmitting tissue cells of the upper style and in the stylar canal. Stigmatic secretion appears between and over the papilla cells, the continuous layer of cuticle having been disrupted by papilla cell expansion (Fig. 4.7F). The secretion stains strongly with Sudan black B and weakly with PAS and Coomassie brilliant blue, but no reaction is obtained with TBO. Copious secretions are seen in *E. spathulata* and *E. cladocalyx* but much less in *E. leptophylla*. The intercellular and stylar canal secretion is Sudan black B positive. Pollen grains adhere to stigmas pollinated at peak receptivity and hydrate in the extracellular secretion on the stigmatic surface, and pollen tubes penetrate the stigma surface between the papilla cells. In the upper style pollen tubes are seen with aniline blue induced fluorescence to grow intercellularly and not in the stylar canal, and intercellular growth continues to the base of the style Ten days after peak receptivity papilla and transmitting cells show intra-cellular degeneration, but retain their external structure. At this stage pollen tubes have reached the ovary and penetrated the ovules.

4.4 DISCUSSION

The generally low levels of seed set resulting from self pollination in *E. spathulata*, *E. cladocalyx* and *E. leptophylla* contrasts with the results of self compatibility trials in other *Eucalyptus* species. Most eucalypts display a mixed mating system, isozyme analyses of open pollinated seed crops of many species reveal approximately thirty percent of seeds result from self pollination (Moran and Bell, 1983). This is however, when self pollen is directly competing with cross pollen for resources in the pistil. Seed set experiments with self and cross pollen in isolation have shown a reduction in seed set with self pollen (Potts and Cauvin, 1988) but rarely to the extent seen in *E. spathulata* and *E. leptophylla*. Potts and Savva (1988) found 80 percent of *E. morrisbyi* trees were self incompatible, however *E. morrisbyi* is a rare Tasmanian endemic, very restricted in range

and population size, and the high degree of self incompatibility may be due to an accumulation of deleterious alleles in the population. Investigations of other species have also identified individual trees that set very low levels of seed on self pollination (Griffin *et al.*, 1987). In this study *E. cladocalyx* also showed much variation in capacity to set self seed between trees.

Observations of pollen tube growth in the style in all three species studied here showed self pollen was capable of reaching the ovary in sufficient numbers to effect full fertilisation. In the ovary, most trees showed no difference between self and cross pollen tube success in ovule penetration, although there were some differences in *E. spathulata*. A reduction of ovule penetrations by self pollen has been reported previously in *E. woodwardii* (Sedgley and Smith, 1989). In *E. regnans* reduced seed set is obtained from self pollinations compared to cross pollination. In mixed pollinations less seed is obtained from self pollen than would be expected from the ratio of pollen types and fitness, indicating selection for outbred zygotes or embryos (Griffin *et al.*, 1987). No differences were found in rates of ovule penetration or in development of embryos to the 16 week stage that would account for the observed preferential outcrossing (Sedgley *et al.*, 1989). This indicated there are post-fertilisation selection mechanisms operating in *E. regnans* and this appears to be the case also in the three species in this study. Although there were good rates of ovule penetration from self pollen in these species very poor self seed set was achieved. Whether this is due to inbreeding depression at the embryo stage or to an active selection mechanism remains unclear (Seavey and Bawa, 1986). Recessive lethal factors at several hundred loci have been suggested to promote outcrossing in *Stylidium* through post-zygotic abortion of embryos homozygous for the lethal elements (Burbidge and James, 1991).

In open pollinations where both cross and self pollen are deposited on stigmas the site and mode of genotype selection can influence reproductive output and seed quality. If selection takes place before the ovule penetration stage, ovule resources are not wasted on unsuitable pollen genotypes which are subsequently aborted. If both self and cross pollen are capable of fertilising ovules and growing to mature seed then the relative fitness of the two types in the pistil will be important in determining their contribution to the seed pool.

In *Eucalyptus*, a genus that shows a greater number of ovules than the maximum number of seeds set, it appears postfertilisation selection is quite common.

A question that remains to be answered is whether the presence of self pollen in post-fertilisation selection systems lowers the reproductive potential of a flower due to the wastage of ovules to self pollinations and subsequent embryo abortion, or does the excess of ovules allow for this eventuality? In *E. regnans* Griffin *et al.*, (1987) found no reduction in the number of seed per capsule in mixed pollinations or open pollinations when compared to controlled cross pollinations, indicating the presence of self pollen, and self pollinated ovules did not lower the reproductive output of the flower. This suggests that although the mechanism for selection of embryo genotypes occurs late in the reproductive cycle the excess of fertile ovules and penetrated ovules in the ovary allows maternal selection of the fittest embryo genotypes without decreasing the reproductive potential of the flower (Stephenson and Bertin, 1983).

To date no evidence has been found of selective stylar inhibition of pollen tube growth as a mechanism to promote outcrossing in any eucalypt species. Such stylar mechanisms have been found in other self incompatible and partially self incompatible species (Hagman 1975; Stott 1972). All *Eucalyptus* species examined show only a proportion of the fertile ovules are ever penetrated by pollen tubes, suggesting competition for ovule resources is not a major limitation to reproductive output, but maternal selection of embryo genotypes is a major determinant of seed quality and thus genetic diversity. Like all eucalypts the three species show a great wastage of reproductive units from floral inititation to seed maturity (Andersen 1989; Ashton, 1975). This is partly due to the long lead time from initiation to anthesis and fruit maturation, and partly due to resource limitation and reproductive selection.

Although there is variability between the three species in overall floral structure the pistil morphology of the three species shows much similarity . Pollen tubes are subjected to similar environments and constraints along the course of their growth. *E. leptophylla* showed much reduced capacity for pollen tube numbers due to the small area of the stigmatic surface and the transmitting tissue. Both morphology and observations of pollen tube growth on maturing stigmas demonstrated an effective system of protandry, helping

to prevent autogamous pollination but still allowing geitonogamous pollination. *E. leptophylla* was the only species with staminodes and differs from many other eucalypt species in that the staminodes are found in the inner whorls of stamens. Davis (1968) found the congenital sterility in *E. melliodora* was a feature of the first formed sporangia, ie the outer whorl of stamens. The pollen-ovule ratio was typical of xenogamous species (Cruden, 1977) with copious pollen grains produced per flower. *E. spathulata* and *E.leptophylla* showed higher pollen grain production per flower than any of the eucalypt species examined by Moncur and Boland (1989).

This study has provided information which supports the emerging picture that eucalypts, while predominantly outcrossers, show wide variability in self and cross fertility. There is clearly no overriding mechanism as manifested in classical self-incompatibility, but rather a variable response which can act at a number of different stages in the breeding cycle. The information to date, from this and other sources, supports a multigenic mechanism which is unlikely to be under major gene control but may act via accumulation of minor genes. The model which best describes the available data is that of inbreeding depression acting at all stages of the female- male interaction, from the ovary to the seedling.

5. THE BREEDING SYSTEM OF *E. LEUCOXYLON*

5.1 INTRODUCTION:

E. leucoxylon (subgenus *Symphyomyrtus*; section *Adnataria*; series *Melliodorae*) is widely planted in southern Australia and in other Mediterranean climate regions world wide as an ornamental. The species ranges in form from a small multistemmed tree to a single stemmed forest tree up to 20m tall (Boland, 1979). Forest provenances have been included in a number of species trials for wood production (Cotterill *et al.*, 1985). Eucalypts have predominantly hermaphrodite flowers and all species examined to date show strong protandry, with male and female phases of the flower separated by several days (Griffin and Hand, 1979; Hodgson, 1976a). Anther shape varies considerably across the genus, but pollen grain numbers are high and pollen to ovule ratios are typical of outcrossers (Cruden, 1977; Moncur and Boland, 1989). In most species pollen is presented on the anthers and readily adheres to pollinators' body parts for transfer to other flowers. Other studies (Boland and Sedgley, 1986; Moncur and Boland, 1989), have noted that the pollen of some species such as *E. melliodora* tends to be sticky and may adhere to the underside of the pin-head stigma in clumps, during filament expansion. Boland and Sedgley (1986) suggested this as a pollen presentation mechanism for collection by insects. This pollen presentation mechanism is also seen in *E. leucoxylon*, *E. sideroxylon* , *E. polyanthemos* and to a limited extent in *E. fasciculosa*. In *E. leucoxylon* the pollen is deposited on the upper style below the pin-head stigma in a large sticky clump.

Departures from the bisexual condition manifested as congenital sterility of male or female structures, have been reported for several species of eucalypt. Carr *et al.*, (1971) observed the occurrence male flowers in several species of the subgenus *Corymbia*. These resulted from bisexual flowers following abortion of the ovary during development. The frequency of male flowers varied from tree to tree with some trees being functionally male. Congenitally sterile anthers also occur in many species, and Carr and Carr (1962) noted that it is common in *Eucalyptus* for the first formed stamens to lack anthers. In chapter four sterile stamens from the inner whorls of *E. leptophylla* were described, apparently due

to incomplete anther development. Davis (1969) found in the three trees of *E. stellulata* she examined, the majority of anthers contained only sterile pollen grains, and in no instance were sterile and fertile pollen grains found in the same anther, or flower. In a study of the breeding system and genetic diversity of *E. pulverulenta*, Peters *et al.*, (1990) found that trees showed differential levels of pollen sterility, with some trees bearing no fertile pollen.

Flowers of *E. leucoxylon* are here reported to fall into two categories, functionally female and hermaphrodite. The hermaphrodite flowers possess normal ovaries and anthers while female flowers have abnormal, shrivelled anthers and no fertile pollen grains. As the two floral forms were never present on the same tree, the population is functionally gynodioecious. Male sterility is of interest to tree breeders for its potential use in tree improvement programmes, and it also has implications for gene flow and reproductive resource allocation in populations. The occurrence of the pollen presentation mechanism and male sterility, and the effect they have on the breeding system are investigated here through morphological observations, anther cytology, female fertility and outcrossing rates.

5.2 MATERIALS AND METHODS

5.2.1 Plant material

Eucalyptus leucoxylon ssp *leucoxylon* trees used in this study were located in a mixed species plantation at Callington (latitude 35' 07", longitude 139' 02") South Australia. Total *E. leucoxylon* population size was approximately one thousand trees over an area of approximately 3km^2. A core of 20 trees (Fig. 5.1) were used for data collection, other trees in the same population were also surveyed when numbers required were greater than 20. Trees were approximately 15 years old, had borne flower and fruit crops for many years, and all trees produced fertile seed. The seed source for the plantation is unknown but plant morphology was identical to the indigenous provenance found in a nearby reserve. Three flower colour morphs were present in the population; red, pink and white.

Figure 5.1 The layout of experimental *E. leucoxylon* trees in a mixed species plantation at Callington, South Australia. Experimental female (F) and hermaphrodite (H) trees are numbered 1-10, other *E. leucoxylon* trees not used in this study (L) and other species (+) are also shown. Rows were approximately six metres apart.

L + H10
+ L + L +
+ + + + +
+ + L + +
H2 F3 F7 H9 + L
+ + + + +
+ + + + + +
L L L
+ L F6 L F10
+ + +
+ + + + +
H1 + + +
+ + + +
+ H4 + L
+ + H8 F8 +
L + F5 L + +
+ H3 + L +
+ + + + L
+ H7 +
+ + H5
+ + + + +
+ F2 +
+ + F4 + F9
L + H6 L +
F1

5.2.2 Floral sequence

Twenty mature buds at operculum lift (anthesis) on one tree were tagged and observed daily to determine the sequence of floral development. Flowers were scored for features including anther dehiscence, pollen deposition, stigma condition and abscission of floral parts. Increase in style length after anthesis was measured on 10 flowers from each of three trees at intervals until day 27. The time of stigmatic receptivity was estimated by observation of the timing of stigmatic exudate production.

5.2.3 Male fertility and pollen presentation

100 flowers per tree for each of two hermaphrodite and two female trees were examined at the stage just prior to full filament extension to determine consistency within trees in pollen presentation on the style, and in male sterility. In addition five flowers from each of 100 trees were examined at the same stage to determine consistency within the population. Flowers were scored for the presence of pollen and the location of pollen masses. The styles and anthers were separated from five flowers per tree for three hermaphrodite and three female trees, and the pollen suspended in 70% alcohol. Four replicate subsamples of pollen grains on the style, and of the anthers of each flower were counted using a haemocytometer.

At least four buds from each of two trees of each morph, at anthesis and at approximately six weeks before anthesis were fixed in 3% glutaraldehyde under vacuum for 24 hours and embedded in glycol methacrylate (GMA) plastic resin, 3.5 μm sections were cut, and stained with Periodic Acid Schiff reagent and Toluidine blue O.

5.2.4 Female fertility

To establish whether pollen presentation on the style resulted in autogamous seed set, mature buds on each of three trees per morph (mean of 122 buds per tree) were bagged prior to anthesis. Assisted self pollinations were conducted in which bags were opened and self pollen from the pollen mass smeared over the stigma during the stigmatic receptive phase. A similar number of open pollinated controls were also set up on each tree. Bags were removed after one month when pistils were no longer receptive, and capsules

harvested one year later at maturity. The number of capsules set and seeds per capsule were analysed by fitting a binomial model followed by chi square analysis.

Twenty mature open pollinated pistils were collected from three female and three hermaphrodite trees, fixed in Carnoys fixative for 24 hours and processed for squash preparation for fluorescence microscopy. The number of pollen tubes in the upper style and lower style of each pistil were counted. Differences in pollen tube growth in the styles of the two morphs, and between styles within morph types were analysed using analysis of variance in a nested treatment structure. Success of pollen tube growth was measured as the ratio of the number of pollen tubes at the base of the style to the number of pollen tubes in the upper style and differences tested using analysis of variance.

25 open pollinated capsules were harvested from three trees of each morph, allowed to dehisce and release their contents and the number of full seed per capsule counted. The number of seeds per capsule in open pollinated capsules of hermaphrodite and female trees were compared using analysis of variance.

5.2.5 Isozyme analysis

Seeds harvested from ten hermaphrodite and ten female trees were germinated on sterile soil under glass and cotyledons harvested before two weeks of age. Cotyledons were ground in 40 µl of 0.05 M tris (pH 8.0) containing 0.15% ascorbic acid, 0.12% cysteine HCl, and 0.1% citric acid, with approximately 1mg of polyvinyl poly pyrrolidone. The crude extract was centrifuged for 10 minutes and the supernatant loaded onto a cellulose acetate gel (Cellogel) and subjected to 200 V at 2° C for 1.5 to 2 hours. Five enzyme systems were assayed; PGI (EC: 5.3.1.9), PGM (EC: 2.7.5.1), LAP (EC: 2.6.1.1), SDH (EC: 1.1.1.25) and 6PGD (EC: 1.1.1.44). Running buffers were 0.05 M tris maleate pH 7.8 for LAP, SDH and 6PGD and 0.025 M tris glycine pH 8.5 for PGI and PGM. Gels were stained according to Richardson *et al.*, (1986) and Jackson and Clarke (1991).

Twenty seedlings per tree were analysed for the five loci. Maternal trees were selected on the basis of proximity to each other (Fig. 5.1) and overlap in flowering season. The experimental trees were surrounded by a large number of other *E. leucoxylon* trees

that could have acted as pollen donors. Maternal genotypes, pollen pool allele frequencies and multilocus estimates of outcrossing rates for the two subpopulations were derived using the methods of Ritland and Jain (1981). Heterozygosities and Wrights fixation index were calculated according to the methods of Brown *et al.*, (1975), Brown (1977) and Hartl and Clarke (1989).

5.3 RESULTS

5.3.1 Floral sequence

Figure 5.2A shows the sequence of floral development in *E. leucoxylon*. Prior to anthesis the *E. leucoxylon* bud is covered by an operculum derived from the perianth, the stamen filaments are tightly inflexed, and the undehisced anthers are held at the base of the floral cup. At anthesis the operculum separates from the hypanthium and is pushed upwards by the elongating style. Stamen filaments begin to extend, lifting the anthers from the base of the hypanthium (Fig. 5.2B). Anther dehiscence occurs during filament extension, at this stage pollinators are excluded from the pollen by the dense dome of filaments. During filament extension each anther brushes against the upper style or stigma and deposits its pollen mixed with anther secretion, in a sticky clump(Fig. 5.2C-D, F). In open pollinated flowers pollen is removed from the style over several days by pollinators. Birds, especially Honeyeaters (*Melliphagidae*), were observed to visit the flowers of *E. leucoxylon*. In male phase some nectar is secreted but peak nectar production occurs when the stigma becomes sticky and expanded and the style ceases to elongate (Fig. 5.2E). After the stigma becomes dry and brown stamen filaments begin to abscise, in later stages of fruit production the style also abscises at the base.

Measurements of style length are shown in Fig. 5.3. There were differences in the length of the style between trees, but all trees showed the same pattern of style length increase and attained maximum length by day 15. Secretion was first observed on stigmas eight days after anthesis, and in bagged flowers secretion volume increased over a period of approximately ten days if not removed by pollinators.

Figure 5.2 Floral development in *E. leucoxylon.*

5.2A. Floral development in open pollinated flowers. (1) Immature bud with intact operculum. (2) Anthesis, operculum starting to lift. (3) Anther dehiscence, filaments extending, style elongating. (4) Pollen deposition, as the filaments extend the dehisced anthers deposit their pollen loads onto the upper style and stigma, pollinators are excluded by the inflexed filaments, style elongating. (5) Male phase, filaments fully extended, style elongating, nectar production starts. (6) Female phase, style fully elongated, pollen mass removed by pollinators, stigma fully expanded with copious secretion, copious nectar production. (7) Senscence, filaments abscissed, stigma brown. Bar represents 5cm.

5.2B. Dissected flower at stage 2, filaments are tightly inflexed in the bud, anthers undehisced. Bar represents 5 mm.

5.2C. Dissected flower at stage 4, as filaments extend all the dehisced anthers brush against the style and deposit pollen in a sticky mass just beneath the stigma. Bar represents 5 cm.

5.2D. Dissected flower at stage 5, filaments fully extended, pollen (p) is presented in a sticky mass at the top of the style just below the stigma. Bar represents 5 mm.

5.2E. Half flower at stage 6, pollen mass has been removed by pollinators, the stigma is covered with secretion and nectar accumulates at the base of the floral cup (n). Bar represents 2 cm.

5.2F. Scanning electron micrograph of pollen masses deposited on the style, pollen grains (p) are mixed with secretion (s). Bar represents 50 μm.

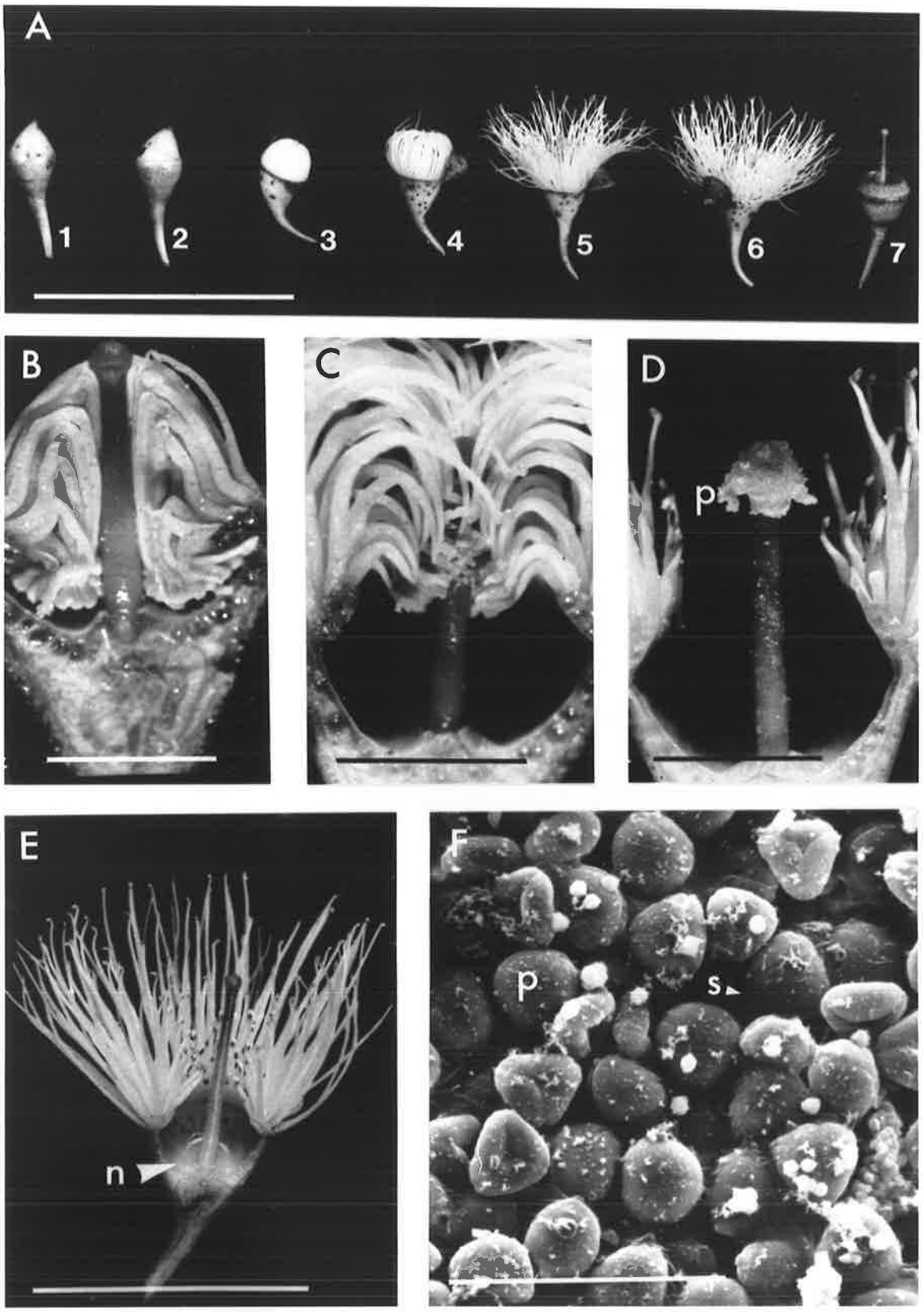
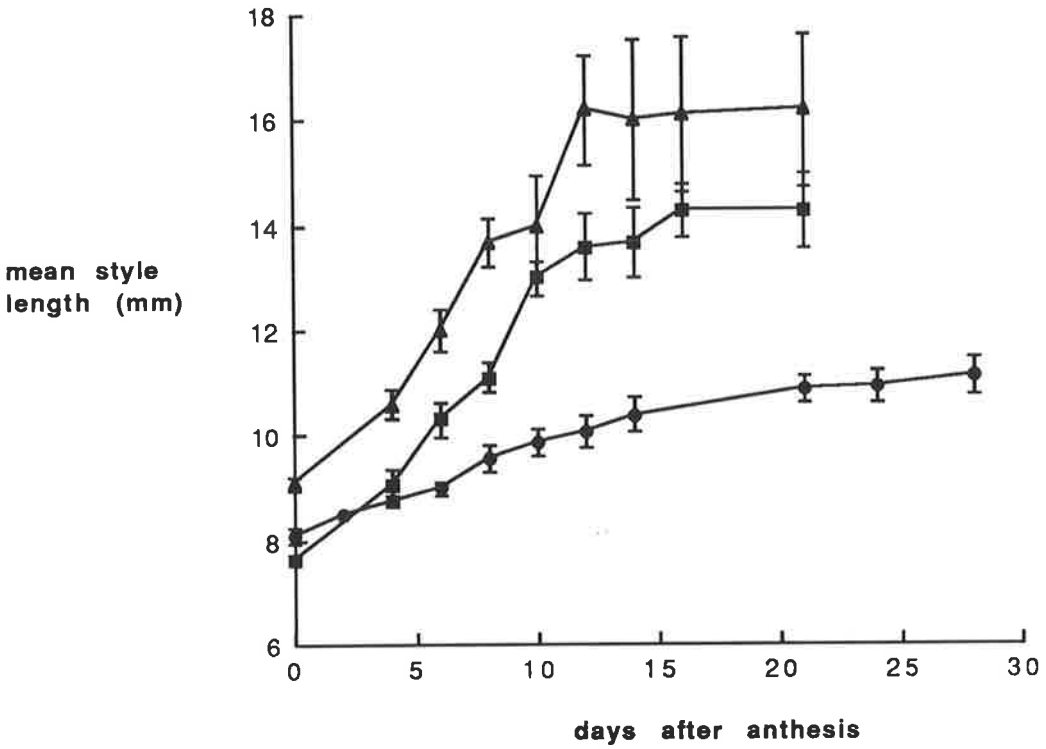

A
1
2
3
4
5
6
7
B
C
D
p
E
n
F
p
s

Figure 5.3. Post anthesis style length increase in three trees of *E. leucoxylon* (mean values of ten styles ± standard error , ▲ tree 1, ■ tree 2, ● tree 3).


mean style length (mm)
18
16
14
12
10
8
6
0
5
10
15
20
25
30
days after anthesis

5.3.2 Male Fertility and Pollen Presentation

The occurrence of sterile anthers or pollen presentation on the style was consistent within a flower and within a tree. No tree sampled showed both male sterile and hermaphrodite flowers. Pollen presentation was also consistent within a tree, all flowers that produced fertile pollen presented pollen on the style. Fifty seven percent of trees were effectively male sterile and forty three per cent hermaphrodite. Trees observed over three flowering seasons were consistent in their sex expression between years. Three colour morphs were present in the population and comprised, red 80 %, pink 12 %, white 8 %. Pollen counts from flowers in the male phase showed that no fertile pollen is produced in female flowers, while in hermaphrodite flowers a mean of 1.74×10^5 pollen grains were produced per flower, 93.2 % of grains were presented on the pollen mass below the stigma and only 6.8 % remained on the anthers (Table 5.1).

5.3.3 Anther and pollen grain development

The *E. leucoxylon* anther consists of two lobes each containing two sporangia. At the base of the anther there is a large gland, which in immature flowers contains large thin walled secretory cells which disintegrate upon anther maturity to form a fluid filled lumen surrounded by the remnants of the secretory cells. Inside the epidermal layer of the anther a single layer of endothecial cells surrounded the sporangia. Two rows of middle layer cells formed the boundary of the sporangium. In fertile anthers approximately six weeks before anthesis the tapetum cells formed a layer of 1-2 cells thickness around the developing microspores (Fig. 5.4A). In some anthers tetrads were present, but in most the microspores were free, possessed a well developed exine, were mononucleate and were surrounded by the intact tapetum. In later stages an increase in starch grain synthesis was accompanied by tapetal breakdown (Fig 5.4B) and division of the microspore nucleus. In mature anthers at anthesis tapetal breakdown was complete, and pollen grains filled the lumen of the sporangia (Fig. 5.4C). The endothecium remained intact and remnants of the middle layers were visible. Pollen grains contained abundant starch grains and showed pronounced thickening of the intine adjacent to the germination pores. Two nuclei were

Table 5.1: Pollen grain counts (x 10^4) on anthers and styles of *E. leucoxylon* flowers from three hermaphrodite and three female trees. (Mean ± standard error).

	Tree 1	Tree 2	Tree 3
Female			
Style	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Anthers	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Hermaphrodite			
Style	16.00 ± 1.41	25.30 ± 4.04	7.45 ± 0.86
Anthers	0.52 ± 0.15	2.05 ± 0.39	1.17 ± 0.49

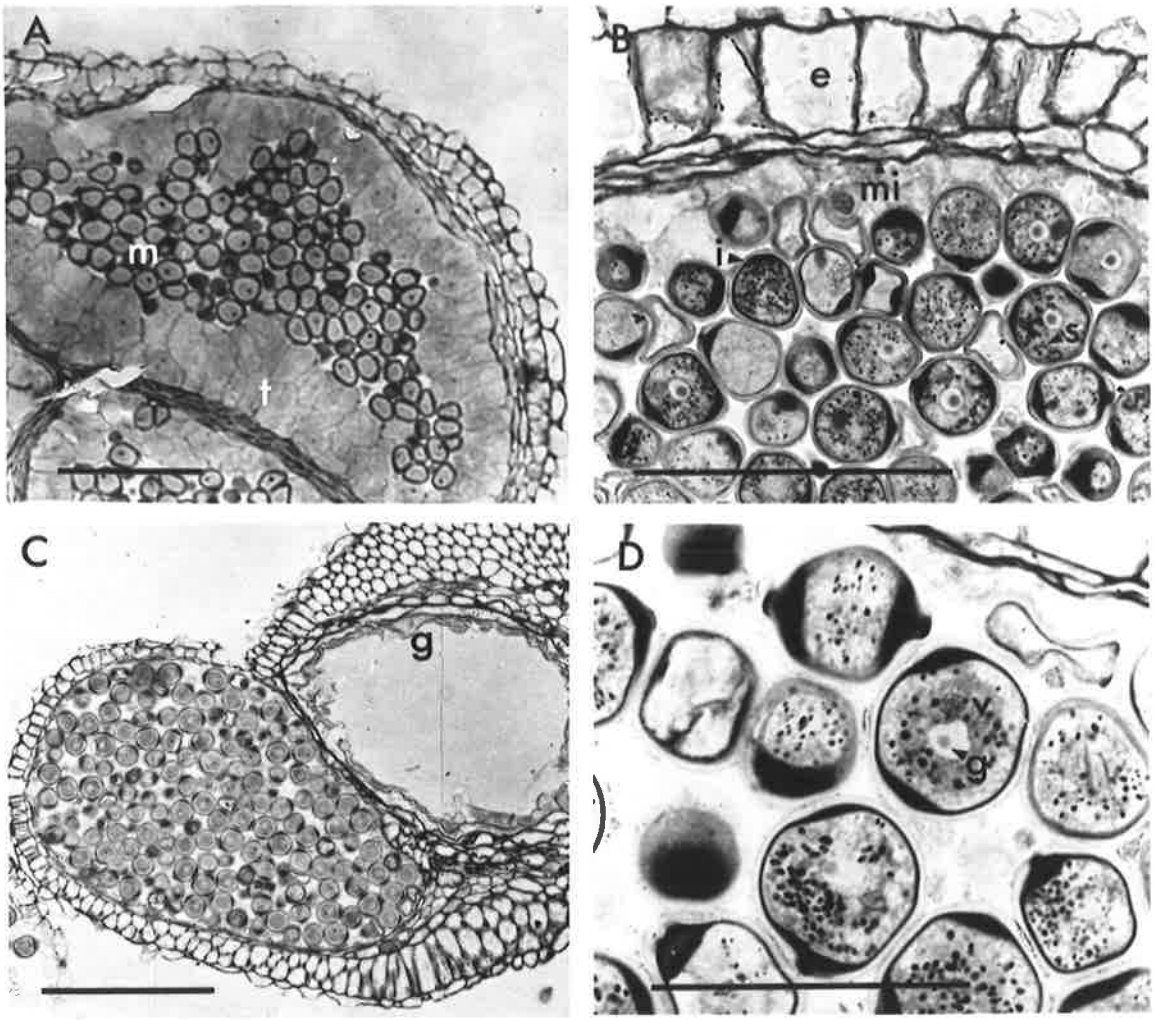
Figure 5.4 Pollen grain development in *E. leucoxylon* . Light micrographs of anthers from hermaphrodite flowers stained with PAS and TBO.

5.4A. Immature anther locule showing developing microspores (m) surrounded by the tapetum (t). Bar represents 20μm.

5.4B. Immature anther locule after degeneration of the tapetum. A single layer of endothecial cells (e) and two layers of middle layer cells (mi) surround the sporangium. Microspores show starch grain synthesis (s) and some intine development (i). Bar represents 100 μm.

5.4C. Mature anther locule at anthesis, showing complete tapetal and middle layer breakdown. Anther gland cells (g) have also broken down leaving a fluid filled lumen. Bar represents 200μm.

5.4D. Mature pollen grains in undehisced anthers at anthesis, showing intine thickenings around the germination pores, abundant starch grains and two nuclei. The generative nucleus (g) is surrounded by an envelope of clear cytoplasm within the vegetative cell (v). Bar represents 50μm.


A
m
t
B
e
mi
i
s
C
g
D
v
g

visible, the generative nucleus was surrounded by an envelope of clear cytoplasm within the cytoplasm of the vegetative cell (Fig. 5.4 D).

In contrast to fertile anthers, anthers from male sterile flowers showed a number of differences (Fig. 5.5A-E). Microspores underwent meiosis and formed tetrads (Fig. 5.5A). Six weeks before anthesis there was little difference in endothecial and middle layer development (Fig. 5.5B), but although microspores had well formed exines, few showed any starch granules (Fig. 5.5C). At anthesis the anther locules and pollen grains had collapsed and were surrounded by an incompletely degenerated tapetum (Fig. 5.5D-E). The pollen grains showed no intine development and few starch granules, and the few pollen grains that did not collapse lacked nuclei and cytoplasm.

5.3.4 Female fertility.

Female fertility was investigated three ways. The effect of pollen dumping on seed set was assessed by comparing seed and capsule set in hermaphrodite flowers (Table 5.2). There was no significant difference between autogamous, assisted self and open pollination treatments in capsule set with little variation between trees. However there was a significant difference in the number of seeds per capsule between the treatments, with open pollinated flowers showing significantly greater numbers of seeds per capsule than either self or autogamous pollinations ($P<0.05$).

Open pollinated capsules were compared between female and hermaphrodite trees for seed numbers. There were found to be significant differences between trees ($P<0.001$) but no differences between tree types. Over all trees examined a mean of 30.7 percent of capsules were barren.

Pollen receipt and efficiency of pollination was tested by measuring the number of pollen tubes in the style of open pollinated flowers from the two morphs (Table 5.3). There was a significant difference in the number of pollen tubes in the upper and lower styles of the two morphs ($P<0.001$). Mean pollen tube numbers were very low in male sterile flowers mainly due to a large number of pistils with zero pollen tubes. Hermaphrodite flowers showed much higher numbers of pollen tubes and few pistils with

Figure 5.5 Light micrographs of sections through male sterile anthers of *E. leucoxylon*, stained with PAS and TBO.

5.5A. Immature anther locule showing tetrads (t) surrounded by the tapetal layer (ta). Bar represents 50μm.

5.5B. Longitudinal Section through an immature anther showing position of anther gland (g) and developing pollen grains (p) in the anther locule. Bar represents 200μm.

5.5C. Immature anther showing endothecial cells (e), middle layer cells (mi), tapetal layer (t) and mononucleate microspores (m), some with starch granules. Bar represents 50μm.

5.5D. Mature anther, pollen grains (p) have collapsed and no nuclei are visible, although some starch granules remain. Bar represents 50μm.

5.5E. Mature anther at anthesis showing collapsed locule (l) and pollen grains and fluid filled lumen of anther gland (g). Bar represents 200μm.

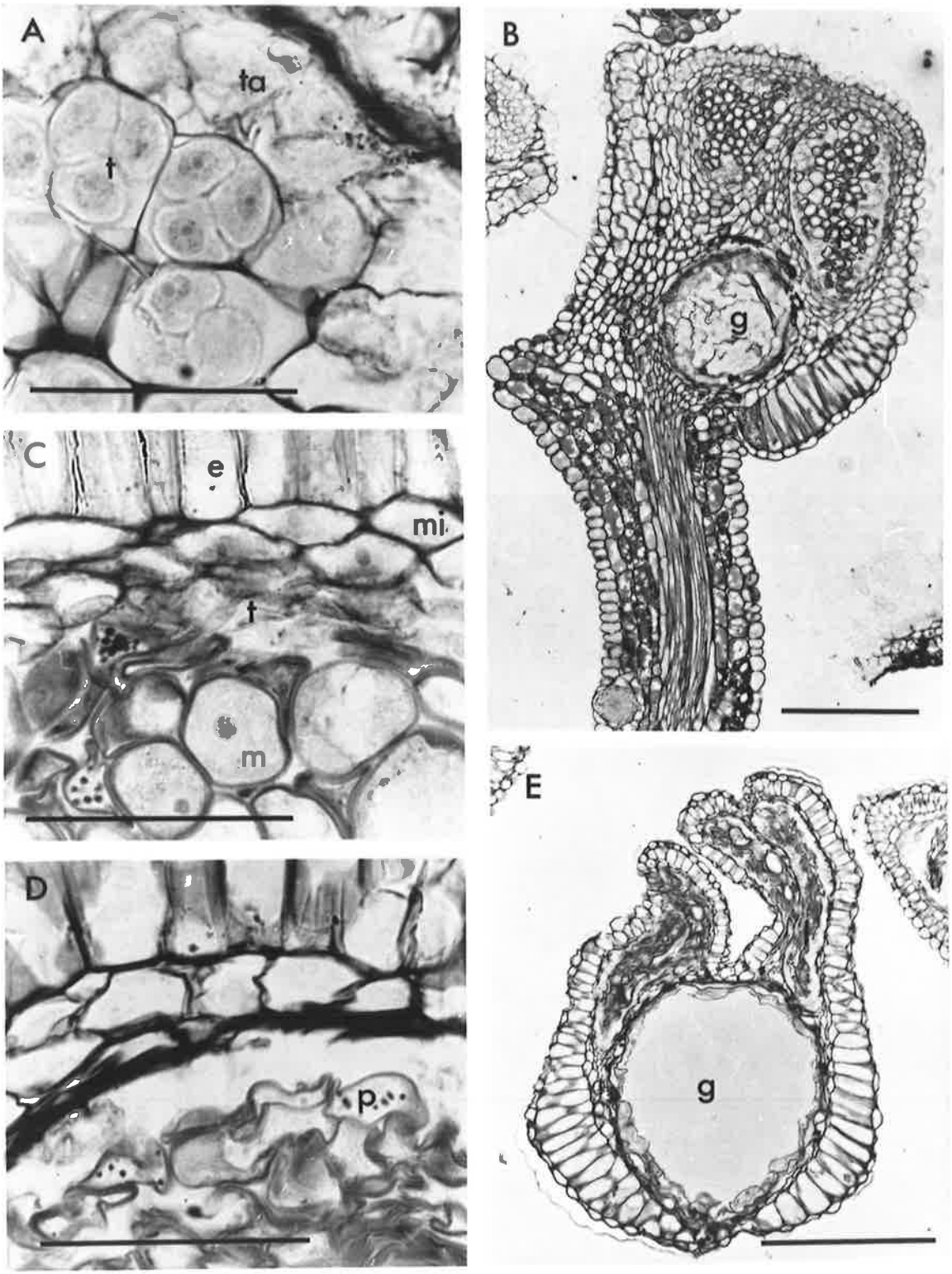

A
ta
t
B
g
C
e
mi
t
m
D
p
E
g

Table 5.2: Capsule and seed set in three hermaphrodite trees of *E. leucoxylon*. (n = number of flowers bagged, % cap set = percent capsule set, S/C = number of seeds per capsule.)

	Tree			
	1	2	3	mean
Autogamous				
% cap	4.7	5.0	7.7	5.7 ± 0.7
S/C	2.8	2.3	0.9	2.0 ± 0.6
n	170	141	119	
Self				
% cap	1.0	2.2	3.5	2.2 ± 0.6
S/C	0.0	2.0	1.5	1.2 ± 0.6
n	97	93	115	
Open				
% cap	5.7	1.4	7.4	4.8 ± 1.0
S/C	5.0	5.0	7.0	5.7 ± 0.6
n	122	148	95	

Table 5.3: Counts of pollen tubes in open pollinated pistils of three hermaphrodite and three female *E. leucoxylon* trees. (Means of twenty pistils ± standard error).

	Hermaphrodite tree			Female tree		
	1	2	3	1	2	3
Upper style	125·4 ± 38·5	127·4 ± 26·1	88·7 ± 25·1	31·4 ± 14·8	6·2 ± 2·8	2·0 ± 0·62
Lower style	70·6 ± 20·1	80·6 ± 22·3	47·6 ± 15·7	18·0 ± 9·5	5·1 ± 6·7	0·84 ± 0·4
Percent success	47·2 ± 8·6	58·2 ± 7·5	47·1 ± 9·6	46·17 ± 10·2	43·0 ± 14·6	58·3 ± 17·5

no pollen tubes. There was no significant difference between the success of pollen tubes in either flower type.

5.3.5 Outcrossing rates

Two zones of activity were found for PGI (Fig. 5.6), PGI-1 was mainly monomorphic with very low levels of polymorphisms, while PGI-2 was highly polymorphic with six alleles occurring in the study population. PGM and 6PGD showed two zones of activity with the faster migrating locus being monomorphic in both cases, and four alleles found for both PGM2 and 6PGD-2. One locus of SDH was found with three alleles identified. Only one strong zone of activity with four alleles was found for LAP probably analagous to the LAP-2 locus described by Moran and Bell (1983). Allele frequencies for the five loci used for mating system estimation are given in Table 5.4. Allele frequencies tended to be similar in the parents, progeny and pollen pool. The greatest differences were evident between the pollen pools of the female and hermaphrodite trees, indicating that the pollen pools available to each morph were different due to the presence of self pollen in the pollen pool of the hermaphrodite trees. Observed levels of heterozygosity (H) were calculated after combining the less common alleles to provide diallelic data (Table 5.5). Expected heterozygosities ($\hat{He}$) were calculated from allelic frequencies according to the Hardy-Weinberg expectation and Wrights fixation index ($\hat{F}$) calculated for each locus using the formula

$$\hat{F} = 1\text{-}H / 2\,pi\,(1 - pi) \qquad \text{(Brown } et\ al.\text{, 1975)}$$

Where pi is the frequency of the common allele at locus i. Variances of $\hat{F}$ were calculated from the formula

$$\text{Var}(\hat{F}) = (1 - 2F).(1 - F)^2 / N + F(-F).(2 - F) / 2.p.q.N \qquad \text{(Brown, 1979)}$$

Where p and q are the frequencies of the common and rare alleles, and N is the sample size. Observed levels of heterozygosity compared closely with expected levels under panmixia although hermaphrodite trees showed a positive overall value of $\hat{F}$ indicating a significant level of inbreeding.

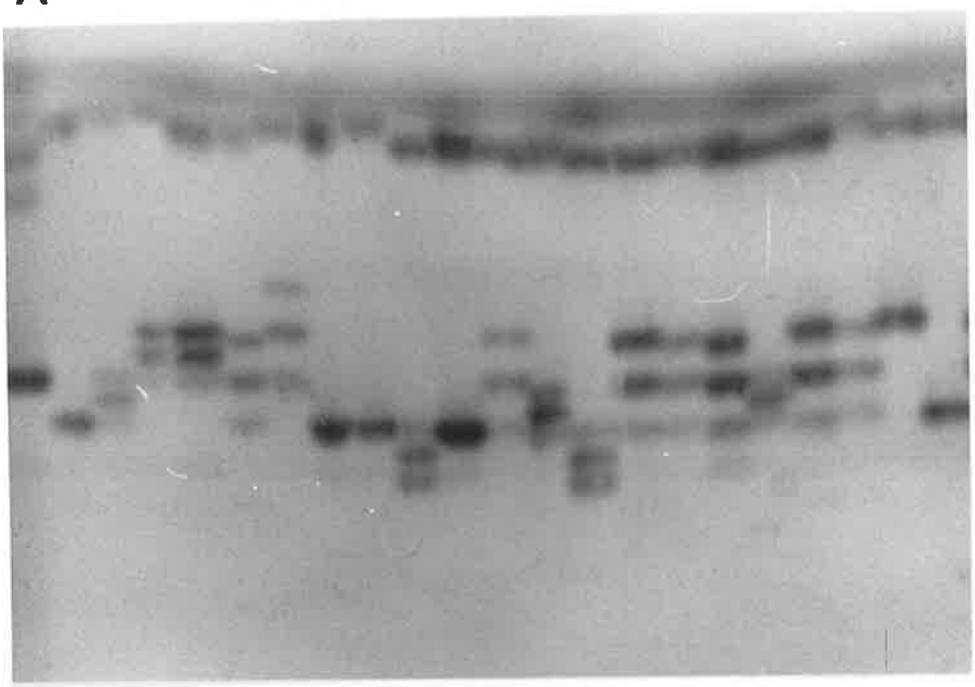
These results are confirmed by estimates of outcrossing rates (Table 5.6). When compared using an unpaired t-test the two morphs showed significant differences in

Figure 5.6. Zymograms of Phospho-Glucose Isomerase in *E. leucoxylon* seedlings.

5.6A. Phospho-Glucose Isomerase zymogram from seedlings of *E. leucoxylon*, showing two zones of activity, PGI-2 shows segregation for five alleles. Lanes 1-13 female tree 2, lanes 14-22 hermaphrodite tree 1.

5.6B. Diagramatic representation of PGI-2 genotypes shown in 5.6A.

A



B

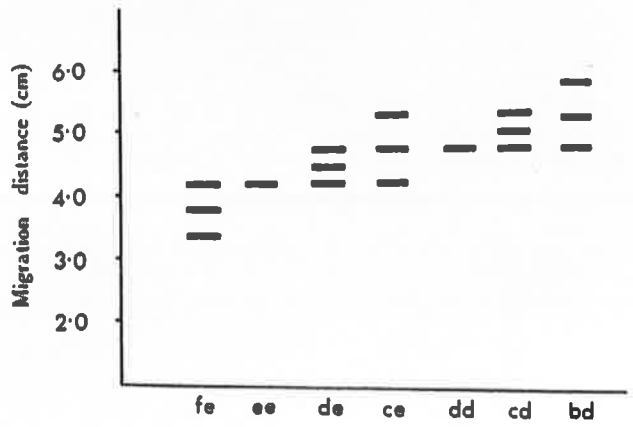


Table 5.4: Allele frequencies at five loci assayed in the progeny of female and hermaphrodite trees. (* frequency calculated for one allele only).

Locus / Allele	Female trees			Hermaphrodite trees		
	progeny	maternal parents	pollen* pool	progeny	maternal parents	pollen* pool
PGI-2						
a	0·000	0·000		0·003	0·000	
b	0·013	0·000		0·064	0·050	
c	0·333	0·350		0·309	0·300	
d	0·184	0·100		0·278	0·300	
e	0·442	0·500	0·265	0·294	0·300	0·189
f	0·028	0·050		0·052	0·050	
PGM-1						
a	0·000	0·000		0·029	0·000	
b	0·767	0·800	0·734	0·714	0·750	0·632
c	0·233	0·200		0·254	0·250	
d	0·000	0·000		0·003	0·000	
LAP-2						
a	0·008	0·000		0·052	0·100	
b	0·836	0·750	0·920	0·819	0·850	0·797
c	0·151	0·250		0·106	0·050	
d	0·005	0·000		0·023	0·000	
SDH-1						
a	0·063	0·000		0·049	0·100	
b	0·894	0·900	0·82	0·879	0·800	0·882
c	0·043	0·100		0·072	0·100	
6PGD-2						
a	0·123	0·050		0·116	0·100	
b	0·877	0·950	0·797	0·863	0·900	0·805
c	0·000	0·000		0·003	0·000	
d	0·000	0·000		0·018	0·000	

Table 5.5: Observed heterozygosities (H), expected heterozygosities ($\hat{H}e$), Wrights fixation index ($\hat{F}$) ± standard error, and inbreeding equilibrium coefficient ($\hat{F}e$) for five loci assayed in the progeny of two morphs of *E. leucoxylon*.. (* standard error undefined).

	Female trees			Hermaphrodite trees		
	H	$\hat{H}e$	$\hat{F}$	H	$\hat{H}e$	$\hat{F}$
PGI	0·51	0·49	-0·05± 5.0×10^{-3}	0·39	0·42	0·06± 5.3×10^{-3}
PGM	0·34	0·36	0·05± 5.2×10^{-3}	0·44	0·41	-0·08± 5.1×10^{-3}
LAP	0·26	0·27	0·05± 5.7×10^{-3}	0·24	0·30	0·19± 6.0×10^{-3}
SDH	0·21	0·19	-0·12 *	0·17	0·21	0·20± 6.8×10^{-3}
6PGD	0·22	0·21	-0·03± 4.6×10^{-3}	0·19	0·24	0·20± 6.6×10^{-3}
mean	0·31	0·30	-0·02	0·29	0·31	0·11
$\hat{F}e$			0·02			0·10

Table 5.6: Outcrossing rates ($\hat{t}$) of ten hermaphrodite and ten female *E. leucoxylon* trees .

Tree number	Female	Hermaphrodite
1	1.000	0.793
2	0.953	0.781
3	0.979	0.644
4	0.938	0.933
5	0.991	0.853
6	0.979	0.683
7	0.977	0.949
8	0.922	0.881
9	0.908	0.895
10	0.952	0.817
mean	0.960 ± 0.010	0.823 ± 0.032

outbreeding rates (P<0.001). Female trees showed a mean $\hat{t}$ of 0.96 which does not differ greatly from zero. This is a conservative estimate of $\hat{t}$ due to the combining of rarer alleles, thus losing some information and detectable outcrosses. Hermaphrodite trees show a mean $\hat{t}$ of 0.82 comparing well with the fixation index value of 0.11 which also showed a significant level of inbreeding. Hermaphrodite trees show more variability between trees in outbreeding rate as would be expected due to differences in opportunities for outcrossing and levels of self compatibility. When outbreeding rates ($\hat{t}$) of all trees are combined and weighted according to the proportion of each morph in the population

$$\hat{t}_{pop} = (\hat{t}_H . \pi_H) + (\hat{t}_F . \pi_F)$$

where $\hat{t}_H$ and $\hat{t}_F$ are the outbreeding rates for hermaphrodite and female trees respectively and π_H and π_F are the proportions of hermaphrodite and female trees in the population. The total $\hat{t}_{pop} = 0.901$. Calculations of the inbreeding equilibrium coefficient ($\hat{F}e$) from the formula

$$\hat{F}_{expected} = \hat{s} / (2 - s) \qquad \text{(Hartl and Clarke, 1989)}$$

where $\hat{s}$ is the selfing rate (1 - $\hat{t}$), are for hermaphrodite trees ($\hat{F}e = 0.10$), female trees ($\hat{F}e = 0.02$) and the whole population ($\hat{F}e = 0.06$) and show the population is in inbreeding equilibrium.

5.4 DISCUSSION

This is the first investigation of gynodioecy in *Eucalyptus*, a large genus noteworthy for its conservation of the hermaphroditic condition. The outcrossing rate calculated for *E. leucoxylon* trees is among the highest reported for eucalypt species. Most other eucalypts have a mixed mating system with up to thirty per cent of open pollinated seed crops resulting from self pollinations (Moran and Bell, 1983). Hermaphrodite *E. leucoxylon* trees achieve a high rate of outcrossing despite the complication of the presence of self pollen on and around the stigmatic area but the female trees have an even higher outcrossing rate. Clearly the mechanisms of protandry and self incompatibility are not totally successful in preventing autogamous pollination. The control of partial self incompatibility in *Eucalyptus* has been demonstrated to occur mainly post-zygotically, after ovule penetration (Sedgley *et al.*, 1989; Sedgley and Smith, 1989),

although in *E. spathulata* and *E. woodwardii,* some pre-fertilisation selection occurs in the ovary prior to ovule penetration (chapter four; Sedgley and Smith, 1989).

Male sterile *E. leucoxylon* trees are pollen limited in the study population. Low pollen tube numbers in female pistils compared with hermaphrodite pistils suggests a large proportion of pollen grains on stigmas of hermaphrodite pistils are either autogamous or geitonogamous. Efficiency of pollen transfer between hermaphrodite and female trees depends upon pollinator abundance and foraging behaviour. The relative frequencies of the two morphs in the population will also determine the efficiency of transfer of pollen to female flowers. As the proportion of hermaphrodites decreases the amount of pollen available to female trees and amount of cross pollen available to hermaphrodites also decreases. Sun and Ganders (1986) found a positive correlation between the frequency of females in eight populations of gynodioecious *Bidens* species and the selfing rates of hermaphrodites in the populations. To assess the effect of gynodioecy on the natural populations, the proportion of each morph in natural stands needs to be investigated.

Differences in the development of microspores in fertile and male sterile anthers of *E. leucoxylon* appear greatest towards the final stages of development. The incomplete degeneration of the tapetum and the lack of intine and starch, implicates functional abnormalities of the tapetum during the critical stages of cytoplasmic synthesis and nuclear division. Tapetal abnormalities have been identified as the cause of male sterility in many other plant groups (Laser and Lersten, 1972; Saini *et al.,* 1984; Sun and Ganders, 1987).

The consistency of male sterility in the population within trees and between years suggests genetic control. Male sterility may be inherited through nuclear genes on the chromosomes, cytoplasmic genes via the plastids or mitochondria or a combination of both (Sedgley and Griffin, 1989). The heritability of this trait in *Eucalyptus* requires further study to determine its implications for gene flow and evolutionary directions and its utility in tree breeding programmes.

Gynodioecy has been suggested as a step towards the evolution of dioecy from bisexual species. Conditions needed for the maintenance and spread of male steriles in a hermaphroditic population are either increased seed production in male sterile plants or a higher fitness of progeny due to obligate outcrossing (Bawa, 1980). The main selective

force is considered to be increase in outcrossing, although some authors have suggested that dioecy is a means of escaping interference between self and cross pollen on the stigma which may lower the chances of cross pollen tubes reaching the ovules and producing seed (Bawa and Opler, 1975; Kikuzawa 1989; Lloyd and Yates 1982). This possibility seems attractive in the case of *E. leucoxylon,* in view of its pollen presentation mechanism, however isozyme studies of open pollinated seed crops show a high level of outbreeding is maintained despite the deposition of self pollen on or near the stigma.

E. leucoxylon and its close relatives are unique among the eucalypts in their pollen presentation mechanism, ensuring some contact between the pistil and self pollen despite temporal differences between the female and male phases in the flower. Bagging experiments have shown that hermaphrodite *E. leucoxylon* trees are capable of setting self seed in the absence of cross pollen. Although most pollen deposition occurs on the style just below the stigmatic surface, enough pollen must come into contact with the papillae to allow autogamous seed set as shown in this study. The semi-pendulous nature of the flower may aid in this transfer of pollen to the stigma surface, especially when stigmatic secretion builds up on the stigma. No differences between success of pollen tube growth in hermaphrodite and female pistils also points to a lack of stylar selection mechanisms, as pollen tubes in hermaphrodite pistils will be a mixture of self and cross pollen tubes. The fact that significantly greater numbers of seed are set when the flowers are open to visits from pollinators and cross pollen, and the high outcrossing estimates for hermaphrodite trees obtained through isozyme analysis indicates that there is selection for outcrossed embryos either through a late acting partial self incompatibility mechanism or through limited numbers of self pollen grains contacting the receptive stigma surface.

The anther gland is particularly prominent in *E. leucoxylon* and is probably the source of the secretion which mixes with the pollen to form the sticky pollen mass essential for the pollen presentation. Similar secretions are found in other Myrtaceous genera. Beardsell *et al.,* (1989) and Slater and Beardsell (1991) found the anther glands in *Thryptomene* and *Chamelaucium* produce lipidic secretion containing small amounts of carbohydrates, proteins and phenolics and may function as a food source to attract pollinators before the onset of peak nectar production.

Griffin (1982) surveyed 101 species of eucalypt and found trends in flowering syndromes, associated with flower size, colour and season of flowering. Coloured flowers occurred predominantly in large flowered species which flowered between July and March. Large floral size and strong colouration are adaptations for bird pollination (Ford and Paton, 1977), as large flowers produce more nectar and can support large pollinators. *E. leucoxylon* , a winter flowering species, which flowers when insect activity is low shows polymorphism in flower colour within and between populations, white, red, pink and yellow forms occurring. Flowering period is long, with trees in flower from April to November, suggesting an adaptation to a vertebrate pollinator.

A question that arises is whether the occurrence of the pollen presentation mechanism, unique in this taxonomic group, is linked to, or coincidental with the instance of gynodioecy. Similar pollen presentation mechanisms have been reported in other genera, notably *Macadamia* (Sedgley *et al.,* 1985), *Banksia* (Collins and Rebelo, 1987) in the Proteacea and *Chamelaucium* (Slater and Beardsell, 1991) in the Myrtaceae. These species maintain a high degree of outcrossing via protandry, pollen removal and partial self incompatibility mechanisms (Fuss and Sedgley, 1991; Sedgley, 1983; Slater and Beardsell, 1991) although facultative autogamous seed set has been noted in *Banksia* (Vaughton, 1988), but male sterility has not been reported.

The possibility of a similar pollen presentation mechanism in other closely related eucalypts deserves more attention. Of these species only *E. melliodora* has received any mention in the literature on breeding systems, and floral morphology and development. Davis (1968) gave a detailed account of gametogenesis in *E. melliodora* which shows great similarity with the observations in normal *E. leucoxylon* anthers, but there has been no report of male sterility in this species. Moncur and Boland (1989) also reported good pollen counts from flowers of all *E. melliodora* trees they examined.

Tree breeders are interested in producing male sterile clones of *Eucalyptus* for use in improved seed production. Improved seed is expensive and time consuming to produce, but in male steriles the lack of contamination from self pollen makes hybrid seed production from mixed species seed orchards a possibility. This technique is used extensively in the horticultural industry for mass production of F1 hybrid seed (Kaul,

1988). Male sterility also ensures that all seeds result from outcrossing, an advantage in species that show inbreeding depression (Griffin and Cotterill, 1988). Current research into transfer of useful gene sequences into the eucalypt genome and subsequent commercial release of genetically engineered clones may also require the production of male sterile lines, to prevent the spread of genetically engineered genomes into natural stands and other plantations through pollen dispersal.

6. POLLEN-PISTIL INTERACTION IN INTERSPECIFIC CROSSES IN *EUCALYPTUS*.

6.1 INTRODUCTION

Interspecific hybridisation is a common phenomenon in woody plant genera which have sympatric species (Burger, 1975; Clausen, 1970). Interspecific *Eucalyptus* hybrids have been reported many times from both natural populations and manipulated crosses, and are of interest to tree breeders as many have potential for inclusion in tree improvement programmes for timber and pulpwood production (Potts and Potts, 1986).

A question that has perplexed ecologists and taxonomists since last century is how so many species can exist, often in mixed species stands, without widespread species breakdown occurring. Temporal isolation in flowering time or lack of common pollen vectors generally prevent species from hybridising but there are, nevertheless, many examples of hybrid zones, clines and introgressions from mixed natural stands (Ashton and Sandiford, 1988; Phillips and Reid, 1980; Potts and Reid, 1985).

The review by Griffin *et al.*, (1988) into the occurrence of natural and manipulated hybrids demonstrated that the frequency of interspecific hybridisation depends on the degree of relationship of species pairs. While the subgenera are reproductively isolated from each other, within subgenera, the frequency of hybrids reflects the hierarchy of relationships and geographical coincidence. This analysis is limited by the incompleteness of its data and depends upon the judgements of previous authors that intermediates were of hybrid origin. Nevertheless it provides a useful background for studies into physiological isolating mechanisms in the genus.

Controlled hybridisations between closely related species have a high degree of success in both seed set and vigour (Tibbits, 1989) while more distant (intersubgeneric) crosses fail to set seed (Griffin *et al.*, 1988). This indicates there may be physiological isolating mechanisms preventing hybridisation between species. Once the nature of such mechanisms is known methods can be developed for by-passing these barriers to allow wide hybridisation as has occurred in other species including *Citrus* L. and *Prunus* (Grosser, Gmitter and Chandler, 1988; Layne and Sherman, 1986).

In chapter four, the partial self incompatibility mechanism was seen to operate in the ovary or at post fertilisation stages of seed development. Gore *et al.*, (1990) found that the unilateral interspecific incompatibility seen between *E. globulus* and *E. nitens* was due to style length disparity; pollen tubes of *E. nitens* did not reach the ovary of *E. globulus*. This study investigates the existence of pre-fertilisation isolation mechanisms in the eucalypt pistil through observation of the interspecific pollen-pistil interaction and their relationship to taxonomic distance between parents.

6.2 MATERIALS AND METHODS

6.2.1 Plant material

Controlled pollinations were performed using three species (*E. spathulata* *E. cladocalyx*, *E. leptophylla*) from the informal subgenus *Symphyomyrtus* section *Bisectaria* (Pryor and Johnson 1971) as female parents with three replicate trees per species. The breeding systems of the female trees have been described in detail in chapter four. Twenty two species representing four sections, three subgenera and two related genera were used as male parents (*E. spathulata*, *E. cladocalyx*, *E. leptophylla*, *E. platypus* var. *heterophylla*, *E. albida*, *E. yalatensis* , *E. melliodora* , *E. viridis* , *E. lansdowneana* , *E. tereticornis*, *E. flindersii*, *E. camaldulensis* , *E. grandis* , *E. botryoides*, *E. obliqua*, *E. pulchella*, *E. diversifolia* , *E. maculata* , *E. citriodora*, *E. ficifolia*, *Angophora costata*, *Melaleuca nesophila*) (Table 6.1). Species were chosen on the criteria of taxonomic position, similar style length, accessibility and commercial potential.

6.2.2 Pollinations

Pollen was collected from flowers just prior to anthesis. Anthers were removed with forceps and desiccated over silica gel for 24-48 hours to promote dehiscence. The anther-pollen mix was stored in eight millimetre diameter gelatine capsules over silica gel at -20^{o}C until needed. Before use, the pollen mix was thawed and rehydrated in a humid environment at room temperature for at least one hour.

Table 6.1: Matrix of intraspecific and interspecific pollinations used to investigate pollen-pistil interactions, showing the proportion of pistils and the number of trees (of three) in brackets, with pistils with penetrated ovules. Taxonomic groupings follow Pryor and Johnson (1971).

Taxonomic group of male parent	Female parent					
	E. spathulata		*E. cladocalyx*		*E. leptophylla*	
Symphyomyrtus						
Bisectaria						
Eucalyptus spathulata	0·90	(3)	0·33	(2)	0	
E . cladocalyx	0·47	(2)	0·60	(3)	0	
E. leptophylla	0		0		0·97	(3)
E. platypus var. heterophylla	0·73	(3)	0·14	(3)	0·03	(1)
E. albida	0		0		0·17	(3)
E. yalatensis	0·07	(1)	0		0·30	(3)
Adnataria						
E. melliodora	0		0		0.07	(3)
E. viridis	0·07	(2)	0·07	(1)	0	
E. lansdowneana	0·37	(2)	0·11	(1)	0·03	(1)
Exsertaria						
E. tereticornis	0		0		0	
E. flindersii	0		0·07	(1)	0	
E. camaldulensis	0		0		0	
Transversaria						
E. grandis	0		0		0	
E. botryoides	0		0		0	
'Monocalyptus'						
E. obliqua	0		0		0	
E. pulchella	0		0		0	
E. diversifolia	0		0		0	
'Corymbia'						
E. maculata	0·23	(1)	0		0	
E. citriodora	0		0		0	
E. ficifolia	0		0		0	
Angophora costata	0		0		0	
Melaleuca nesophila	0		0		0	
Control	0		0		0	

Buds were prepared for controlled pollination as described in section 4.2.3. At peak pistil receptivity (see section 4.3.2), bags were removed and buds pollinated directly from gelatine capsules containing a mix of pollen from three other trees of the same species. All buds within a bag received the same treatment. Branches were then rebagged until harvesting ten days later. Controls were treated similarly but without pollination.

6.2.3 Sample preparation and analysis

Thirty styles per treatment (numbers were lower in some cases due to bud abscission) were viewed and photographed under fluorescence microscopy. Pollen tubes were counted at four levels in the pistils of *E. spathulata* and *E. cladocalyx*; stigma surface, upper style, mid style and base of the style. Pollen germination was quantified for one female tree per treatment. Styles of *E. leptophylla* were not amenable to squashing due to a heavy layer of cuticle and rigid epidermis. In this species, pollen tube quantification was limited to presence or absence at the four levels. Observations were also made on pollen tube morphology. If pollen tubes were viewed at the base of the style the ovary was also processed. The hypanthium was softened whole in 0·8 N NaOH at 60°C for one hour and all ovules of one locule per ovary dissected, stained with decolourised aniline blue, squashed in glycerol and viewed under fluorescence microscopy for evidence of pollen tube penetration of the micropyle.

6.2.4 Pollen storage and viability

Not all species flowered at the same time and pollen was stored for up to six months. To test the effect of storage at -20°C on pollen viability and pollen tube growth *in vivo*, intraspecific cross pollinations of *E. spathulata* using fresh pollen applied by anther, pollen stored for six weeks and pollen stored for one year were compared for pollen tube morphology and success of ovule penetrations.

6.2.5 Floral measurements

Measurements of floral dimensions were made of all species used, to test the effect of style length on pollination success. Style length was measured from the tip of the

stigma to the junction of the style and hypanthium, pistil length was from stigma tip to ovary base. Mature flowers in the receptive phase were used for all measurements. Temperature records during the flowering season were obtained from the Department of Meteorology records for Adelaide or Hobart for each species. Mean monthly maximum temperatures were used for analysis to test the effect of flowering temperature on pollination success.

6.2.6 Statistical analysis

The number of pollen tubes at each of the three levels of the style was analysed for each replicate and treatment. The data were also summarised as the number of pistils per treatment in which pollen tubes had successfully penetrated to the upper style, mid style or base of the style. Results were analysed using a binomial model by Chi square analysis. The probability of pollen tubes reaching the upper style, mid style or the base of the style was calculated for each cross.

Regression analyses were performed on the data to determine whether style length and temperature at the time of flowering of the male parent influenced the pollen-pistil interaction. Ovule penetration data from the pollen storage experiment were also analysed using a binomial model and Chi square.

6.3 RESULTS

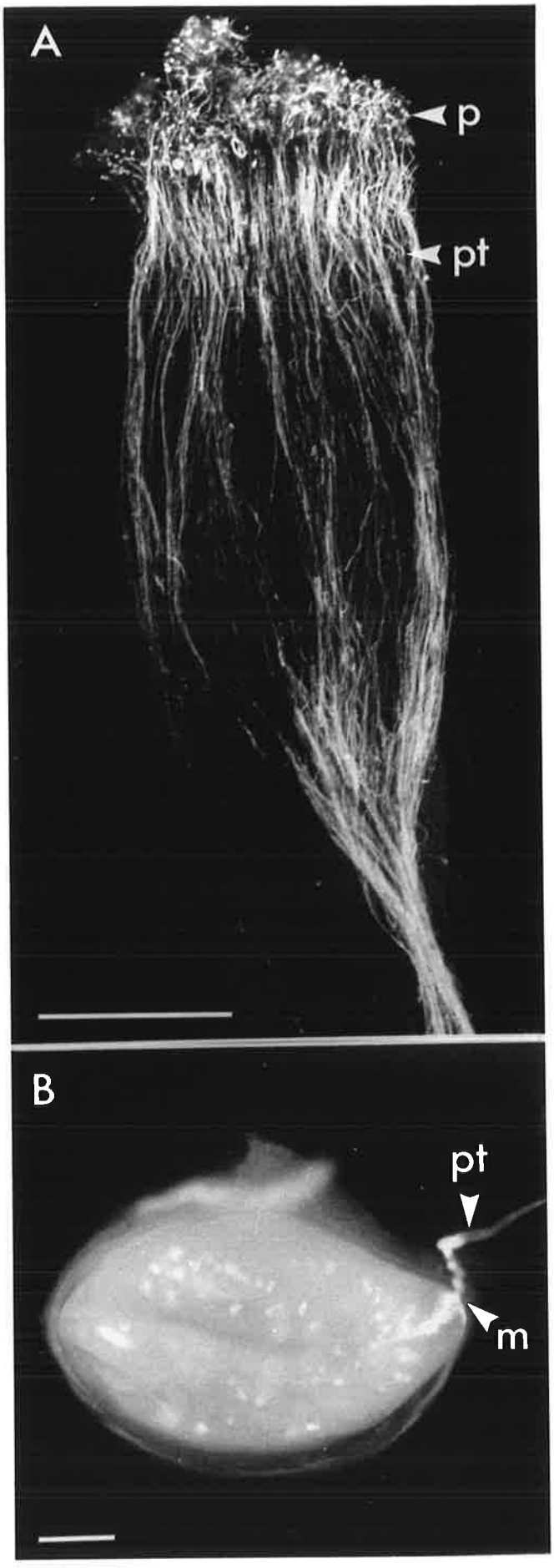
6.3.1 Intraspecific pollination

All intraspecific pollinations showed healthy pollen tube growth, (Fig. 6.1A-B) with only a small percentage of pollen tubes in these crosses showing abnormalities. Control unpollinated pistils showed no contamination by stray pollen and thus no pollen tubes. Pollen tubes penetrated the upper style between the papilla cells of the stigma. In the style, pollen tube growth was intercellular and did not follow the short stylar canal. A maximum of 21 percent of the fertile ovules were penetrated by pollen tubes, a single pollen tube penetrating the micropyle of each ovule (Fig. 6.1B)

Figure 6.1. Fluorescence micrographs of intraspecific pollen tube growth in *Eucalyptus* pistils.

6.1A. Squash preparation of *E. spathulata* style 10 days after intraspecific cross pollination, stained with aniline blue, showing abundant pollen germination (p) and healthy pollen tube growth. Pollen tubes (pt) show directional growth, roping towards the base of the style. Bar represents 1mm.

6.1B. Dissected ovule of *E. cladocalyx* 10 days after intraspecific cross pollination, stained with aniline blue, showing a pollen tube (pt) penetrating the micropyle (m). Bar represents 100μm.


A
p
pt
B
pt
m

6.3.2 Interspecific pollination

Pollen of all male species was observed to adhere and germinate on the stigma and penetrate the stigma of all three female species. Although variation between male species was high, there was no relationship between taxonomic group and percent pollen germination or penetration of the stigma (Table 6.2). Both intraspecific pollen, and pollen of very distantly related species, showed similar germination percentages on *E. spathulata* and *E. cladocalyx* stigmas.

Both the numerical pollen tube data and the presence/absence data showed similar trends (Figs 6.2-3). In all cases except in the upper style of *E. spathulata*, there was a highly significant difference ($p<0{\cdot}001$) between male species. Replicate trees of the three female species showed slight differential fertility but when the three female species were analysed together there was found to be a significant male-female interaction ($p>0{\cdot}001$). There was also a significant difference between the success of the taxonomic groups as male parents, and a female-taxonomic group interaction at all levels in the style ($P>0{\cdot}001$). In *E. spathulata* and *E. cladocalyx* the probability of reaching the base from the midstyle was greater than the probability of reaching the midstyle from the upper style (Fig. 6.2)

Although there was a significant female-taxonomic group interaction, the three female species showed similar trends with respect to combining ability with the eight taxonomic groups (Figs 6.2-3). Closer crosses have the highest probability of penetrating the lower style and thus the ovary.

Most of the crosses in which ovule penetrations were observed were intrasectional or between sections *Bisectaria* and *Adnataria.* Only two crosses fell outside these groups (Table 6.1). *Eucalyptus spathulata* x *E. maculata* was unusual in being an intersubgeneric cross, but ovules were penetrated in pistils from one female tree only and could be the result of a unique genotype interaction. Table 6.3 presents rates of ovule penetration in pistils in which pollen tubes reached the base of the style. The highest rates of ovule penetration were in intraspecific crosses (with the exception of *E. cladocalyx* x *E. spathulata*) with distant crosses showing low ovule penetration rates and high variability between trees.

Table 6.2: Mean percentage germination and stigma penetration of intra and interspecific pollen on eucalypt stigmas. (n = number of pistils examined).

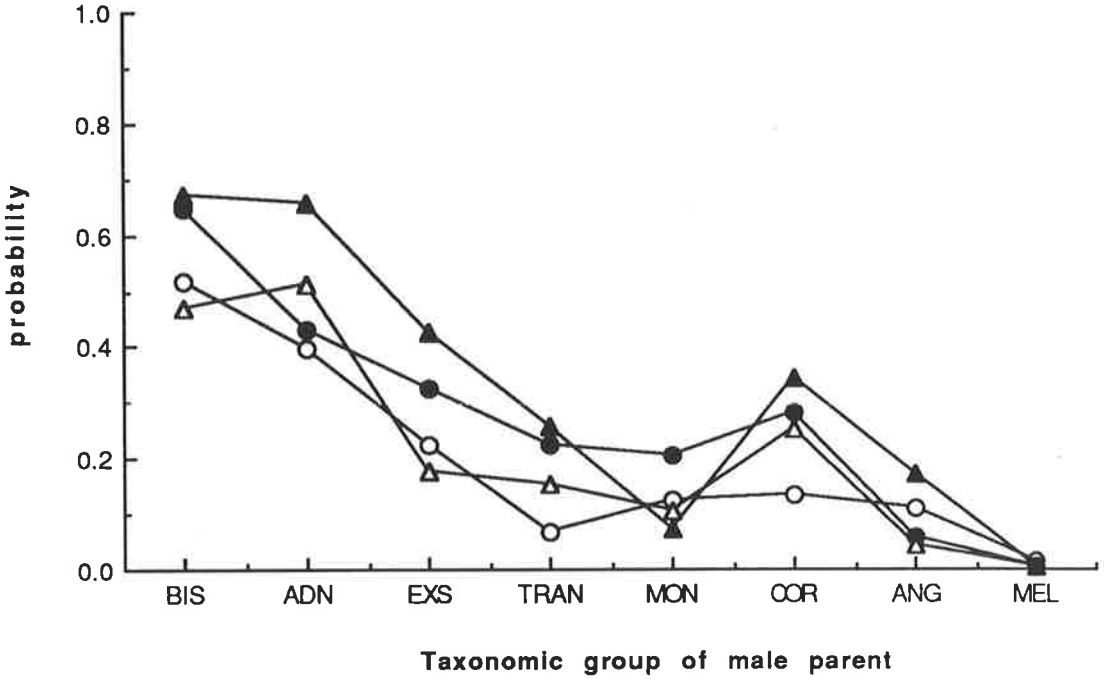
	Female Parent					
	Eucalyptus spathulata			*Eucalyptus cladocalyx*		
Taxonomic group of male parent	Pollen Germination	Stigma Penetration	n	Pollen Germination	Stigma Penetration	n
Symphyomyrtus						
Bisectaria						
Eucalyptus spathulata	65·1	74·3	10	43·3	74·7	10
E. cladocalyx	75·7	59·0	10	94·5	72·0	10
E. leptophylla	73·2	84·9	9	98·6	87·5	10
E. platypus	85·1	100	10	89·3	84·6	6
E. albida	53·2	66·6	10	54·8	68·7	10
E. yalatensis	77·8	73·9	10	82·3	76·2	10
Adnataria						
E. melliodora	94·6	78·6	10	21·1	71·0	10
E. viridis	54·4	76·5	10	62·3	90·2	10
*E. lansdowneana**	-	-	-	-	-	-
Exsertaria						
E. tereticornis	88·6	43·8	10	62·0	78·3	10
E. flindersii	75·0	56·0	10	30·9	72·9	5
E. camaldulensis	71·7	59·0	10	15·6	74·6	5
Transversaria						
E. grandis	88·3	49·2	10	32·7	26·7	10
E. botryoides	50·6	71·1	10	54·6	67·8	10
'Monocalyptus'						
E. obliqua	82·5	48·8	10	46·6	72·4	10
E. pulchella	52·3	73·5	10	18·9	45·0	10
E. diversifolia	78·0	51·2	10	98·5	86·0	10
'Corymbia'						
E. maculata	68·5	66·0	10	35·5	74·0	10
E. citriodora	67·7	68·0	10	80·6	92·5	10
E. ficifolia	83·7	58·1	10	66·9	88·5	10
Angophora costata	79·3	30·7	10	83·0	83·7	10
Melaleuca nesophila	64·2	43·4	10	56·2	32·2	10

* *E. lansdowneana* omitted due to inadequete number of replicates

Figure 6.2. The effect of taxonomic distance on the success of pollen tube growth in intra and interspecific crosses of *Eucalyptus* using *E. spathulata* and *E. cladocalyx* (*Symphyomyrtus*, section *Bisectaria*) as female parents. Probability of pollen tubes continuing growth from the upper style to the mid style of *E. spathulata* (△), mid style to the base of the style of *E. spathulata* (▲), upper style to mid style of *E. cladocalyx* (○), mid to base of the style of *E. cladocalyx* (●). Based on the number of pollen tubes at each level. Taxonomic groups of male parents, *Symphyomyrtus* sections: *Bisectaria* (BIS), *Adnataria* (ADN), *Exsertaria* (EXS), *Transversaria* (TRAN); informal subgenera, '*Monocalyptus* ' (MON), '*Corymbia* ' (COR); genera, *Angophora* (ANG), *Melaleuca* (MEL).

Figure 6.3. The effect of taxonomic distance on the probability of pollen tubes reaching the base of the style in intra and interspecific crosses in *Eucalyptus* . Female parents, *E. spathulata* (□), *E. cladocalyx* (○), *E. leptophylla* (△). Based on presence/absence data. Taxonomic groups of male parents as in Figure 6.2.

6.2


1.0
0.8
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0.4
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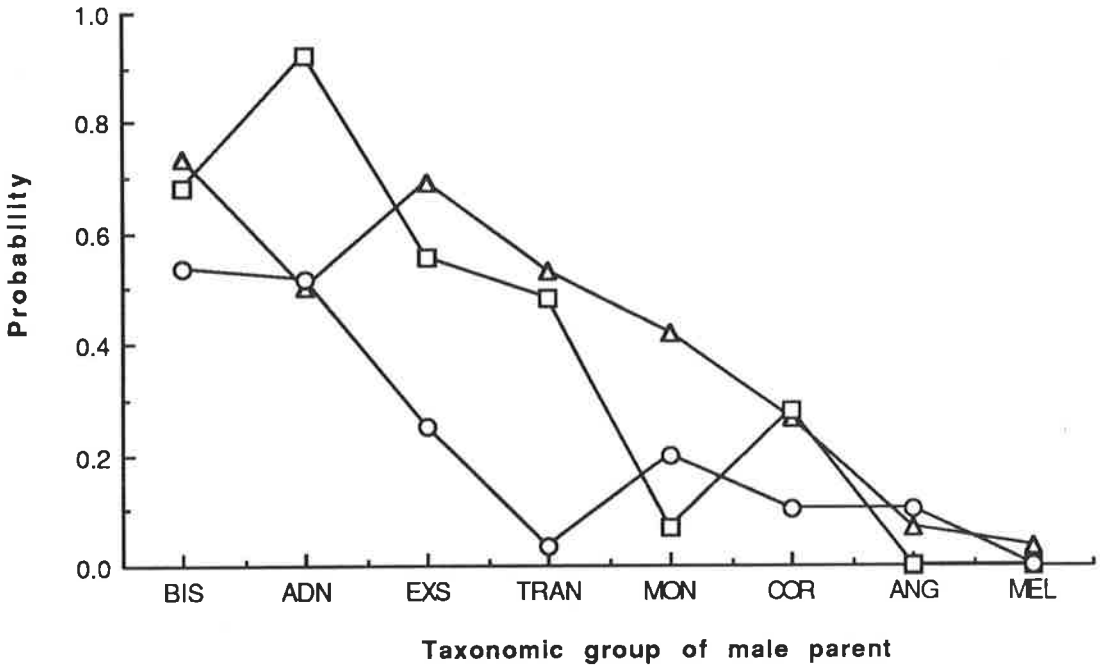
probability
BIS
ADN
EXS
TRAN
MON
COR
ANG
MEL
Taxonomic group of male parent

6.3


1.0
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Probability
BIS
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ANG
MEL
Taxonomic group of male parent

Table 6.3: Mean percentage of penetrated ovules in intra and interspecific crosses in *Eucalyptus*. (n = number of pistils examined).

Taxonomic group of male parent	Female parent *E. spathulata* Pen. ovules	n	*E. cladocalyx* Pen. ovules	n	*E. leptophylla* Pen. ovules	n
Symphyomyrtus						
Bisectaria						
E. spathulata	20·59	30	15·33	30		
E. cladocalyx	7·64	30	11·55	30		
E. leptophylla					17·98	30
E. platypus	19·24	30	0·94	28	0·26	30
E. albida					1·24	30
E. yalatensis	0·57	30			4·32	30
Adnataria						
E. lansdowneana	9·00	30	0·31	18	0·30	30
E. viridis	0·51	30	2·71	30		
E. melliodora					0·61	30
Exsertaria						
E. flindersii			0·33	15		
'Corymbia'						
E. maculata	1·41	30				

6.3.3 Pollen tube abnormalities

Some interspecific crosses produced apparently normal pollen tubes, but most showed some pollen tube growth abnormalities (Fig 6.4A-F). Six categories of abnormality (Table 6.4) were observed, four of which occurred in the style and four in the ovary. Most abnormalities occurred in the upper stylar region and the occurrence of particular abnormalities and their frequencies were consistent between replicate pistils within crosses. More non-directional pollen tubes and tube distortions were observed in wider crosses, and intergeneric crosses with *Angophora* and *Melaleuca* showed gross abnormalities. In general there was no association between type of abnormality and taxonomic group but some crosses showed a greater proportion of one abnormality type. *Eucalyptus spathulata* x *M. nesophila* (Fig. 6.4A) showed directionless pollen tube growth, and *E. albida* pollen tubes produced characteristic swollen, bulbous tips in the upper style of *E. spathulata* (Fig. 6.4B). There was no correlation between the number of pollen tubes in a style and the health or success of the tubes.

6.3.4 Pollen storage and viability

In vivo trials of fresh and stored pollen (Table 6.5) showed that storage at -20°C had no detrimental effect on pollen tube growth. Counts of ovule penetrations in pistils pollinated with fresh, six week old or one year old pollen showed no significant reduction in pollen viability with length of storage, and one year old pollen actually showed the highest mean penetrated ovule count. Observation of pollen tube morphology in the three treatments also showed no detrimental effect of storage. Healthy pollen tubes were observed in all treatments.

6.3.5 Floral measurements

There was found to be no significant relationship between the success of a cross and style or pistil length of the male parent (the largest r^2 was 0·11 for *E. spathulata*), or the mean maximum temperature of flowering season of the male parent ($r^2 = 0{\cdot}04$).

Figure 6.4. Fluorescence micrographs of squash preparations of *Eucalyptus* styles and ovules stained with aniline blue.

6.4A. Style of *E. spathulata* 10 days after pollination with *Melaleuca nesophila* pollen, showing non-directional pollen tubes (pt). Bar represents 200µm.

6.4B. Upper style of *E. spathulata* 10 days after pollination with *E. albida* pollen, showing pollen tubes with bulbous swellings (b). Bar represents 100µm.

6.4C. Upper style of *E. cladocalyx* 10 days after pollination with *E. obliqua* pollen, showing thickened pollen tube growth (t). Bar represents 100µm.

6.4D. Upper style of *E. cladocalyx* 10 days after pollination with *E. pulchella* pollen, showing forked pollen tube growth (f). Bar represents 100µm.

6.4E. Dissected ovule of *E. spathulata* 10 days after pollination with *E. platypus* pollen showing penetration of the micropyle by three pollen tubes. Bar represents 100µm.

6.4F. Dissected ovule of *E. spathulata* 10 days after pollination with *E. maculata* pollen, showing thickening of pollen tube (t) after entry of the micropyle. Bar represents 100µm.

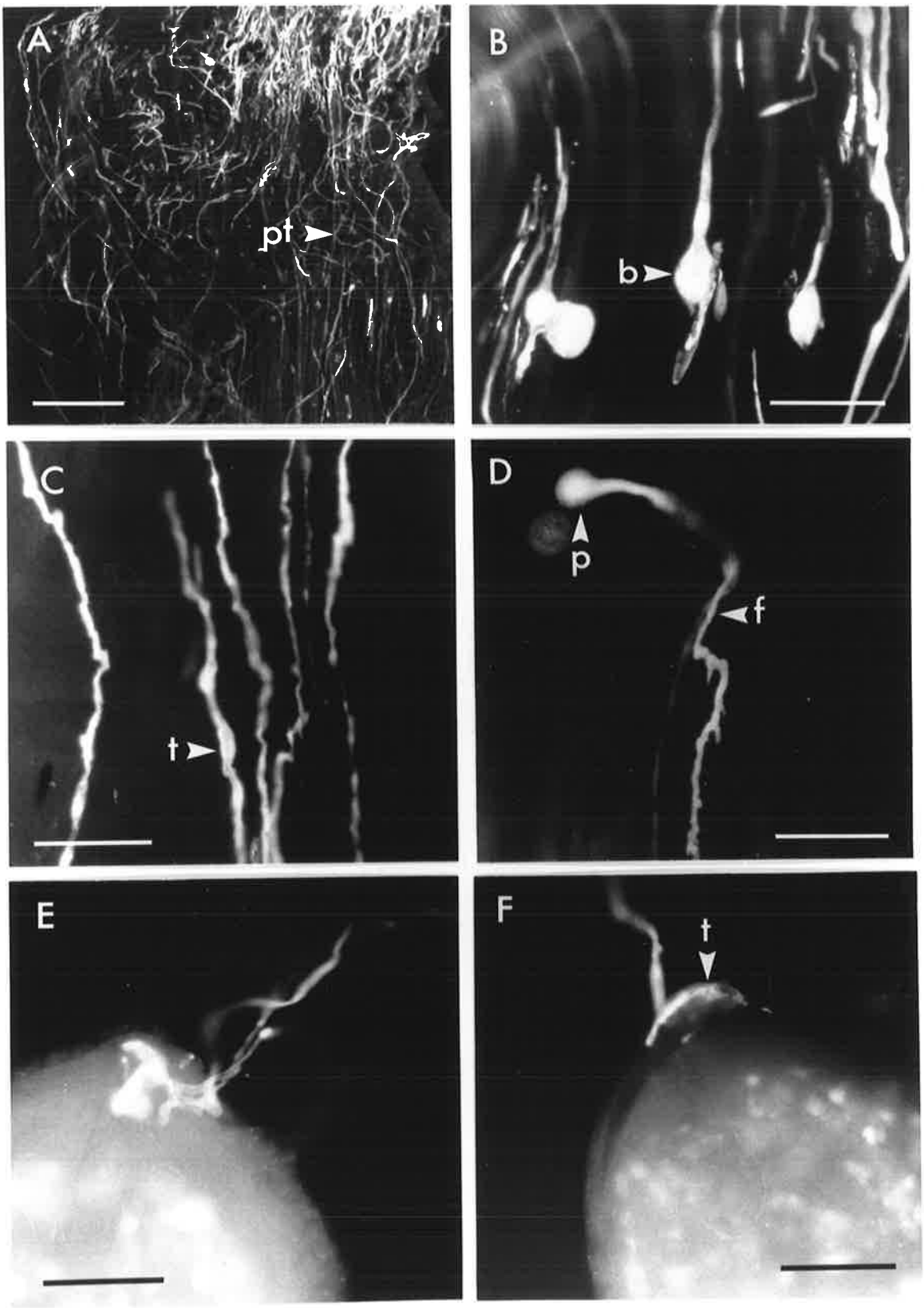
A
pt
B
b
C
t
D
p
f
E
F
t

Table 6.4: Pollen tube abnormalitites observed in interspecific crosses of *Eucalyptus*, Locations; S, stigma; US, upper style; MS, mid style; BS, base of style; O, ovary.

Pollen tube abnormality	Location				
	S	US	MS	BS	O
Non-directional growth (Fig. 6.4A)	+	+	+	-	+
Bulbous swellings (Fig. 6.4B)	-	+	+	-	-
Thickened walls (Fig. 6.4C)	+	+	+	+	+
Forked growth (Fig. 6.4D)	+	+	+	+	+
Multiple penetration of ovule (Fig. 6.4E)	-	-	-	-	+
Thickened after entry into the micropyle (Fig. 6.4F)	-	-	-	-	+

Table 6.5: The effect of storage on in vivo viability of *E. spathulata* pollen in intraspecific pollinations (mean percent penetrated ovules , n = number of ovules examined).

Pollen treatment	penetrated ovules	n
Fresh	19·1	422
6 week old	19·9	409
1 year old	25·7	397

6.4 DISCUSSION

This study is the first to demonstrate the existence of interspecific reproductive isolation mechanisms in the eucalypt pistil. This effect is shown to be related to taxonomic distance between male and female parents, and manifests as a number of pollen tube abnormalities and a lowering of the probability of ovule penetration.

The upper style was the major point of arrest of interspecific pollen tubes. Most abnormalities occurred in the upper style, and there was a higher probability of pollen tube arrest in the upper than lower style. In many other genera including *Petunia* Juss. and *Prunus* the style is the major site of action of the gametophytic self incompatibility response (de Nettancourt, 1977), and glycoproteins involved in the S gene action have been detected in the stylar matrix (Knox and
Williams, 1986). The S gene has also been implicated in rejection of interspecific pollen tubes (Pandey, 1977). McClure *et al.*, (1989) suggested a mechanism for action of the S gene in unilateral interspecific incompatibility, but involvement of the S gene complex in rejection of foreign pollen tubes has yet to be proven. No evidence for S gene mediated self incompatibility has been found in *Eucalyptus* although self pollination results in reduced seed set compared with cross pollination (Griffin *et al.*, 1987; Potts and Cauvin, 1988). The ovary was the other major site of selection. Pollen tubes in many crosses were seen to reach the base of the style and in some cases the placenta and ovule surface, but failed to penetrate the ovules.

The eucalypt stigma was not a barrier to interspecific pollen tube growth. Potts and Marsden-Smedley (1989) found significant differences in *in vitro* germination requirements of four eucalypt species in respect to sucrose and boric acid concentrations. This may reflect different conditions on the stigma surface of each species and could explain the occurrence of the pollen tube abnormalities seen on the stigma surface in this study. This absence of strong interspecific or intergeneric barriers to pollen tube growth at the level of the stigma is consistent with findings in other woody plant genera with wet stigmas (Sedgley and Griffin, 1989). Similar pollen tube abnormalities to those reported in this study have been reported in *Rhododendron* , a woody plant genus that shows some

similarities with *Eucalyptus* including a wet stigma, a mixed mating system, protandry, a large number of species and frequent interspecific hybridisation, but differs in having a mucilage filled canal for much of the length of the style (Williams *et al* 1982).

The nature of reproductive isolation demonstrated here in *Eucalyptus* is consistent with the concept of interspecific incongruity as suggested by Hogenboom (1975, 1984). Wide hybridisations were seen to display more pollination-pathway malfunctions and thus, earlier pollen tube arrest than close hybridisations, which showed a higher probability of a congruous pollen-pistil interaction. If the theory of incongruity through evolutionary divergence is applied, then observations of interspecific pollen-pistil interaction can be used to reflect on evolutionary relationships between species or taxonomic groups. Pryor and Johnson's (1971) discussion of their treatment of the genus suggests an affinity between sections *Bisectaria* and *Transversaria* not supported by our data. The ovule penetration data corresponds well with the stylar data, with males mainly from sections *Bisectaria* and *Adnataria* succeeding in ovule penetration of the section *Bisectaria* females. This system of affinities is supported by the cladistic analysis of *Symphyomyrtus* sections by Chappill (1988), which indicates a close affinity between sections *Bisectaria* and *Adnataria* but shows section *Transversaria* diverging early in the evolutionary sequence. Brooker (1979) raised doubts as to whether *E. cladocalyx* was a true member of section *Bisectaria*. However, the pollen-pistil data shows that responses of *E. cladocalyx* to interspecific pollination are similar to those of the other two section *Bisectaria* females, thus supporting an affinity with this section.

Most of the combinations in this study are not geographically possible in natural populations (Chippendale, 1988) and so can occur only through co-cultivation or via manipulated pollinations. Griffin *et al.*, (1988) reported a low rate of hybridisation both within section *Bisectaria* and in combination with other *Symphyomyrtus* sections, yet our manipulated pollinations within section *Bisectaria*, and between sections *Bisectaria* and *Adnataria*, show a high rate of ovule penetration. The only previously reported hybrids from this combination is *E. cladocalyx* x *camaldulensis* (Griffin *et al.*, 1988), but this report has not been confirmed in this study and the original trees of putative hybrid origin are no longer in existence (L. Pryor personal communication). In this study this

combination failed during pollen tube growth in the style, although it is possible that other genotype combinations may prove successful. The combination *E. platypus* x *E. spathulata* ssp *grandiflora* has also been reported (Griffin *et al.,* 1988), and a similar cross between *E. spathulata* ssp *spathulata* and *E. platypus* ssp *heterophylla* in this study showed high levels of ovule penetration.

This study monitored the pollen-pistil interaction only to the stage of ovule penetration, pollen tube growth in the embryo sac and fusion of gametes was not studied but are further stages at which breakdown is possible (Williams *et al.,* 1986).

7. SEED SET AND EARLY SEEDLING CHARACTERISTICS IN HYBRID CROSSES IN *EUCALYPTUS*

7.1 INTRODUCTION

In chapter six many species combinations were found to have compatible pollen-pistil interactions, although some showed reduced fitness in terms of pollen tube success and ovule penetration. While this information is useful in describing the interaction between the female sporophyte and the male gametophyte and has implications for the probability of successful hybridisation, the interaction between gametophyte genotypes, and subsequent embryo development and germination are also limiting steps in the process of interspecific hybridisation. Some self-incompatibility and interspecific isolation systems have been shown to operate at the post-fertilisation stage (Franken *et al.*, 1988; Seavey and Bawa, 1986) and genotype selection through maternal resource allocation may also influence which fertilised ovules develop into mature seed (Stephenson and Bertin, 1983).

Hybrid seed set resulting from controlled pollinations have been measured in other species combinations in the genus, such as combinations involving *E. nitens* (Gore *et al.*, 1990; Tibbits 1989) and *E. gunnii* (Potts *et al.*, 1987), but apart from the detection of unilateral cross compatibility in *E. nitens* x *E. globulus* (Gore *et al.*, 1990), no other studies have combined the analysis of pre-fertilisation relationships with post-zygotic development. After analysis of the pollen-pistil interaction, the opportunity exists to determine the extent to which interspecific isolation is controlled by interactions during seed and capsule development and early seedling growth, and the correlation with taxonomic distance. This section investigates the interspecific relationship at the seed set, seedling germination and early seedling stages, in combinations that showed successful ovule penetration in chapter 6.

7.2 MATERIALS AND METHODS

7.2.1 Plant material

Following analysis of pollen-pistil interactions in 1988-89 a further series of interspecific pollinations was performed in 1989-90 of combinations that showed ovule penetrations in at least one female tree. Female and male parents used were the same as those described in section 6.2.1, but fresh collections of pollen were made and stored using the methods described in 6.2.4. Pollinations of *E. cladocalyx* with *E. lansdowneana* pollen were not repeated due to limited availability of *E. lansdowneana* pollen. Two trees of *E. spathulata* were also pollinated with *E. sargentii* and *E. occidentalis* (section *Bisectaria*) pollen. These species were included in the schedule due to their salt tolerance and the potential for the transmission of this character to hybrid progeny.

7.2.2 Intra and interspecific ovule penetration and seed set

A single pollination was applied to pistils at peak stigma receptivity (mean sample size was 70 pistils per treatment per tree), and bags were left in place until after style abscission. Control treatments that received no pollen were also set up for each maternal tree. Capsules were harvested one year later at maturity and allowed to dehisce their contents of chaff and seed in the laboratory. Capsule contents were classified into the three categories of, full seed, aborted seed and chaff, with the numbers in the first two categories being recorded for each capsule. Aborted seed was shrunken, contained no white embryo tissue, but was significantly more developed than the chaff particles originating from the ovulodes, unfertilised ovules and ovules aborted at early post zygotic stages. Fertile seeds were separated and weighed in lots of fifteen. Capsule and seed set statistics were compared with ovule penetration rates in the same genotype combinations in pollinations from the previous season (chapter six). Differences in ovule penetration rates, capsule and seed set, and seed weights were tested between genotypes using analysis of variance.

7.2.3 Germination trials

To test seed viability samples of 15 seeds per maternal tree per cross, and open pollinated seed from trees used as male parents was collected (sample sizes were smaller where seed numbers were limiting). Seed was surface sterilised in two percent sodium hypochlorite solution containing a drop of wetting agent (Tween 20) for ten minutes, then rinsed three times in sterile water. Seeds were transferred to sterile low salt nutrient agar (0.7 % agar with half strength Murashige and Skoog (1962) nutrients), seeds were kept at constant temperature (25°C) in the laboratory and scored daily for germination and cotyledon expansion. Seeds were scored as germinated when the radicle was about 0.5mm long. After 40 days any ungerminated seed was tested for viability by squashing and seeds were scored as viable if they contained a firm white embryo. Differences in germination times between seed genotypes were tested for using analysis of variance. After germination seedlings were transferred to sterile potting soil (University of California mix), fertilised fortnightly with soluble fertiliser.and placed in a glasshouse (maximum temperature 25°C, minimum temperature 15°C) until the two leaf stage when plants were transferred outdoors to full sunlight and grown for 6 months (February-August).

7.2.4 Hybrid verification

Cotyledon morphology of newly germinated parental and hybrid seedlings were recorded. Morphological measurements were taken from five seedlings per cross at five months. Measurements were taken of one leaf from each of the fifth and tenth leaf pairs counting the cotyledons as zero. When leaves were alternate, the uppermost leaf was used for measurement. Variables measured were, leaf length, leaf width at the widest point, petiole length, angle at leaf tip (measured 10mm from tip), the distance from the leaf base to the widest point, the distance from the fifth to the tenth node, the node at which leaves became alternate, the number of branches/number of nodes, leaf glaucousness (scale of 0-5, 0=most glaucous), red pigmentation of young stems (scale of 0-5, 5=most pigmented), the log ratio of leaf length to width was also calculated and used in the analysis. To verify parentage of seed from controlled pollinations, data from the two parental species and the putative hybrid progeny were compared through Principle Component Analysis (Johnson

and Wichem, 1982). This allowed reduction of a complex data set to a small number of principle components that account for much of the variability in the original data, allowing separation of morphologically distinct genotypes on a two dimensional axis.

7.3 RESULTS

7.3.1 Intra and interspecific ovule penetration and seed set.

In tables 7.1-7.3 data on ovule penetration rates from the experiments in chapter six is presented, percent ovule penetration and mean number of ovules per locule are calculated from pistils with at least one ovule penetrated. Total ovule penetration is derived from mean number of ovules penetrated per locule and mean number of locules per flower (chapter four). There were significant differences between the numbers of pistils with penetrated ovules between treatments in all three maternal species ($P<0.001$). Although numbers of penetrated ovules were generally lower in interspecific crosses, there was no significant difference between treatments. This indicates most of the reduction in ovule penetration in interspecific crosses occurs due to the poor numbers of pistils in which pollen tubes reach the ovules.

Data on capsule set, seeds per capsule and contents of capsules are listed in tables 7.4-7.6 for the female parents *E. spathulata, E. cladocalyx* and *E. leptophylla.* Observations of capsule development suggested that most immature fruit abscission occurs in the first month after pistil receptivity, and that any capsules left after this period have a high probability of developing to maturity. At maturity, capsules contained three types of contents, full seed, aborted seed and chaff . Seed was classified as aborted if it was shrunken and when cut in half contained no white embryo tissue. Barren capsules contained only aborted seed and/or chaff particles. No capsules were set in the unpollinated control treatment, confirming the efficiency of the emasculation and bagging technique.

Pollinations using *E. spathulata* as a female parent showed significant differences between crosses in the percentage of capsules set ($P<0.05$) and the numbers of seed per capsule ($P<0.05$). Intraspecific cross and self pollinations are more fully discussed in chapter four, but provide a background for interpretation of the results of interspecific

Table 7.1 Intra and interspecific ovule penetrations in *E. spathulata*. (mean ± standard error).

Taxonomic group	Male parent	Female tree	% pistils with penetrated ovules	% ovule penetration	Mean number of penetrated ovules per locule	Total penetrated ovules
Bisectaria						
	E. spathulata	1	90	0.23 ± 0.04	2.78 ± 0.46	9.7
	Cross	2	90	0.27 ± 0.05	2.67 ± 0.41	9.3
		3	90	0.22 ± 0.03	2.63 ± 0.42	9.2
		mean	90	0.24 ± 0.02	2.69 ± 0.24	9.4
	E. cladocalyx	1	0	- -	- -	-
		2	90	0.17 ± 0.03	1.89 ± 0.26	6.6
		3	50	0.15 ± 0.02	2.20 ± 0.49	7.7
		mean	47	0.16 ± 0.02	2.00 ± 0.23	7.0
	E. platypus	1	80	0.19 ± 0.06	2.50 ± 0.80	8.8
		2	90	0.41 ± 0.06	4.78 ± 0.70	16.7
		3	50	0.09 ± 0.01	1.20 ± 0.20	4.2
		mean	73	0.26 ± 0.04	3.14 ± 0.51	11.0
	E yalatensis	1	0	- -	- -	-
		2	20	0.09 ± 0.01	1.00 ± 0.00	3.5
		3	0	- -	- -	-
		mean	7	- -	- -	-
Adnataria						
	E. viridis	1	10	0.08 ± 0.00	1.00 ± 0.00	3.5
		2	0	- -	- -	-
		3	10	0.07 ± 0.00	1.00 ± 0.00	3.5
		mean	7	0.07 ± 0.00	1.00 ± 0.00	3.5
	E. lansdowneana	1	100	0.26 ± 0.04	3.40 ± 0.58	11.9
		2	0	- -	- -	-
		3	10	0.10 ± 0.00	1.00 ± 0.00	3.5
		mean	37	0.25 ± 0.01	3.18 ± 0.57	11.1
'Corymbia'						
	E. maculata	1	0	- -	- -	-
		2	70	0.22 ± 0.04	2.71 ± 0.57	9.5
		3	0	- -	- -	-
		mean	23	- -	- -	-

Table 7.2: Intra and interspecific ovule penetrations in *E. cladocalyx*. (mean ± standard error).

Taxonomic group	Male parent	Female tree	% pistils with penetrated ovules	% ovule penetration	Mean number of penetrated ovules per locule	Total penetrated ovules
Bisectaria						
	E. cladocalyx	1	70	0.20 ± 0.04	3.86 ± 1.01	10.8
	Cross	2	50	0.23 ± 0.07	2.20 ± 0.58	7.0
		3	60	0.20 ± 0.03	3.00 ± 0.58	9.6
		mean	60	0.20 ± 0.03	3.11 ± 0.47	10.0
	E. spathulata	1	0	- -	- -	-
		2	80	0.51 ± 0.07	7.25 ± 0.90	23.2
		3	20	0.07 ± 0.00	1.00 ± 0.00	3.2
		mean	33	0.42 ± 0.08	6.00 ± 1.10	19.2
	E. platypus	1	10	0.05 ± 0.00	1.00 ± 0.00	3.2
		2	50	0.07 ± 0.00	1.00 ± 0.00	3.2
		3	10	0.09 ± 0.00	1.00 ± 0.00	3.2
		mean	23	0.07 ± 0.01	1.00 ± 0.00	3.2
Adnataria						
	E. viridis	1	0	- -	- -	-
		2	50	0.17 ± 0.03	2.40 ± 0.40	7.7
		3	0	- -	- -	-
		mean	17	- -	- -	-
Exsertaria						
	E. flindersii	1	0	- -	- -	-
		2	20	0.08 ± 0.00	1.0 ± 0.00	3.2
		3	0	- -	- -	-
		mean	7	- -	- -	-

Table 7.3: Intra and interspecific ovule penetrations in *E. leptophylla* (mean ± standard error).

Taxonomic group	Male parent	Female tree	% pistils with penetrated ovules	% ovule penetration	Mean number of penetrated ovules per locule	Total penetrated ovules
Bisectaria						
	E. leptophylla	1	100	0.16 ± 0.03	5.67 ± 1.09	19.3
	Cross	2	90	0.23 ± 0.03	6.60 ± 0.82	22.4
		3	90	0.22 ± 0.04	5.90 ± 1.12	20.1
		mean	93	0.20 ± 0.02	6.07 ± 0.57	20.6
	E. platypus	1	0	- -	- -	-
		2	10	0.08 ± 0.00	1.00 ± 0.00	3.4
		3	0	- -	- -	-
		mean	3	- -	- -	-
	E. albida	1	10	0.07 ± 0.00	1.00 ± 0.00	3.4
		2	20	0.07 ± 0.00	1.00 ± 0.00	3.4
		3	20	0.15 ± 0.05	1.50 ± 0.50	5.1
		mean	17	0.10 ± 0.02	1.20 ± 0.20	4.1
	E. yalatensis	1	30	0.18 ± 0.06	2.67 ± 0.88	9.1
		2	10	0.09 ± 0.00	1.00 ± 0.00	3.4
		3	50	0.13 ± 0.04	1.40 ± 0.40	4.8
		mean	30	0.14 ± 0.03	1.78 ± 0.40	6.1
Adnataria						
	E. melliodora	1	0	- -	- -	-
		2	10	0.09 ± 0.00	1.00 ± 0.00	3.4
		3	10	0.09 ± 0.00	1.00 ± 0.00	3.4
		mean	7	0.09 ± 0.00	1.00 ± 0.00	3.4
	E. lansdowneana	1	0	-	-	-
		2	0	-	-	-
		3	10	0.09 ± 0.00	1.00 ± 0.00	3.4
		mean	3	- -	- -	-

Table 7.4. Intra and interspecific seed set in *E. spathulata*. (means ± standard error, n = number of flowers pollinated).

Taxonomic group	Male parent	Female tree	Percent capsule set	Seeds per capsule	Percent barren capsules	Aborted seeds per capsule	Seeds per pollination	n
Bisectaria								
	E. spathulata							
	Cross	1	91.3	10.9 ±0.47	0	0.55 ±0.10	9.95	81
		2	70.6	5.3 ±0.35	0	1.00 ±0.14	3.72	102
		3	64.6	11.4 ±0.66	0	1.00 ±0.19	6.34	116
		mean	75.5 ±8.1	9.2 ±2.00	0	0.85 ±0.20	6.67± 1.81	
	E. spathulata							
	Self	1	8.8	2.3 ±0.47	0	0.33 ±0.21	0.18	77
		2	12.0	1.2 ±0.13	0	0.40 ±0.16	0.15	83
		3	0.0	- -	-	- -	0	100
		mean	6.9±3.59	1.8 ±0.60	0	0.37 ±0.04	0.37 ±0.06	
	E. cladocalyx							
		1	25.8	5.5 ±0.77	0	0.35 ±0.12	1.41	66
		2	55.8	2.6 ±0.40	0	0.63 ±0.18	1.44	77
		3	15.9	4.8 ±0.90	0	0.60 ±0.34	0.76	63
		mean	32.5 ±12.0	4.3 ±0.90	0	0.53 ±0.10	1.20 ±0.22	
	E. platypus							
		1	81.8	7.1 ±0.69	0	0.69 ±0.19	5.80	66
		2	82.9	5.2 ±0.40	3	0.79 ±0.14	3.95	76
		3	20.3	10.8 ±1.45	0	2.33 ±0.97	2.20	59
		mean	61.7 ±20.7	7.7 ±1.64	1 ±1.0	1.27 ±0.53	3.98 ±1.04	
	E. yalatensis							
		1	38.0	9.0 ±0.99	0	0.68 ±0.20	3.42	50
		2	47.0	6.7 ±0.57	0	0.82 ±0.15	3.14	83
		3	8.6	5.0 ±0.73	0	0	0.43	70
		mean	21.2 ±11.59	6.9 ±1.2	0	0.50 ±0.20	2.33 ±0.95	
	E. occidentalis							
		1	79.2	7.4 ±0.54	0	0.54 ±0.12	5.89	72
		2	76.6	5.0 ±0.46	3	0.56 ±0.12	3.82	77
		mean	77.9 ±1.3	6.2 ±1.20	1.5 ±1.5	0.55 ±0.01	4.86 ±1.04	
	E. sargentii							
		1	2.6	12.0 ±4.0	0	1.50 ±1.50	0.32	76
		2	76.2	6.8 ±0.43	0	0.64 ±0.11	5.14	84
		mean	39.4 ±36.8	9.4 ±2.60	0	1.07 ±0.43	2.73 ±2.41	
Adnataria								
	E. viridis							
		1	16.7	7.8 ±1.14	0	0.50 ±0.22	1.30	60
		2	80.2	4.1 ±0.3	0	0.42 ±0.09	3.30	86
		3	24.7	5.0 ±0.74	0	0.63 ±0.26	1.23	97
		mean	40.5 ±20.0	5.6 ±1.1	0	0.52 ±0.10	1.94 ±0.68	
	E. lansdowneana							
		1	40.5	4.0 ±0.37	0	0.27 ±0.10	1.62	74
		2	77.5	5.0 ±0.38	2	0.65 ±0.12	3.86	71
		3	27.6	4.7 ±0.88	0	0.25 ±0.14	1.29	58
		mean	48.5 ±15.0	4.6 ±0.30	0.7 ±0.7	0.39 ±0.10	2.26 ±0.81	
'Corymbia'								
	E. maculata							
		1	0.0	-	-	-	0	64
		2	7.1	0.5 ±0.5	50	0.50 ±0.50	0.04	28
		3	0.0	-	-	-	0	60
		mean	2.4 ±2.4	- -	-	- -	0.01 ±0.01	

Table 7.5: Intra and interspecific seed set in *E. cladocalyx*. (means ± standard error, n = number of flowers pollinated).

Taxonomic group	Male parent	Female tree	Percent capsule set	Seeds per capsule	Percent barren capsules	Aborted seeds per capsule	Seeds per pollination	n
Bisectaria								
	E. cladocalyx							
	Cross	1	7.4	1.83 ± 0.31	0	0.83 ± 0.31	0.15	81
		2	56.7	2.09 ± 0.32	37	0	1.18	120
		3	5.0	1.00 ± 0.0	0	3.06 ± 0.28	0.05	2
		mean	23.0 ± 16.9	1.64 ± 0.3	12.3 ±12.3	1.30 ± 0.91	0.46 ± 0.36	
	E. cladocalyx							
	Self	1	10.3	1.50 ± 0.48	20	0.30 ± 0.15	0.15	97
		2	63.9	0.04 ± 0.03	97	0.63 ± 0.18	0.02	72
		3	7.8	0.00	100	1.33 ± 0.12	0	102
		mean	27.3 ± 18.3	0.51 ± 0.50	72.3 ± 26.2	0.75 ± 0.30	0.06 ± 0.05	
	E. spathulata							
		1	7.7	1.0 ± 0.77	60	1.40 ± 0.60	0.08	61
		2	50.7	1.29 ± 0.60	38	1.00 ± 0.0	0.65	134
		3	4.7	0	100	2.43 ± 0.22	0.03	64
		mean	21.0 ± 14.9	0.70 ± 0.40	66 ± 18.2	1.61 ±0.70	0.25 ± 0.20	
	E. platypus							
		1	0	-	-	-	0	53
		2	87.3	2.16 ± 0.29	32	1.60 ± 0.16	1.92	79
		3	64.0	2.37 ± 0.42	30	2.90 ± 0.26	1.46	50
		mean	50.4 ± 26.1	2.27 ± 0.10	31.0 ± 1.0	2.25 ± 0.65	1.13 ± 0.58	
Adnataria								
	E. viridis							
		1	0	-	-	-	0	43
		2	35.8	0.13 ± 0.09	92	1.26 ± 0.23	0.05	67
		3	45.1	0.91 ± 0.20	35	1.54 ± 0.39	0.43	51
		mean	27.0 ± 13.8	0.52 ± 0.21	63.5 ± 25.9	1.40 ± 0.60	0.24 ± 0.14	
Exsertaria								
	E. flindersii							
		1	0	-	-	-	0	55
		2	1.5	0	100	0	0	65
		3	0	-	-	-	0	39
		mean	0.5 ± 0.5	-	-	-	0	

Table 7.6: Intra and interspecific seed set in *E. leptophylla*. (means ± standard error, n = number of flowers pollinated).

Taxonomic group	Male parent	Female tree	Percent capsule set	Seeds per capsule	Percent barren capsules	Aborted seeds per capsule	Seeds per pollination	n
Bisectaria								
	E. leptophylla							
	Cross	1	36.2	2.2 ±0.29	0	0.35 ±0.24	0.79	47
		2	63.4	3.1 ±0.17	2.4	0.08 ±0.03	1.95	134
		3	66.7	2.7 ±0.16	1.5	0.19 ±0.05	1.78	102
		mean	55.4 ±9.7	2.6 ±0.30	1.3 ±0.7	0.21 ±0.08	1.51 ±0.36	
	E. leptophylla							
	Self	1	2.8	1.0 ±0.00	0	0	0.03	72
		2	26.2	0.9 ±0.17	35	0.47 ±0.15	0.23	65
		3	2.8	0.5 ±0.50	50	1.00 ±0.0	0.01	74
		mean	10.6 ±7.8	0.8 ±0.20	28.3 ±14.8	0.49 ±0.30	0.09 ±0.07	
	E. albida							
		1	3.4	0.5 ±0.50	50	0	0.02	59
		2	6.9	1.2 ±0.58	40	0.60 ±0.40	0.08	72
		3	1.3	0 ±1.00	100	1.00 ±0.00	0	78
		mean	3.9 ±1.7	0.6 ±0.40	63.3 ±18.6	0.53 ±0.30	0.03 ±0.03	
	E. yalatenis							
		1	55.6	2.6 ±0.33	0	0.17 ±0.07	1.46	54
		2	50.0	1.8 ±0.21	0	0	0.90	58
		3	61.0	2.3 ±0.16	2	0.18 ±0.07	1.41	82
		mean	55.5 ±3.2	2.3 ±0.25	0.7 ±0.7	0.12 ±0.10	1.26 ±0.18	
	E. platypus							
		1	5.3	0	100	0.33 ±0.29	0	57
		2	26.7	0.3 ±0.11	75	1.13 ±0.29	0.07	60
		3	1.3	0	100	1.00 ±0.00	0	80
		mean	11.1 ±7.9	0.1 ±0.10	91.7 ±8.3	0.82 ±0.30	0.02 ± 0.02	
Adnataria								
	E. melliodora							
		1	8.3	0	100	0	0	48
		2	1.8	1	0	0	0.02	56
		3	3.0	0	100	0	0	67
		mean	4.4 ±2.0	0.3 ±0.30	66.7 ±33.3	0	0.01 ± 0.01	
	E. lansdowneana							
		1	5.2	0.8 ±0.48	50	0	0.04	77
		2	13.5	0.9 ±0.14	14	0	0.12	52
		3	0	-	-	-	0	46
		mean	6.2 ±3.9	0.8 ±0.1	32.0 ±18.0	0	0.05 ±0.04	

pollinations. *E. spathulata* intraspecific cross pollinations showed high levels of capsule set and numbers of fertile seeds per capsule, and all capsules contained some fertile seeds, with a mean of 0.85 aborted seeds per capsule. Self pollinations also showed no barren capsules despite a low number of fertile seeds per capsule and much reduced capsule set. Numbers of aborted seeds per capsule were similar but lower than in intraspecific cross pollinations. Interspecific pollinations using *E. spathulata* as a female parent showed varying levels of success in capsule and seed set, with most combinations showing a decrease in both capsule and seed set compared to cross intraspecific pollinations. Some combinations set levels of capsules and seeds approaching those resulting from intraspecific cross pollinations, notably combinations with *E. platypus* and *E. occidentalis*, both members of the section *Bisectaria*, and closely allied to *E. spathulata.* Most combinations outperformed self intraspecific pollinations in terms of both capsule set and number of seeds per capsule. Interspecific crosses were characterised by high variability between female trees in capsule and seed set rates. All three female trees used in interspecific pollinations set hybrid seed in all combinations except with *E. maculata* (an intersubgeneric cross), although *E. spathulata* tree 2 set lower numbers of seeds per capsule in most combinations indicating tree to tree variation in potential reproductive output. The proportion of barren capsules did not vary greatly between crosses (*E. maculata* pollinations showed 50% barren capsules but sample size was small), and there were no significant differences in numbers of aborted seeds per capsule.

In *E. cladocalyx,* capsule and seed set was low in both intraspecific and interspecific pollinations with no significant differences in capsule set between treatments, due to the variability between maternal trees, but there were significant differences in the numbers of seeds per capsule ($P<0.05$) between treatments. Some interspecific combinations yielded higher numbers of capsules and seeds per capsule than intraspecific pollinations, but were characterised by high proportions of barren capsules. Numbers of aborted seeds per capsule were not significantly different between treatments. Some female *E. cladocalyx* trees showed a complete failure to set seed in some hybrid

combinations, and although capsules were produced, pollinations with *E. flindersii* pollen produced no fertile seed.

In *E. leptophylla* there were significant differences between the percentage capsule set ($P<0.001$) and the number of seeds per capsule ($P<0.001$) between crosses. *E. leptophylla* intraspecific cross pollinations showed high levels of capsule set, few barren capsules and low numbers of aborted seeds per capsule. Intraspecific self pollinations yielded lower numbers of capsules and lower seed to capsule ratios than cross pollinations. There were also significant differences in the number of barren capsules between crosses ($P<0.05$) but no significant difference in the number of aborted seeds per capsule. The closely related *E. yalatensis* set comparable levels of capsules and seeds to intraspecific cross pollinations in combination with *E. leptophylla*, but all other combinations showed lower capsule and seed set than intraspecific crosses. Some interspecific crosses showed very high levels of barren capsules. Crosses with *E. albida*, *E. platypus*, *E. melliodora* and *E. lansdowneana* were characterised by very low numbers of fertile seed.

When capsule and seed set data for individual trees of the three species used as females are compared with ovule penetration rates from the same crosses from the previous season (table 7.7) it is apparent that there is variability from year to year (and perhaps with pollen batches) in cross compatibility. Some trees that showed no ovule penetration in the 1988-9 pollination season showed good seed set in the same combination in the 1989-90 season (tables 7.1-7.6). *E. spathulata* intraspecific cross pollinations show similarity between the percentage of pistils with penetrated ovules and percent capsule set, indicating there is little loss of flowers with fertilised ovules. The mean number of penetrated ovules per flower also agrees closely with the number of seeds per capsule in the same cross suggesting little post-zygotic abortion after the stage of ovule penetration. Capsule and seed set in some other combinations with *E. spathulata* such as *E. platypus* also shows similarity with ovule penetration rates. Other combinations show lower numbers of seeds and capsules resulting from high ovule penetration rates such as *E. spathulata* x *maculata*.

E. cladocalyx and *E. leptophylla* showed a higher reduction than *E. spathulata* in capsule and seed yield compared to ovule penetration rates in intraspecific crosses. In

Table 7.7: Summary of mean ovule penetrations (1988-89) and seed set (1989-90) in intraspecific and interspecific crosses. (*results from one tree only).

		Female parent					
		E. spathulata		*E. cladocalyx*		*E. leptophylla*	
Tax. group	Male parent	total ovule penetrations	seeds per capsule	total ovule penetrations	seeds per capsule	total ovule penetrations	seeds per capsule
Bisectaria							
	E. spathulata	9.4	9.2	19.2	0.7	-	-
	E. cladocalyx	7.0	4.3	10.0	1.6	-	-
	E. leptophylla	-	-	-	-	20.6	2.6
	E. platypus	11.0	7.7	3.2	2.3	3.4*	0.1
	E. albida	-	-	-	-	4.1	0.6
	E. yalatensis	3.5*	6.9	-	-	6.1	2.3
Adnataria							
	E. melliodora	-	-	-	-	3.4	0.3
	E. viridis	3.5	5.6	7.7*	0.5	-	-
	E. lansdowneana	11.1	4.6	-	-	3.4*	0.8
Exsertaria							
	E. flindersii	-	-	3.2*	0.0*	-	-
'Corymbia'							
	E. maculata	9.5*	0.5*	-	-		-

interspecific crosses there was less difference between the two measures mainly due to lower ovule penetration rates, although there was still a reduction in penetrated ovules prior to capsule set and seed maturation. Sample sizes were larger in the seed set experiments and thus could detect a lower rate of seed set. In addition, in the pollen-pistil investigations only one locule per flower was examined for evidence of ovule penetration, thus reducing the probability of detecting ovule penetrations at very low frequencies.

7.3.2 Seed weights

Seed weight varied considerably within crosses between maternal trees of the same species (Table 7.8). All cross combinations with *E. spathulata*, *E. cladocalyx* and *E. leptophylla* as female parents showed no significant differences in seed weight. There was no reduction in seed weight in the more distant as compared with the close crosses, and the paternal parents did not appear to influence seed weight. The combinations of *E. spathualata* x *maculata* and *E. leptophylla* x *platypus*, which produced much lighter seeds which later proved to be inviable.

7.3.3 Germination and viability

There were significant differences ($P<0.001$) in the time taken for seed to germinate between the parental species (Table 7.9) under the conditions of the experiment. When germination times of hybrid seed was compared with that of parental seed it was found that of fourteen interspecific combinations that germinated , eight showed intermediate germination times, four took longer to germinate and two germinated faster than the parental species. Most gentoypes tested had high germination rates and viability levels, except for some combinations with *E. platypus* and *E. yalatensis*. The intersubgeneric cross *E. spathulata* x *maculata* and the intrasectional cross *E. leptophylla* x *platypus* failed to germinate, all seeds proved to be inviable.

7.3.4 Seedling survival and vigour

The combinations *E. spathulata* x *lansdowneana*, *E. leptophylla* x *lansdowneana* and *E. leptophylla* x *melliodora*, all of which had good germination rates, showed hybrid

Table 7.8: Seed weights of parental and F1 hybrid seed (g x 10^{-4}). (Taxonomic groups; B = *Bisectaria*; A = *Adnataria*, C = *Corymbia*. n = number of seeds).

Taxonomic Group	Genotype	Maternal Tree 1	2	3	mean	n
B	*E. spathulata*	2.73	3.00	2.93	2.89 ± 0.08	45
B x B	*E. spathulata x cladocalyx*	3.07	3.27	2.73	3.02 ± 0.16	45
B x B	*E. spathulata x platypus*	2.73	2.13	2.60	2.49 ± 0.18	45
B x B	*E. spathulata x yalatensis*	2.60	2.93	2.60	2.71 ± 0.11	45
B x B	*E. spathulata x occidentalis*	3.27	4.00	-	3.63 ± 0.37	30
B x B	*E. spathulata x sargentii*	1.67	2.53	3.33	2.51 ± 0.48	30
B x A	*E. spathulata x viridis*	2.00	4.13	2.40	2.84 ± 0.65	45
B x A	*E. spathulata x lansdowneana*	2.13	3.87	3.00	3.00 ± 0.50	45
B x C	*E. spathulata* x *maculata*	-	1.50	-	1.50 -	1
B	*E. cladocalyx*	11.40	8.07	-	9.73 ± 1.67	25
B x B	*E. cladocalyx x spathulata*	14.80	8.93	-	11.87 ± 2.93	20
B x B	*E. cladocalyx x platypus*	-	7.00	7.93	7.47 ± 0.47	30
B x A	*E. cladocalyx x viridis*	-	6.33	8.73	7.53 ± 1.20	18
B	*E. leptophylla*	3.67	2.33	2.40	2.80 ± 0.43	45
B x B	*E. leptophylla x albida*	3.00	1.75	-	2.38 ± 0.63	5
B x B	*E. leptophylla x yalatensis*	4.13	2.73	3.27	3.38 ± 0.41	45
B x B	*E. leptophylla x platypus*	-	1.50	-	1.50 -	2
B x A	*E. leptophylla x melliodora*	-	2.00	-	2.00 -	1
B x A	*E. leptophylla x lansdowneana*	2.60	3.00	-	2.80 ± 0.02	8
B	*E. platypus*	9.33	6.67	8.27	8.09 ± 0.78	45
B	*E. albida*	9.07	8.07	7.33	8.16 ± 0.50	45
B	*E. yalatensis*	3.87	5.93	4.33	4.64 ± 0.67	45
B	*E. occidentalis*	6.80	7.40	-	7.10 ± 0.30	30
B	*E. sargentii*	4.60	3.27	3.53	3.80 ± 0.41	45
A	*E. viridis*	1.87	1.60	1.47	1.64 ± 0.12	45
A	*E. lansdowneana*	4.77	7.53	10.13	7.48 ± 1.55	45

Table 7.9: Germination and viability of seed from parental and hybrid seed lots. (means ± standard error, n = number of seeds). (Taxonomic groups, B = *Bisectaria*, A = *Adnataria*, C = *Corymbia*).

Taxonomic Group	Genotype	# female trees	n	Days to germination	Percent germination	Percent viability
B	*E. spathulata* Cross	3	45	5.9 ± 1.0	93.3 ± 6.7	100 ± 0.0
B	*E. spathulata* Self	1	14	6.5 ± 0.9	93 -	93 -
B x B	*E. spathulata* x *cladocalyx*	3	45	11.2 ± 3.1	82 ± 5.9	82 ± 5.9
B x B	*E. spathulata* x *platypus*	3	45	19.3 ± 6.8	62.3 ± 12.5	71.1 ± 9.8
B x B	*E. spathulata* x *yalatensis*	3	45	26.6 ± 3.8	71.1 ± 4.4	91.1 ± 8.9
B x B	*E. spathulata* x *occidentalis*	2	30	5.1 ± 0.3	90 ± 10.0	93.4 ± 6.7
B x B	*E. spathulata* x *sargentii*	2	30	4.0 ± 0.3	86.7 ± 13.4	90 ± 10.0
B x A	*E. spathulata* x *viridis*	3	41	4.6 ± 0.6	81.1 ± 15.7	83.5± 13.3
B x A	*E. spathulata* x *lansdowneana*	3	43	5.4 ± 1.1	85.3 ± 8.9	85.3 ± 8.9
B x C	*E. spathulata* x *maculata*	1	1	-	0	0
B	*E. cladocalyx* Cross	2	25	16.4 ± 5.7	80 ± 20.0	100 ± 0.0
B	*E. cladocalyx* Self	1	15	16.1 ± 3.9	60 -	100 -
B x B	*E. cladocalyx* x *spathulata*	2	20	13.3 ± 7.2	100 ± 0.0	100 ± 0.0
B x B	*E. cladocalyx* x *platypus*	2	30	8.4 ± 0.7	100 ± 0.0	100 ± 0.0
B x A	*E. cladocalyx* x *viridis*	2	18	3.3 ± 0.3	100 ± 0.0	100 ± 0.0
B	*E. leptophylla* Cross	3	45	10.4 ± 1.2	96.7 ± 2.7	96.7 ± 2.7
B	*E. leptophylla* Self	3	13	4.8 ± 1.5	100 ± 0.0	100 ± 0.0
B x B	*E. leptophylla x albida*	2	6	9.8 ± 2.6	100 -	100 -
B x B	*E. leptophylla x yalatensis*	3	44	22.3 ± 6.5	60 ± 16.3	100 ± 0.0
B x B	*E. leptophylla x platypus*	1	2	- -	0 -	0 -
B x A	*E. leptophylla x melliodora*	1	1	3.0 -	100 -	100 -
B x A	*E. leptophylla* x *lansdowneana*	2	8	16.6± 11.4	100 ± 0.0	100 ± 0.0
B	*E. platypus*	3	45	22.4 ± 2.7	50.0 ± 8.2	93.4 ± 5.5
B	*E. albida*	3	45	18.8 ± 4.0	93.4 ± 5.3	100 ± 0.0
B	*E. yalatensis*	3	45	26.3 ± 2.9	90.4 ± 8.2	100 ± 0.0
B	*E. occidentalis*	2	30	4.3 ± 0.4	100 ± 0.0	100 ± 0.0
B	*E. sargentii*	3	45	3.3 ± 1.5	100 ± 0.0	100 ± 0.0
A	*E. viridis*	3	45	4.0 ± 0.5	100 ± 0.0	100 ± 0.0
A	*E. lansdowneana*	3	45	3.7 ± 0.1	86.7 ± 4.4	86.7 ± 4.4

breakdown at the early seedling stage. Healthy cotyledons were produced but all seedlings withered before production of the first leaf pair. These three combinations are all distant crosses between the section *Bisectaria* and section *Adnataria*. All other combinations produced healthy seedlings but with variation within families for vigour.

7.3.5 Hybrid verification and early seedling characteristics

Cotyledon shape is characteristic of particular taxonomic groups. Cotyledons of members of the section *Bisectaria* are typically deeply bisected and bi-lobed, while members of the section *Adnataria* have kidney shaped cotyledons. Thus cotyledon shape can be used to identify intermediates between taxonomic groups. Cotyledon shapes of the genotypes under investigation are shown in Figure 7.1. Interspecific hybrid seedlings between members of the sections *Bisectaria* and *Adnataria* showed intermediate cotyledon lobe length and shape. A putative spontaneous hybrid was detected in an open pollinated seed batch of *E. sargentii* which had less pronounced cotyledon lobes than the parental species, suggesting a hybrid with another *Symphyomyrtus* section.

Juvenile leaf morphology of parental species and interspecific hybrids is shown in Figs. 7.2-7.4. Leaf morphology changes at each node with growth of the seedling so measurements of leaf characteristics were standardised at the fifth and tenth leaf pairs, (tables 7.10-12). All putative hybrids had intermediate leaf morphology between the parental species (Fig. 7.5), and the spontaneous putative hybrid of *E. sargentii*, identified by its cotyledon characteristics, also showed different leaf morphology from its siblings (Fig. 7.6). Figures 7.7-7.12 show the results of principle component analysis on parental and putative hybrid data sets. In most cases the two parental species show discontinuous distributions allowing genotype discrimination, and hybridity is confirmed for progeny of controlled pollinations through intermediacy between parental genotypes. In cases such as *E. spathulata* x *E. viridis* (Fig. 7.8A) where overlap between parental distributions occurs discrimination of hybrid progeny is less distinct, and in this case the putative hybrids display morphology closer to the male parent, at the extreme range of *E. spathulata*. The variables measured give poor genotype discrimination for *E. leptophylla*, *E. yalatensis* and the putative hybrid (Fig. 7.12B). *E. spathulata* x *occidentalis* (Fig. 7.9B) and

Figure 7.1 Cotyledon morphology of eleven species and fifteen putative interspecific hybrids of *Eucalyptus*. Bar represents 10 mm.

a, *E. spathulata*;
b, *E. cladocalx*;
c, *E. leptophylla*;
d, *E. platypus*;
e, *E. yalatensis*;
f, *E. albida*;
g, *E. sargentii*;
h, *E. occidentalis*;
i, *E. lansdowneana*;
j, *E. viridis*;
k, *E. melliodora*;
l, *E. spathulata* x *platypus*;
m, *E. spathulata* x *cladocalyx*;
n, *E. spathulata* x *yalatensis*;
o, *E. spathulata* x *occidentalis*;
p, *E. spathulata* x *sargentii*;
q, *E. spathulata* x *lansdowneana*;
r, *E. spathulata* x *viridis*;
s, *E. cladocalyx* x *spathulata*;
t, *E. cladocalyx* x *platypus*;
u, *E. cladocalyx* x *viridis*;
v, *E. leptophylla* x *albida*;
w, *E. leptophylla* x *yalatensis*;
x, *E. leptophylla* x *lansdowneana*;
y, *E. leptophylla* x *melliodora*;
z, *E. sargentii* spontaneous hybrid.

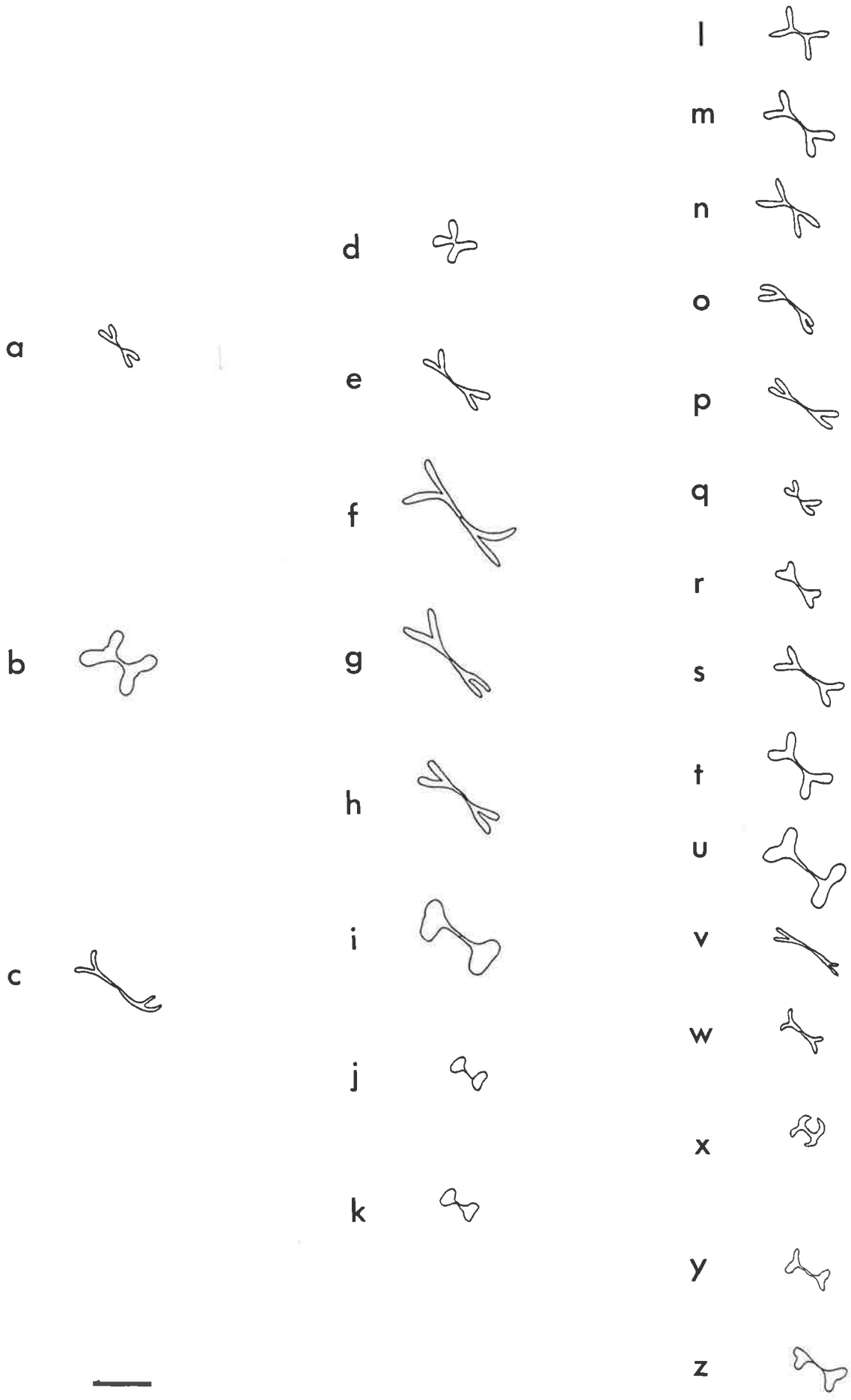
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Figure 7.2 Morphology of leaves from the 10th node of seedlings of *Eucalyptus* species and putative interspecific hybrids with *E. spathulata*. Bar represents 5cm.

a, *E. spathulata*;

b, *E. platypus*;

c, *E. viridis*;

d, *E. yalatensis*;

e, *E. sargentii*;

f, *E. occidentalis*;

g, *E. spathulata* x *platypus;*

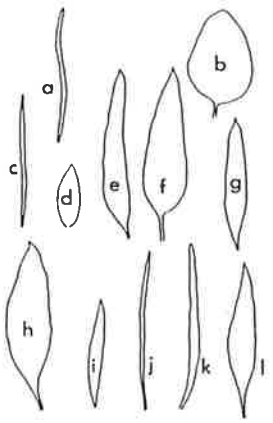
h, *E. spathualata* x *cladocalyx*;

i, *E. spathulata* x *yalatensis*;

j, *E. spathulata* x *viridis*;

k, *E. spathulata* x *sargentii*;

l, *E. spathulata* x *occidentalis*.



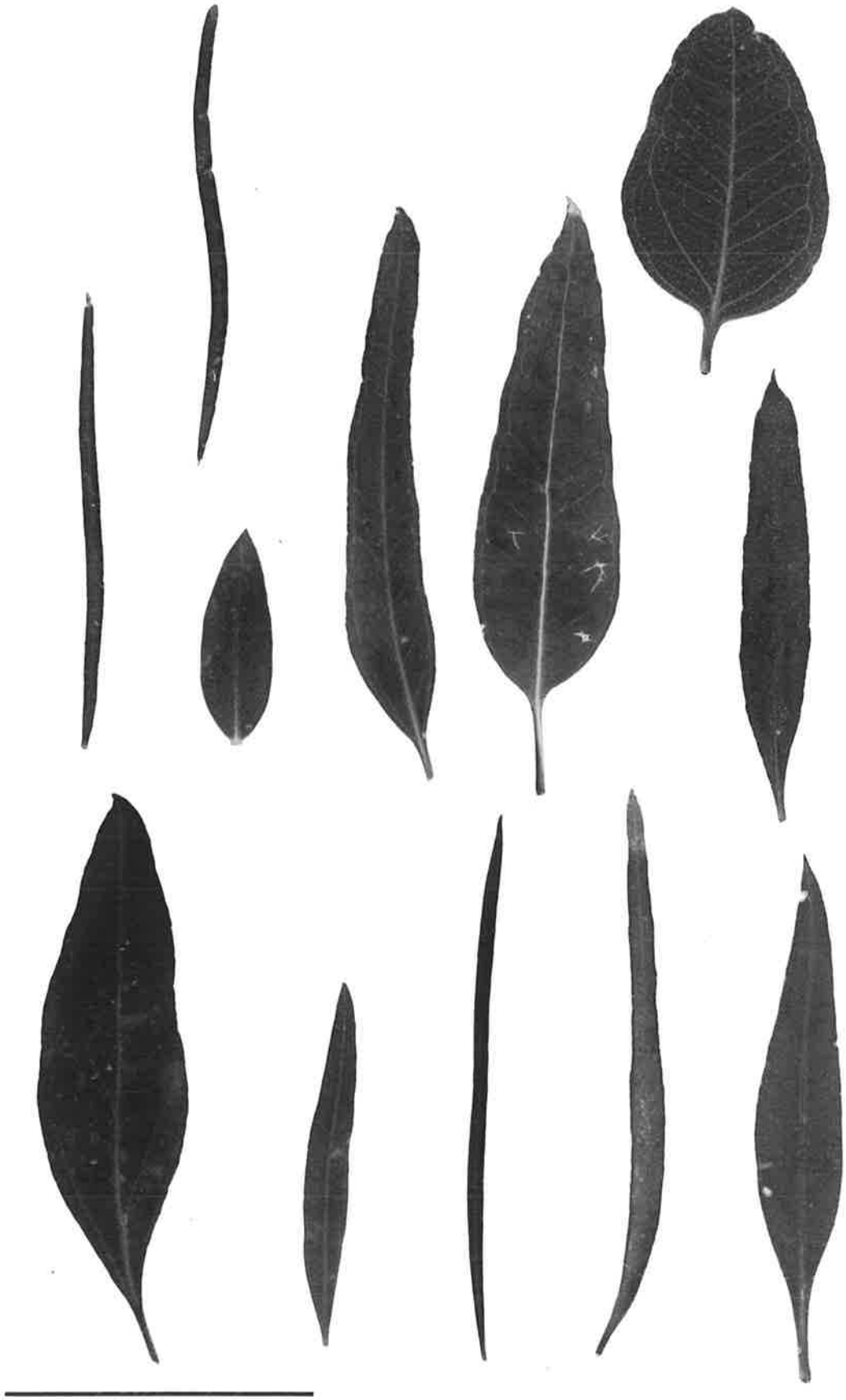


Figure 7.3 Morphology of leaves from the 10th node of seedlings of *Eucalyptus* species and putative interspecific hybrids with *E. cladocalyx*. Bar represents 5cm.

a, *E. cladocalyx,*

b, *E. platypus*;

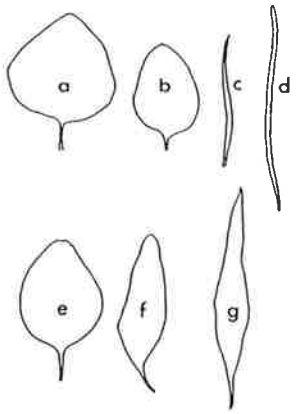
c, *E. spathulata*;

d, *E. viridis,*

e, *E. cladocalyx* x *platypus*;

f, *E. cladocalyx* x *spathulata*;

g, *E. cladocalyx* x *viridis.*



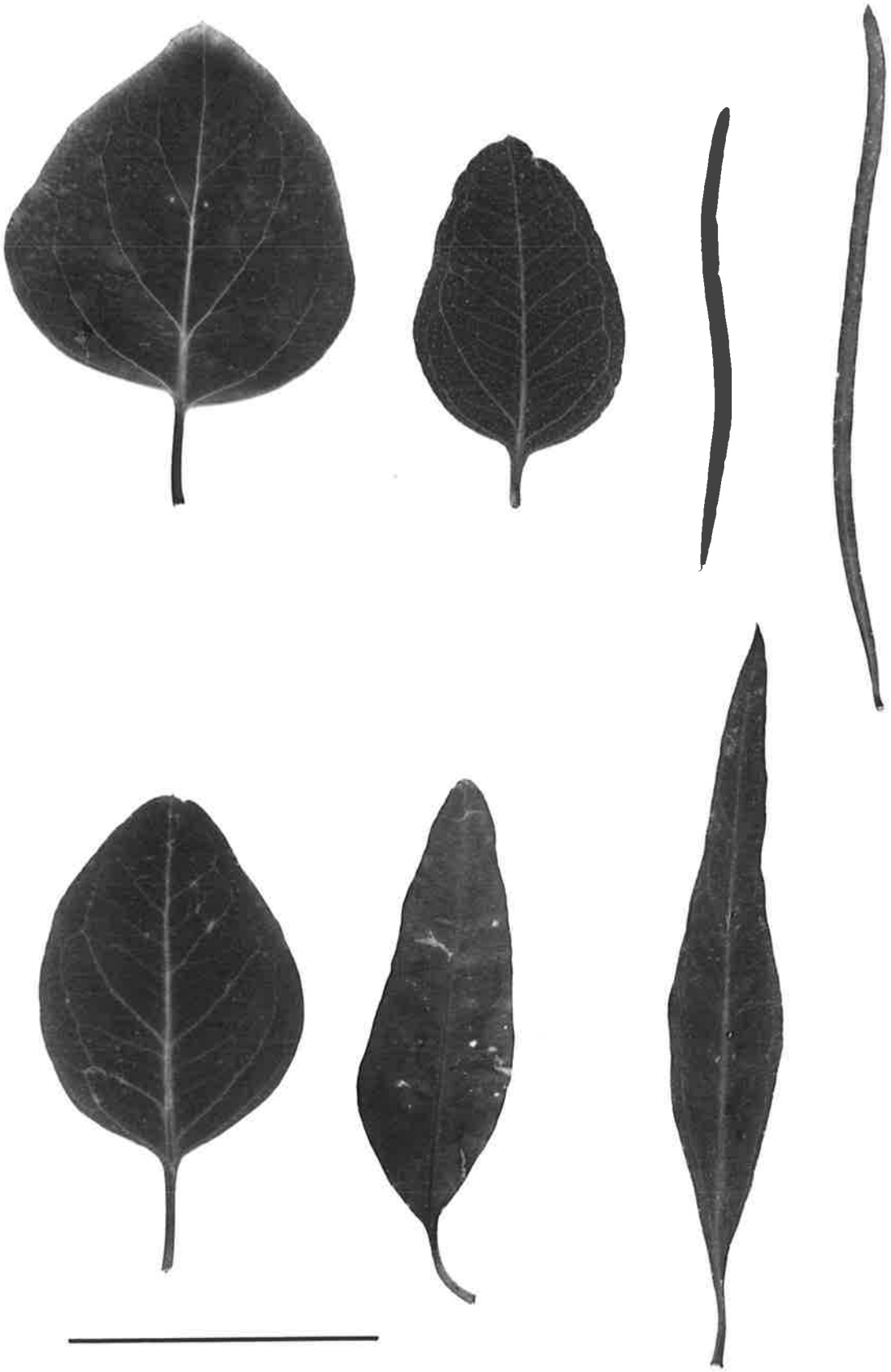


Figure 7.4 Morphology of leaves from the 10th node of seedlings of *Eucalyptus* species and putative interspecific hybrids with *E. leptophylla*. Bar represents 5cm.

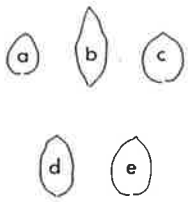
a, *E. leptophylla*;

b, *E. yalatensis*;

c, *E. albida*;

d, *E. leptophylla* x *yalatensis*;

e, *E. leptphylla* x *albida*.



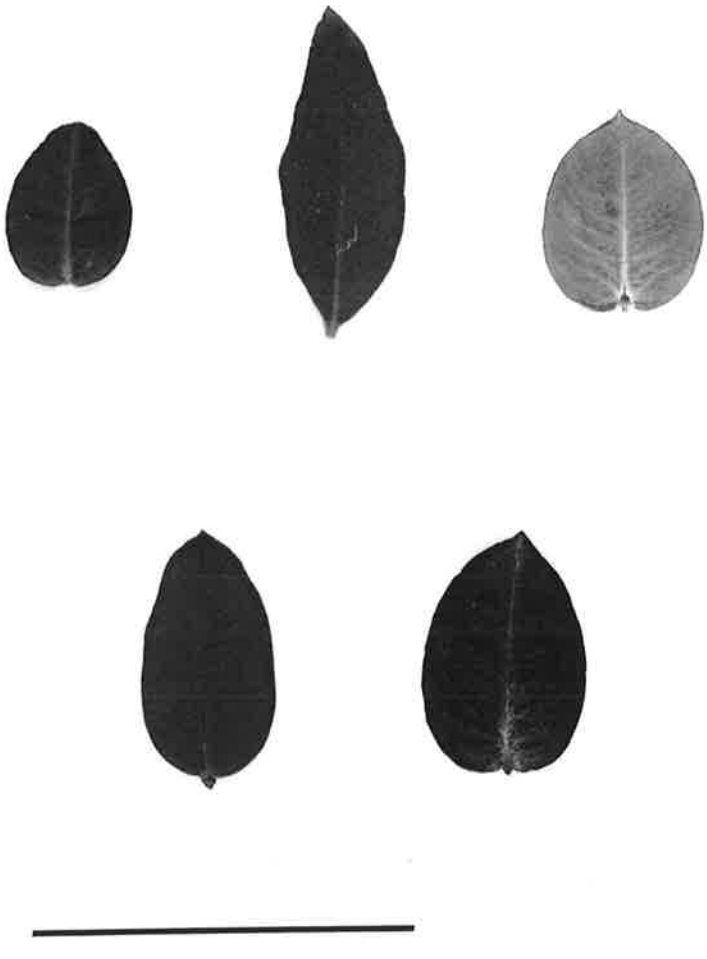


Table 7.10: Morphological measurements of five month old seedlings from parental and hybrid seed lots from *E. spathulata* female parents. (L = leaf length, W = leaf width, Pw = distance from leaf base to the widest point, Pe = petiole length, A = angle at leaf tip, D = distance from 5th node to 10th node, Br/N = number of branches/number of nodes, N = node at which leaves become alternate, G = glaucousness, R = red pigmentation of stems, n = sample size). (means ± standard errors). (* not available).

Genotype	5th leaf										10th leaf					
	L	W	Pw	Pe	A	D	Br/N	N	G	R	L	W	Pw	Pe	A	n
E. spathulata	72.5 ± 2.7	7.5 ± 0.8	34.6 ± 3.0	1.4 ± 0.2	28.1 ± 2.0	217 ± 6.8	0.7 ± 0.04	3.4 ± 0.3	0	2.4 ± 0.3	70.4 ± 2.6	3.1 ± 0.3	40.1 ± 2.9	1.0 ± 0.1	14.5 ±1.2	14
E. spathulata x *cladocalyx*	51.3 ± 5.0	25.9 ± 2.4	18.3 ± 1.8	9.9 ± 0.7	65.5 ± 1.8	215 ± 12.1	0.6 ± 0.05	3.7 ± 0.6	0	2.6 ± 0.5	57.0 ± 4.3	21.3 ± 1.9	20.4 ± 0.8	11.4 ± 1.2	63.4 ±3.1	8
E. spathulata x *platypus*	54.7 ± 2.1	28.0 ± 0.8	20.8 ± 1.0	8.2 ± 0.4	70.6 ± 2.1	231 ± 5.8	0.7 ± 0.02	4.1 ± 0.3	0	2.6 ± 0.2	60.4 ± 3.5	11.2 ± 0.5	21.1 ± 1.6	3.0 ± 0.4	34.3 ± 2.1	8
E. spathulata x *yalatensis*	39.2 ± 1.6	14.0 ± 0.6	13.6 ± 0.5	1.0 ± 0.0	49.0 ± 1.0	169 ± 10.9	0.6 ± 0.07	8.4 ± 1.4	0	2.4 ± 0.2	48.0 ± 2.6	15.6 ± 0.9	17.2 ± 1.5	2.2 ± 0.7	52.8 ± 2.2	5
E. spathulata x *occidentalis*	77.4 ± 2.8	23.3 ± 0.8	22.1 ± 1.9	9.4 ± 0.5	53.7 ± 1.8	274 ± 14.9	0.7 ± 0.04	3.7 ± 0.2	0	0	*	*	*	*	*	10
E. spathulata x *sargentii*	88.6 ± 4.2	16.2 ± 0.8	28.9 ± 1.8	5.6 ± 0.7	38.6 ± 2.7	264 ± 13.8	0.7 ± 0.03	3.9 ± 0.16	0	2.9 ± 0.3	81.9 ± 2.5	7.2 ± 0.9	24.9 ± 2.4	3.4 ± 0.4	22.2 ± 1.6	14
E. spathulata x *viridis*	78.3 ± 4.0	10.1 ± 0.6	27.8 ± 1.8	0.0	38.0 ± 3.3	221 ± 12.1	0.7 ± 0.06	4.4 ± 0.3	0	2.7 ± 0.2	102.0 ±6.3	4.3 ± 0.9	39.2 ± 5.9	0.0	15.1 ± 1.5	10

Table 7.11: Morphological measurements of five month old seedlings from parental and hybrid seed lots from *E. cladocalyx* and *E. leptophylla* female parents. (L = leaf length, W = leaf width, Pw = distance from leaf base to the widest point, Pe = petiole length, A = angle at leaf tip, D = distance from 5th node to 10th node, Br/N = number of branches/number of nodes, N = node at which leaves become alternate, G = glaucousness, R = red pigmentation of stems, n = sample size). (means ± standard errors). (* not available).

Genotype	5th leaf										10th leaf					
	L	W	Pw	Pe	A	D	Br/N	N	G	R	L	W	Pw	Pe	A	n
E. cladocalyx	40.2 ± 1.5	36.8 ± 0.9	13.9 ± 0.6	15.9 ± 0.8	127.0 ± 2.7	227 ± 15.3	0.6 ± 0.02	4.1 ± 0.3	0	3.5 ± 0.3	53.8 ±2.0	53.8 ± 2.0	18.4 ± 1.6	16.6 ± 0.9	137.0 ± 6.5	10
E. cladocalyx x *spathulata*	49.4 ± 2.8	26.6 ± 1.2	17.7 ± 0.8	10.0 ± 0.8	79.1 ± 4.3	208 ± 27.9	0.7 ± 0.03	4.6 ± 0.3	0	3.6 ± 0.2	62.8 ± 3.8	20.8 ± 1.2	22.9 ± 1.8	9.8 ± 0.8	54.1 ± 4.1	9
E. cladocalyx x *platypus*	46.6 ± 2.3	45.1 ± 2.1	14.7 ± 1.1	12.0 ± 1.3	117.0 ± 5.8	296 ± 25.3	0.8 ± 0.03	3.6 ± 0.2	0	2.4 ± 0.3	51.9 ± 2.7	46.5 ± 2.4	18.5 ± 0.9	13.7 ± 1.1	111.0 ± 4.0	10
E. cladocalyx x *viridis*	52.7 ± 2.3	25.0 ± 1.0	19.9 ± 1.0	10.6 ± 0.7	82.1 ± 4.7	196 ± 20.0	0.5 ± 0.03	4.6 ± 0.2	0	3.3 ± 0.3	86.3 ± 8.6	20.9 ± 2.0	34.1 ± 2.6	11.4 ± 0.5	49.1 ± 6.2	7
E. leptophylla	28.9 ± 0.8	14.2 ± 0.7	9.7 ± 0.4	0.0	54.7 ± 2.7	97.7 ± 4.2	0.7 ± 0.1	*	2 ± 0.0	1.9 ± 0.2	25.4 ± 0.6	19.7 ± 0.6	8.8 ± 0.4	0	90.1 ± 4.0	15
E. leptophylla x *albida*	28.0 ± 4.0	13.0 ± 1.0	11.5 ± 0.5	0.0	60.5 ± 5.5	63.5 ± 9.5	1.0 ± 0.01	*	3	1.0 ± 1.0	22.5 ± 1.5	16.0 ± 3.0	13.0 ± 0.0	0	89.5 ± 10.5	2
E. leptophylla x *yalatensis*	24.4 ± 1.0	11.1 ± 1.6	7.6 ± 0.6	0.0	57.1 ± 8.7	101.0 ± 4.8	0.6 ± 0.05	14.0	0.3 ± 0.2	3.9 ± 0.5	28.6 ± 1.7	19.6 ± 1.4	8.4 ± 0.7	0	87.5 ± 6.8	8

Table 7.12: Morphological measurements of five month old seedlings from parental seed lots. (L = leaf length, W = leaf width, Pw = distance from leaf base to widest point, Pe = petiole length, A = angle at leaf tip, D = distance from 5th node to 10th node, Br/N = number of branches/number of nodes, N = node at which leaves become alternate, G = glaucousness, R = red pigmentation of stems, n = sample size). (means ± standard errors). (* not available).

	5th leaf										10th leaf					
Genotype	L	W	Pw	Pe	A	D	Br/N	N	G	R	L	W	Pw	Pe	A	n
E. platypus	43.5 ± 0.7	39.3 ± 0.5	15.3 ± 0.6	7.8 ± 0.4	122 ± 3.3	170.0 ± 10.7	0.8 ± 0.02	5.2 ± 0.4	0	0.2 ± 0.2	38.5 ± 1.3	29.2 ± 1.5	14 ± 0.8	6.7 ± 0.4	100 ± 3.3	11
E. yalatensis	25.6 ± 1.0	13.5 ± 1.1	8.4 ± 0.5	0	59.9 ± 4.3	124.0 ± 13.7	0.4 ± 0.05	9.4 ± 0.5	0.5 ± 0.1	3.4 ± 0.2	32.3 ± 1.5	21.3 ± 1.2	10.2 ± 0.7	0.14 ± 0.1	78.6 ± 4.1	14
E. albida	27.2 ± 1.4	15.5 ± 1.3	10.3 ± 0.7	0	65.9 ± 2.3	72.5 ± 3.8	0.6 ± 0.04	*	5	0.6 ± 0.2	26.1 ± 0.8	22.1 ± 0.7	9.2 ± 1.1	0	104 ± 5.2	11
E. occidentalis	64.2 ± 2.9	32.2 ± 1.9	17.3 ± 0.6	12.2 ± 1.0	62.0 ± 3.1	202.0 ± 9.0	0.6 ± 0.06	3.5 ± 0.2	0	4 ± 0.4	67.3 ± 2.3	27.8 ± 2.3	16 ± 0.8	12.3 ± 1.0	55.7 ± 5.8	6
E. sargentii	88.5 ± 3.1	16.3 ± 1.5	21.5 ± 1.2	7.6 ± 0.5	39.3 ± 2.1	283.0 ±± 10.5	0.8 ± 0.03	3.5 ± 0.1	0	3.1 ± 0.3	86.6 ± 4.6	8 ± 0.8	25.8 ± 3.4	6.4 ± 0.4	23.5 ± 1.7	15
E. sargentii spontaneous hybrid	100	46	26	15	70	26.0	0.3	2.0	2	4	105	41	21	14	65	1
E. viridis	55.1 ± 2.1	9 ± 0.8	29.5 ± 1.0	0.1 ± 0.4	40.8 ± 2.1	183.0 ±5.8	0.5 ± 0.02	4.9 ± 0.26	0	2.9 ± 0.2	109 ± 3.5	5.3 ± 0.5	85.6 ± 1.6	0.5 ± 0.4	26 ± 2.1	15

Figure 7.5 Five month old seedlings of *E. cladocalyx* (left), *E. cladocalyx* x *viridis* (centre) and *E. viridis* (right). Tube diameter 6cm.

E. cladocalyx

E. cladocalyx x viridis

E. viridis

Figure 7.6 Five month old seedlings of *E. sargentii* (right) and putative spontaneous hybrid (left) identified in an open pollinated seed batch of *E. sargentii*. Tube diameter 6cm.

E. sargentii (spontaneous hybrid)

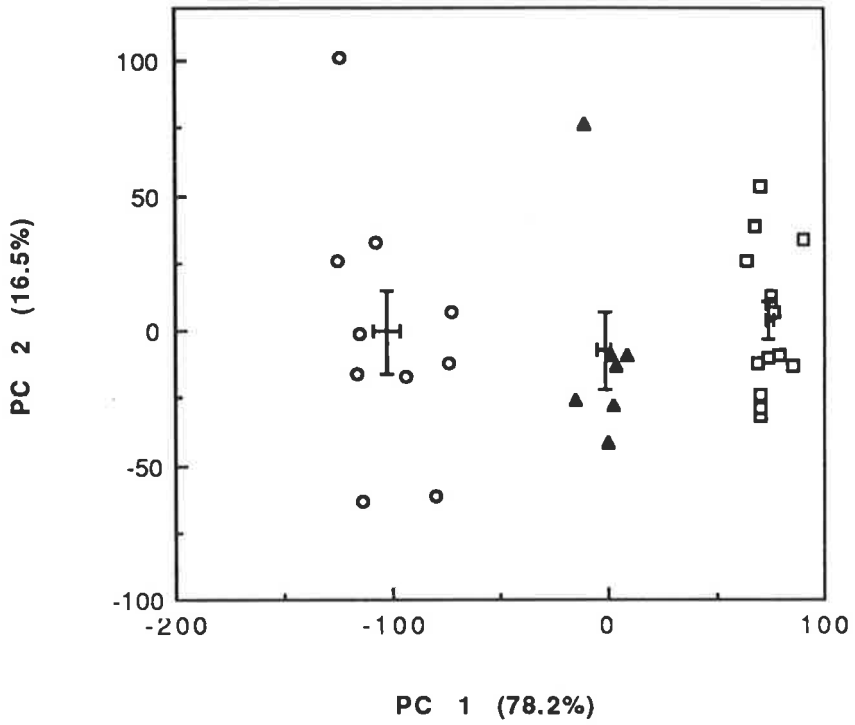
E. sargentii

Figure 7.7 Principle component ordination based on 15 morphological measurements of five month old *Eucalyptus* seedlings. Means and standard errors are shown for each species and putative hybrid genotype.

7.7A *E. spathulata* (□), *E. cladocalyx* (○), *E. spathulata* x *cladocalyx* (▲).

7.7B *E. spathulata* (□), *E. platypus* (○), *E. spathulata* x *platypus* (▲).

7.7A


100
50
0
-50
-100
-200
-100
0
100
PC 2 (16.5%)
PC 1 (78.2%)

7.7B

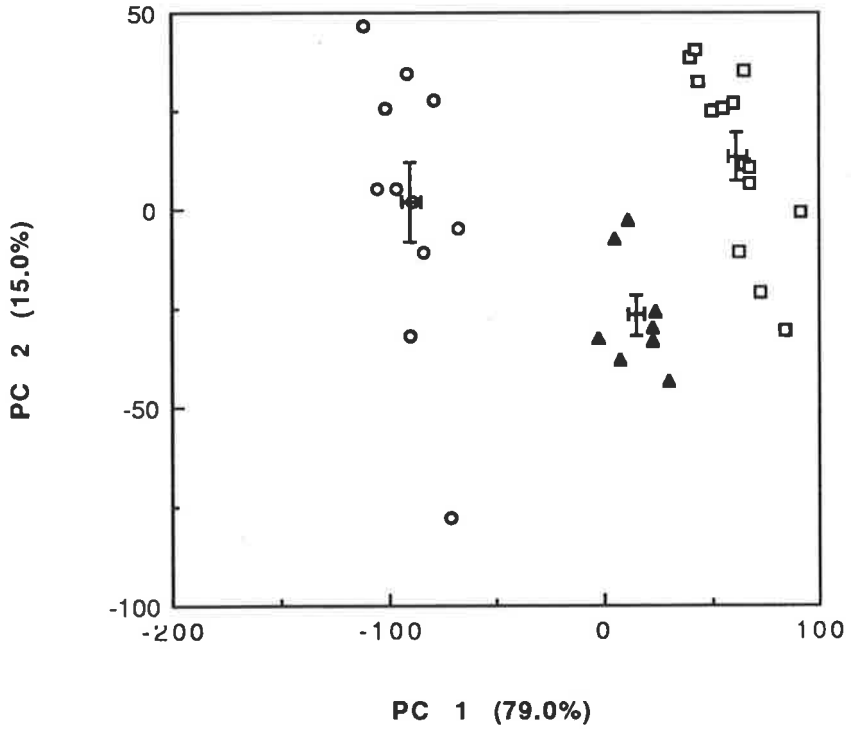
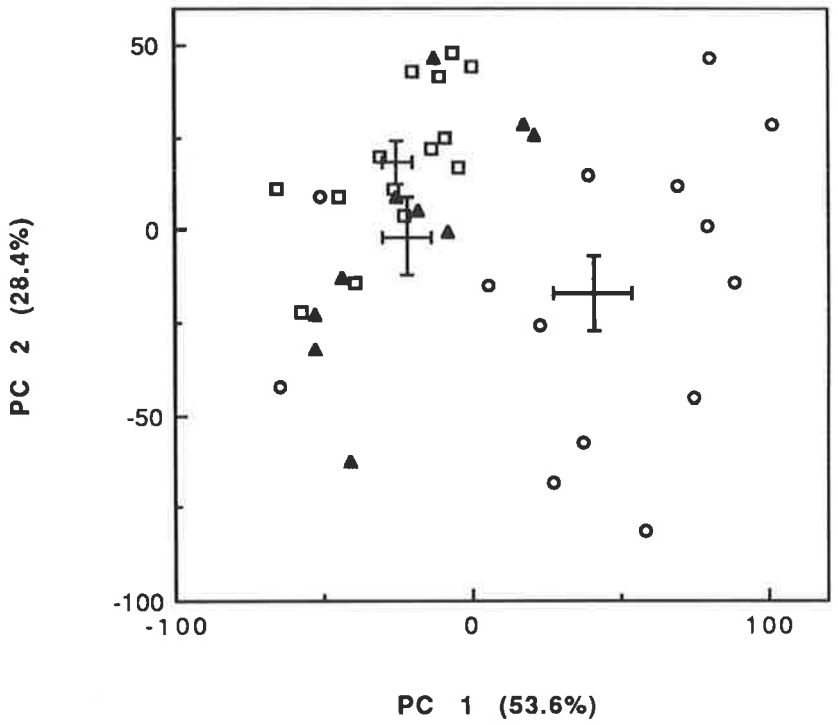

50
0
-50
-100
-200
-100
0
100
PC 2 (15.0%)
PC 1 (79.0%)

Figure 7.8 Principle component ordination based on 15 morphological measurements of five month old *Eucalyptus* seedlings. Means and standard errors are shown for each species and putative hybrid genotype.

7.8A *E. spathulata* (□), *E. viridis* (○), *E. spathulata* x *viridis* (▲).

7.8B *E. spathulata* (□), *E. yalatensis* (○), *E. spathulata* x *yalatensis* (▲).

7.8A


50
0
-50
-100
-100
0
100
PC 2 (28.4%)
PC 1 (53.6%)

7.8B

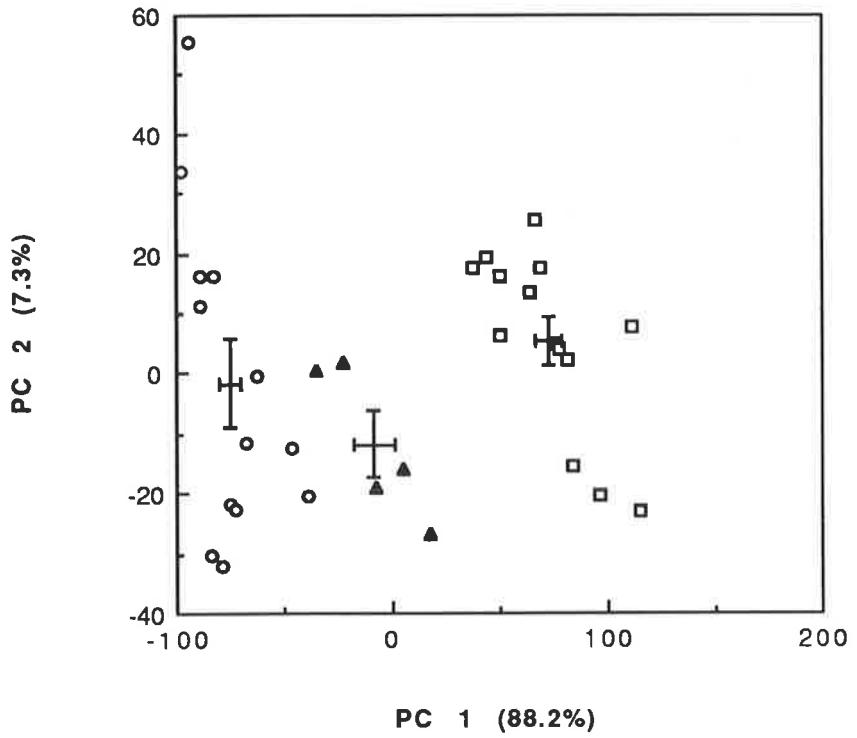
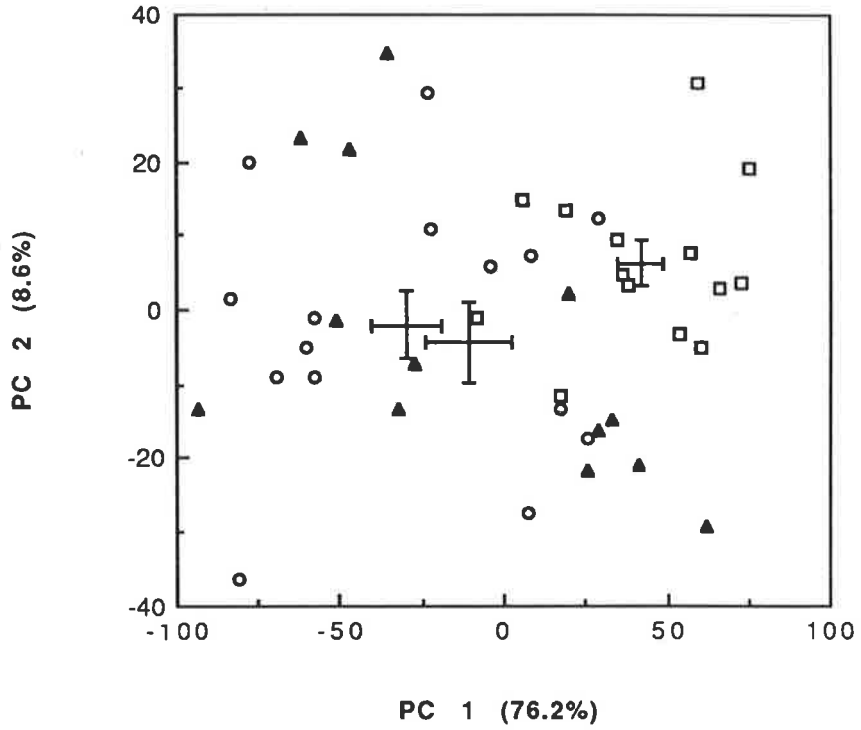

60
40
20
0
-20
-40
-100
0
100
200
PC 2 (7.3%)
PC 1 (88.2%)

Figure 7.9 Principle component ordination based on 15 morphological measurements of five month old *Eucalyptus* seedlings. Means and standard errors are shown for each species and putative hybrid genotype.

7.9A *E. spathulata* (□), *E. sargentii* (○), *E. spathulata* x *sargentii* (▲).

7.9B *E. spathulata* (□), *E. occidentalis* (○), *E. spathulata* x *occidentalis* (▲).

7.9A


40
20
0
-20
-40
-100
-50
0
50
100
PC 2 (8.6%)
PC 1 (76.2%)

7.9B

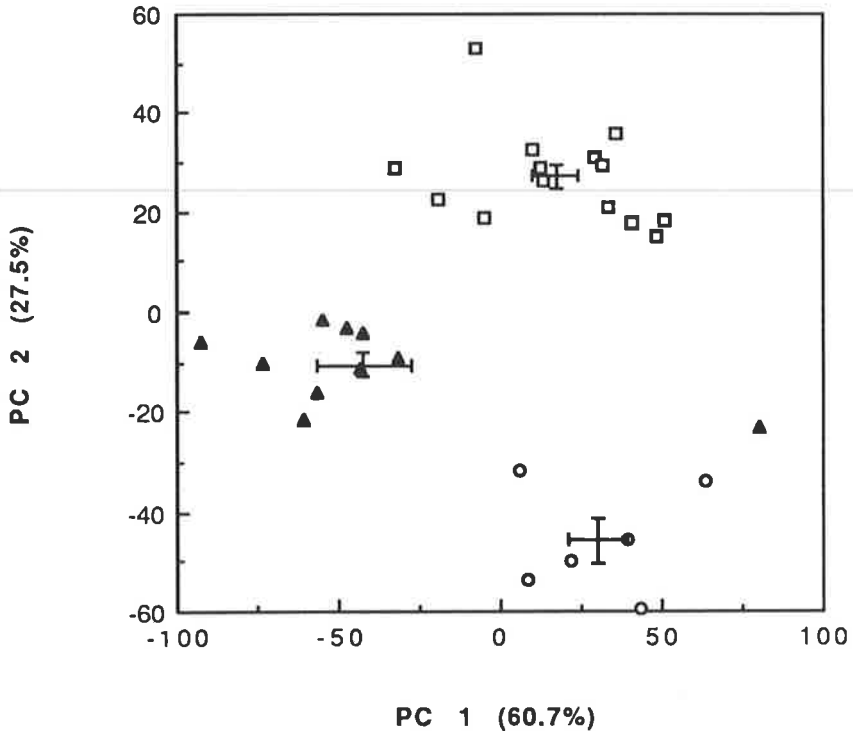
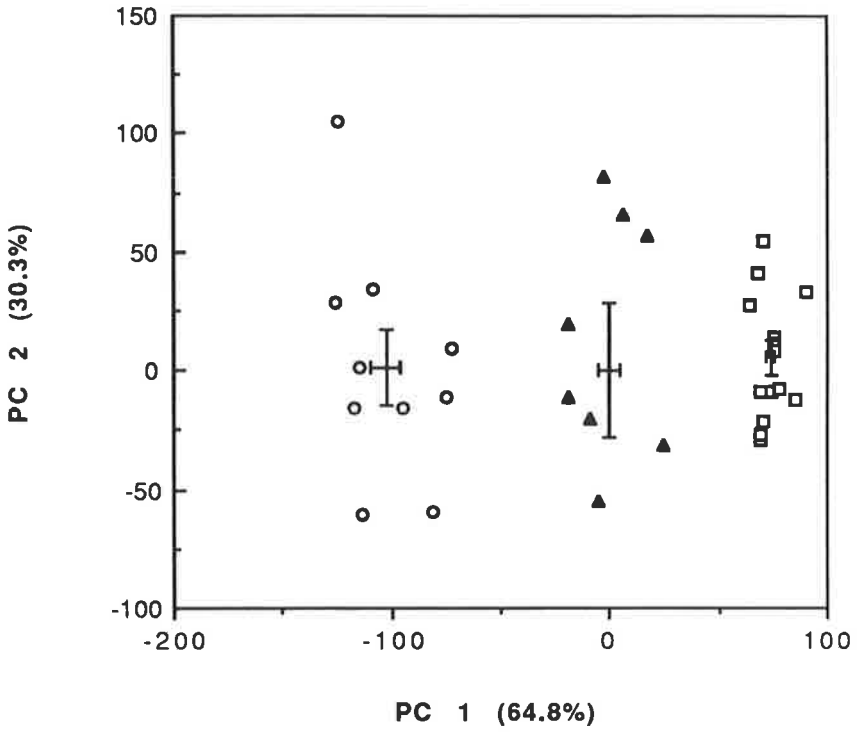

60
40
20
0
-20
-40
-60
-100
-50
0
50
100
PC 2 (27.5%)
PC 1 (60.7%)

Figure 7.10 Principle component ordination based on 15 morphological measurements of five month old *Eucalyptus* seedlings. Means and standard errors are shown for each species and putative hybrid genotype.

7.10A *E. cladocalyx* (□), *E. spathulata* (○), *E. cladocalyx* x *spathulata* (▲).

7.10B *E. cladocalyx* (□), *E. platypus* (○), *E. cladocalyx* x *platypus* (▲).

7.10A


150
100
50
0
-50
-100
-200
-100
0
100
PC 2 (30.3%)
PC 1 (64.8%)

7.10B

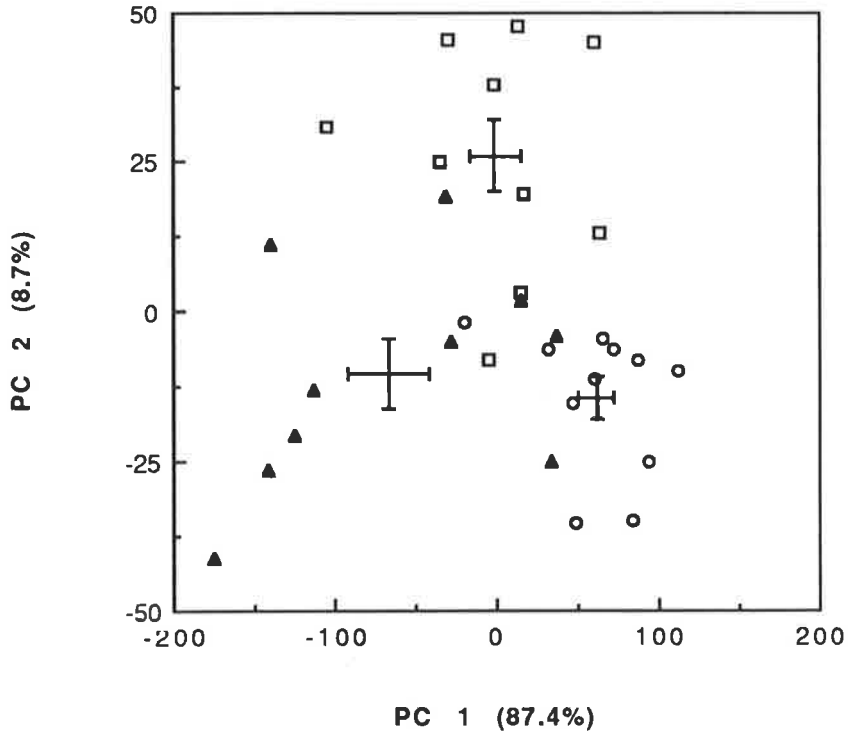
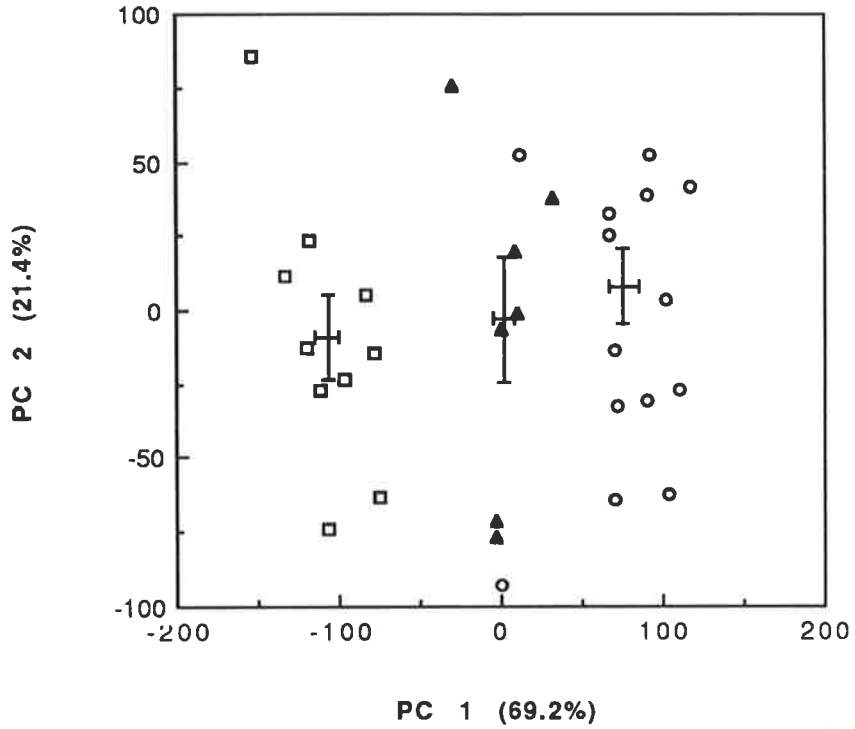

50
25
0
-25
-50
-200
-100
0
100
200
PC 2 (8.7%)
PC 1 (87.4%)

Figure 7.11 Principle component ordination based on 15 morphological measurements of five month old *Eucalyptus* seedlings. Means and standard errors are shown for each species and putative hybrid genotype.

7.11A *E. cladocalyx* (□), *E. viridis* (○), *E. cladocalyx* x *viridis* (▲).

7.11B *E. sargentii* (□), *E. sargentii* spontaneous hybrid (▲).

7.11A


100
50
0
-50
-100
-200
-100
0
100
200
PC 2 (21.4%)
PC 1 (69.2%)

7.11B

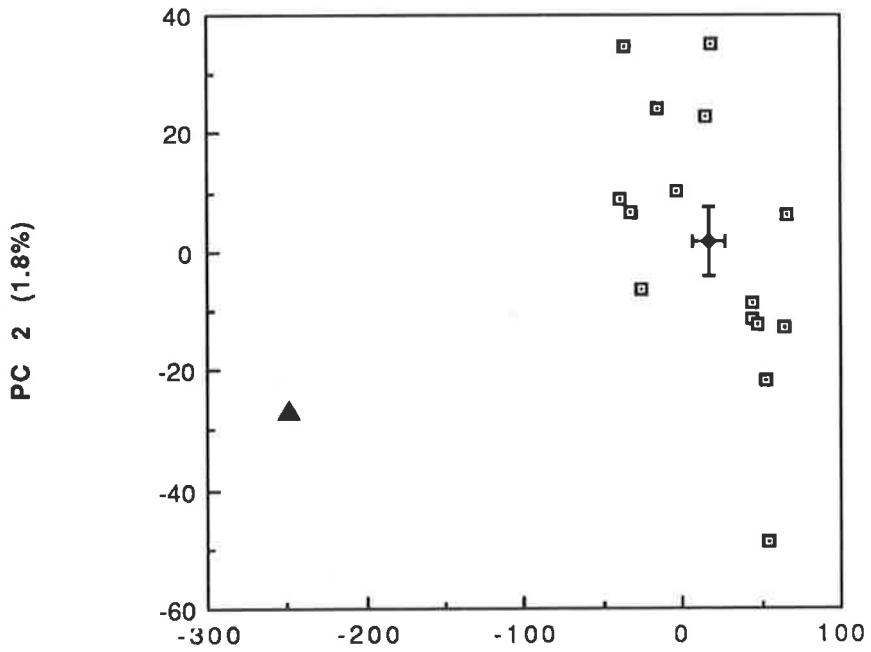
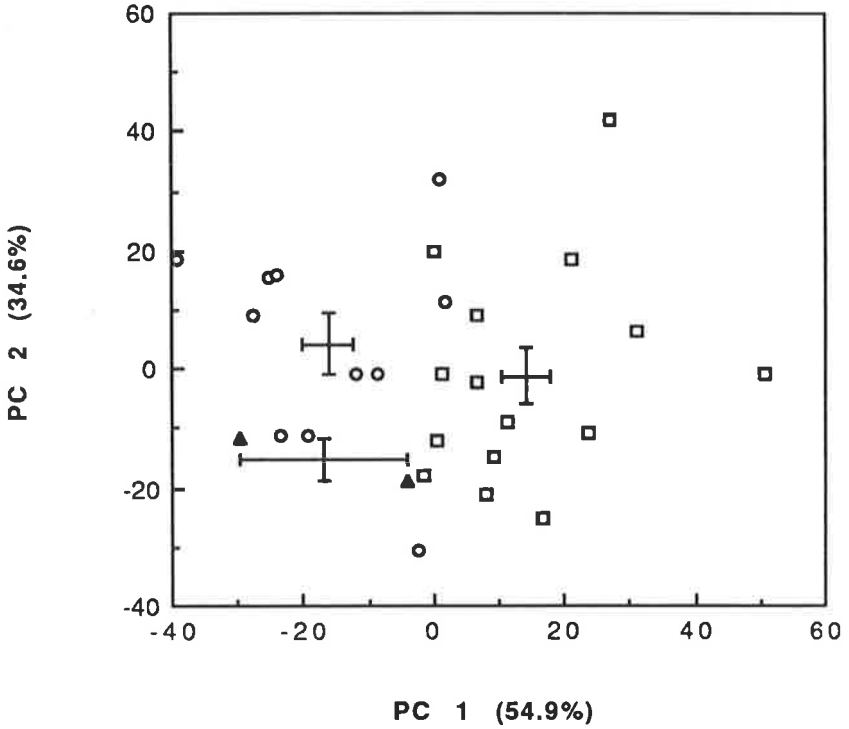

40
20
0
-20
-40
-60
-300
-200
-100
0
100
PC 2 (1.8%)
PC 1 (86.2%)

Figure 7.12 Principle component ordination based on 15 morphological measurements of five month old *Eucalyptus* seedlings. Means and standard errors are shown for each species and putative hybrid genotype.

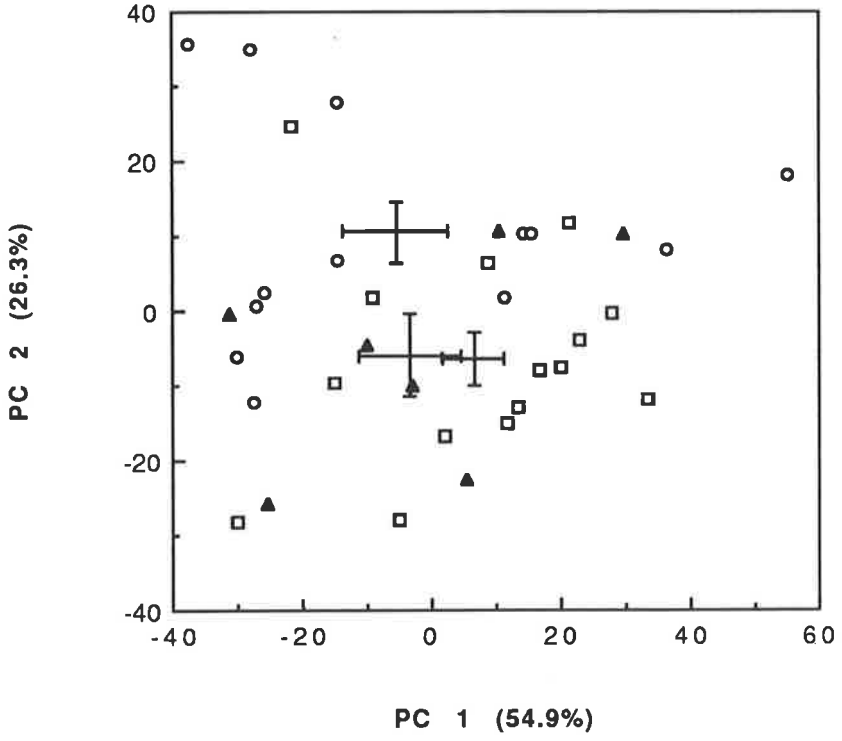
7.12A *E. leptophylla* (□), *E. albida* (○), *E. leptophylla* x *albida* (▲).

7.12B *E. leptophylla* (□), *E. yalatensis* (○), *E. leptophylla* x *yalatensis* (▲).

7.12A


60
40
20
0
-20
-40
-40
-20
0
20
40
60
PC 2 (34.6%)
PC 1 (54.9%)

7.12B


40
20
0
-20
-40
-40
-20
0
20
40
60
PC 2 (26.3%)
PC 1 (54.9%)

E. cladocalyx x *platypus* (Fig. 7.10B) hybrids show differences from both parents along the PC1 axis providing evidence of creation of new traits through recombination of parental characterisitics. The confirmation of hybridity through intermediate morphology and the complete absence of capsule set in the controls shows the reliability of the technique of controlled pollination employed in this study for the production of interspecific hybrids without contamination from intraspecific pollen.

7.4 DISCUSSION

This study has demonstrated that interspecific hybrids can be produced by controlled pollination of members of the section *Bisectaria*, including intersectional hybrids with the section *Adnataria*. Griffin *et al.*, (1988) reported only 25 hybrid combinations within the section *Bisectaria*, of which, three were intraseries hybrids, 17 interseries and five intersectional hybrids. Intersectional hybrids between sections *Bisectaria* and section *Adnataria* are rare, and there is only one naturally occurring combination reported, despite 423 co-occurring species pairs of the lowest geographical rank (Griffin *et al.*, 1988). The combination of *E. gracilis* (*Bisectaria*) x *E. largiflorens* (*Adnataria*) was reported from herbarium specimens intermediate in morphology between the two putative parent species, but no further progeny testing or controlled crossing has been performed to verify their hybrid origin. Beardsell *et al.*, (1979) reported success with manipulated crosses between *E. caesia* as a pollen parent and *E. leucoxylon* and *E. sideroxylon* (section *Adnataria*), but a lack of success when *E. caesia* was used as a female parent. They attributed this to the low pollen viability in *E. leucoxylon* and *E. sideroxylon*, although other factors could have been involved.

Interspecific hybrid seed set in *Eucalyptus* can be predicted to some extent by observations of ovule penetrations. Differences in observed levels of ovule penetration and resultant seed set may be accounted for by between flower variability in pollen or ovule fertility, by failure of gamete union in the ovule or by post-zygotic abortion of the developing embryos. As this reduction is seen in both cross intraspecific and interspecific pollinations, and as the remains of aborted seeds are present in the capsules much of this reduction may be due to post-zygotic abortion. Results from microscopical observation of

ovule penetrations can be obtained soon after flowering, thus gaining a year over seed set data in the prediction of success of new combinations.

Female parent species show differences in the tendency for post-zygotic abortion of both intraspecific cross fertilised ovules and ovules fertilised by interspecific pollen. *E. spathulata* which was shown in chapter four to possess pre-fertilisation selection mechanisms for discriminating against self pollen tubes in the ovary, shows little post-fertilisation abortion of fertilised ovules in intraspecific cross pollinations. *E. cladocalyx* and *E. leptophylla* however showed higher ovule penetrations than mature seed set. Drake (1975) identified aborted seeds among the contents of mature capsules of nine eucalypt taxa. Two general forms of aborted seeds containing some embryonic tissue were recognised, although there was a continuous gradation between morphological types. Some of these aborted seeds were capable of germination but few produced normal seedlings. The continuous array of forms of aborted seeds with varying amounts of embryo tissue and displaying differing levels of external development suggests a continuous series of abortions during capsule development. Fertilised ovules aborted soon after fertilisation would go undetected in capsule contents.

The presence of high levels of barren capsules in *E. cladocalyx* and in some interspecific crosses with *E. leptophylla* suggest that fertilised ovules are aborted after capsule development has been initiated. No evidence for parthenocarpy has been found in *Eucalyptus* and developing seeds may be aborted for a number of reasons, including competition for limited space and resources and may depend on the fitness of embryo genotypes. The mechanisms determining which embryos continue development remain unclear. In *E. regnans* Sedgley *et al.*, (1989) found no relationship between ovule placement on the placenta and the likelihood of maturing into full seed. Evidence for selective abortion of both fruits and ovules on the basis of embryo genotype and number of fertilised ovules per flower has been found in herbaceous plants (Casper, 1988; Stephenson and Winsor, 1986) with natural ovule abortion leading to an increase in remaining seed and seedling fitness.

The sample sizes used in the pollen-pistil interaction studies in chapter six and in the seed set experiments may be too low to detect very low rates of ovule penetration or

hybrid seed set, but have relevance for practically based improvement programmes or seed set in natural situations where very low levels of hybrid seed set are commercially or ecologically unimportant. Large differences between years, with some maternal trees showing poor ovule penetration in some crosses, while showing much improved seed set the following year in the same combinations, exemplifies the variable nature of the reproductive interactions. Between year variations may be due to climatic conditions, possibly affecting pollen or pistil viability and factors causing variations in fruit crop densities may also play a role. Within this seasonal variability lies the potential for producing low numbers of hybrids of relatively incongruous combinations. The intersubgeneric cross *E. spathulata* x *E. maculata* was successful in ovule penetration in female tree 2 in the 1988-89 pollinations and set some capsules and seed in 1989-90 suggesting a unique genotype interaction not shared by the other two female trees. While this combination may never produce viable seedlings due to the evolutionary distance between the parental species, other more closely related combinations may be produced by performing a large number of pollinations over several flowering seasons.

The poor rates of development of penetrated ovules to mature seed in some crosses allows for the possibility of embryo rescue, a technique used in other genera for the production of hybrid plants where hybrid embryos are aborted during development (Raghavan, 1977). Vamaguchi *et al.*, (1987) used this technique to produce plants of the hybrid *Camelia japonica* x *C. crysantha*. However some combinations proved inviable at the early seedling stage. These combinations were intersectional suggesting genetic disharmony between the parental genotypes (Stebbins, 1958). The viability of the seedling may be a limiting factor to production of wide hybrids via embryo rescue. Pryor (1957) noticed that wide hybrids in *Eucalyptus* displayed a lack of vigour and it is possible that disharmonies in chromosome number may be responsible. The usual chromosome number for *Eucalyptus* is 2N=22 with some species 2N=24. Ruggeri (1961) reported *E. cladiocalyx* (sic) to have 2N=24. Evidence from interspecific hybridisation from this study casts doubt on this observation.

This study has confirmed and extended findings of chapter six that a number of interspecific combinations can be generated within the *Bisectaria*, but that wider crosses have an increasingly reduced chance of success.

8. GENERAL DISCUSSION

The maintenance of genetic diversity and long term viability of natural populations depends on gene flow via cross pollination and outbreeding. *Eucalyptus* species show a number of floral and ecological adaptations promoting outcrossing. Floral features such as protandry effectively prevent autogamous pollination (Griffin and Hand, 1979; Hodgson 1976a), and style elongation, which occurs in many species (Griffin, 1982), helps to separate male and female structures within flowers. The partial self incompatibility mechanism investigated in this study also helps to maintain high outcrossing rates by selecting against self pollinated progeny. The relative contributions of these mechanisms to maintaining outcrossing in natural situations remains unclear, but a major factor appears to be the partial self incompatibility mechanism and post-zygotic selection of outcrossed progeny at several stages of the life cycle. Preferential selection of outcrossed embryos was demonstrated in *E. regnans* by Griffin *et al.,* (1987) and post-zygotic selection for outcrossed embryos was indicated in *E. spathulata* , *E. cladocalyx* and *E. leptophylla* in this study. Phillips and Brown (1977) found an increase in mean heterozygosity through the life cycle of *E. pauciflora* from seed, seedling to adult trees, presumably due to selection for outcrossed or heterozygous individuals.

Eucalyptus species differ in genetic variability both within and between populations (Moran and Hopper, 1987), with more widespread species showing high levels of genetic variability within populations (Coates and Sokolowski, 1989), while localised species show higher interpopulational variation (Moran and Hopper, 1983; Sampson *et al.,* 1988). In situations where land clearance has led to the dissection of widespread species into small populations (Prober *et al.,* 1990), changes in population genetic structure can be expected in future generations. A knowledge of both population genetic variability and breeding system is vital for the prediction of the composition and quality of future seed crops, and thus for the formulation of optimum conservation strategies.

Outcrossing rates are of importance to producers of high quality seed for plantation establishment, and can provide an estimate of heterozygosity in seed crops, and

conversely expected losses of vigour due to inbreeding. Seed orchard design attempts to maximise the chances of outcrossing through the spatial arrangement of genotypes, but variables other than the degree of self incompatibility of a species may influence the amount of heterozygosity. Differences in flowering times may limit interbreeding between provenances, while more subtle differences in the timing of peak anthesis may be important in determining patterns of gene flow. Fripp *et al.*, (1986) showed differences in peak flowering, times of *E. regnans* trees in a seed orchard and consequent variation in allele frequencies in the pollen pool over time. In this situation where there is some overlap in flowering the first trees to flower will receive more cross pollen due to protandry and the receipt of pollen during the stigmatic receptive phase from later flowering individuals in the male phase.

This investigation has shown that ovule numbers are not limiting to seed set, but the capacity of flowers to set seed from late cross pollinations, after pre-emptive self pollinations early in the receptive phase, has not been determined. This ability depends on the capacity of the stigma and stylar transmitting tissue to support further pollen tube growth, and of the maternal parent to impose post-zygotic genotype selection in favour of late fertilised ovules.

As relatively few eucalypt species have been studied for breeding system variables there needs to be a greater body of information before correlations with life strategies can be made. Within the genus, species have distinct habitat preferences and adopt a variety of survival and recruitment strategies in response to environmental and ecological variables such as fire, disturbance or successional changes in vegetation. Some ecological traits have been correlated to taxonomic groups (Noble, 1989). Mallee species are known to recruit very infrequently requiring a combination of fire and good rainfall events to establish the conditions necessary for seedling establishment (Wellington and Noble, 1985a). The potential for seed storage in the canopy in mature fruits also varies between species (seed storage in the soil is minimal in Myrtaceous species) with some species retaining capsules for many years, while others shed seeds annually. Thus differences in the control of the breeding system may be linked to life strategy and taxonomy.

Research on the nature and control of breeding systems in *Eucalyptus* has centred on four points, the capacity to set self seed, the proportion of inbred seed in natural seed crops, the location and extent of the self incompatibility mechanism and the relative fitness of self and cross pollen in mixed pollinations. However there is a notable lack of information on aspects such as the role of the inflorescence (umbel) in embryo genotype selection and maternal resource allocation between flowers, comparative growth rates of self and cross pollen in the pistil, the genetic basis of self incompatibility, the effect of self pollen in mixed pollinations on total reproductive output, the effect of mixed pollinations on outcrossing in species that show some pre-fertilisation control of self incompatibility (eg *E. woodwardii*), and the contribution of late pollinations to seed set when preceded by earlier pollinations. As information is gathered on an increasing number of species we can collate a picture of the types of breeding systems, the degree of self compatibility and the mechanisms of genotype selection in the genus, and these can then be related to life history and recruitment strategies. To complete the picture, work on breeding systems and seed production must be seen in conjunction with research on the dynamics of recruitment and the genetic processes involved.

Effective pre-zygotic and post-zygotic interspecific isolation was seen to occur in the genus in this study. Previously little attention was paid to the reproductive interactions between species in maintaining species integrity, but research concentrated on isolation due to flowering time, ecological factors, hybrid fitness and F1 reproductive output (Drake, 1981a,b; Hopper *et al.*, 1978; Pryor, 1976; Rogers and Westman, 1979). The existence of physiological pre-fertilisation isolation mechanisms have implications for reproductive outputs and gene flow in situations where two or more co-occurring species flower synchronously, including, natural stands, mixed species plantations, or introductions of non-indigenous species near natural stands.

The success of interspecific and intergeneric pollen tube growth in the eucalypt stigma and style demonstrated here, coupled with pollen tube arrest either further down the style or in the ovary in most wide crosses, has implications for the reproductive fitness of flowers that receive interspecific or intergeneric pollen. If interspecific pollination precludes further intraspecific pollen tube growth and seed set by competition

for stylar and ovule resources then interspecific pollination will lower the reproductive output. Even in cases where viable hybrid seed is set, failure at the early seedling stage may lower the effective reproductive output of the maternal parent through wastage of reproductive units and allocation of resources to non-viable progeny. Due to the non-specific nature of eucalypt pollinating agents most open pollinations will contain mixtures of pollen. These factors could be a strong selective force for the evolution of separate flowering seasons for co-occurring species. The effect of mixed intraspecific and interspecific pollinations has not been determined and may lead to either a majority of seed being intraspecific through competitive superiority of intraspecific pollen, an increase in interspecific seed set through the mentor effect (Knox, 1972a), or be a reflection of the independent survival probabilities of both pollen types. Pryor (1976) noted that the frequency of interspecific hybrids is higher in disturbed natural stands than in pristine stands. Disruption of flowering patterns and pollinator behaviour may lead to a higher frequency of interspecific pollination and seed set, or disturbance may create new habitats suitable for the establishment of hybrid progeny previously excluded by niche complementarity. In stands disturbed by clearance or logging the reduction of intraspecific mating partners and a proportional increase in self pollen in the pollen pool may lead to an increase of fitness of interspecific pollen, leading to a higher proportion of hybrid seed in the crop. This possibility is supported by the results of this investigation which demonstrated the higher fitness of interspecific over intraspecific self pollen in some combinations.

This feature of higher fitness of close interspecific combinations compared to self pollen could have potential in mass production of F1 hybrid seed for use in tree improvement programmes. In mixed species seed orchards where individual trees of one species are exposed to the pollen pool of another compatible species most seed will be of hybrid origin. Seedlings could be screened at the early seedling stage for intraspecific contaminants, most likely to be self pollinated progeny. The use of self incompatible, or male sterile clones would further reduce intraspecific contamination. Techniques such as this would reduce labour and financial inputs and allow mass production of hybrid seed for commercial plantation establishment.

This study identified the pre-fertilisation limiting steps in interspecific pollen tube growth in *Eucalyptus* section *Bisectaria*. This information can now be used to design and test methods for improving hybrid seed set in interspecific crosses. Pryor and Willing (1974) claimed the application of hexane to eucalypt stigmas improved hybrid seed yield in a number of intersectional crosses with the section *Adnataria*. This treatment has been used successfully in plants with dry stigmas such as poplar (Whitecross and Willing, 1975) but as the stigma surface was not a barrier to interspecific or intergeneric pollen tube germination or growth in *Eucalyptus* no mechanism for the action of this treatment can be seen from the results of this study. Potts and Cauvin (1988) described treatments used in *E. gunnii* to promote interspecific hybridisation with *E. ovata*. The most successful treatment involved amputating the style and pollinating the cut stump, reducing the distance pollen tubes must grow to the ovules. This treatment has not succeeded in breaking the unilateral interspecific incompatibility between *E. globulus* and *E. nitens* (Gore *et al.*, 1990). Other manipulative techniques such as mentor pollination (Knox *et al.*, 1972) may be useful in improving hybrid seed set.

The results of this study have demonstrated that in *Eucalyptus* the partial self incompatibility mechanism which operates mainly post-zygotically and maintains a high level of outcrossing, is balanced by physiological interspecific isolating mechanisms in the pistil, and post-zygotic selection against wide hybridisation. These mechanisms act together to maintain both high levels of heterozygosity and define an upper limit to hybridisation (de Nettancourt, 1977), thus preserving genetic integrity and ensuring the maintenance of useful gene combinations that could be lost through wide hybridisation.

9. CONCLUSIONS:

This study has added to the understanding of the mechanisms controlling breeding systems and interspecific hybridisation in *Eucalyptus*, and illustrates the diversity of floral form and function in the genus. In particular it has been shown that,

- *Eucalyptus spathulata*, *E. cladocalyx* and *E. leptophylla* (subgenus *Symphyomyrtus* section *Bisectaria*) set low levels of seed upon self pollination, but individual trees differ in the degree of self compatibility.

- The partial self incompatibility mechanism in these species operates mainly through post-zygotic abortion of self fertilised ovules, with some pre-zygotic selection against self pollen tubes seen in the ovary of *E. spathulata.*

- The breeding system of *E. leucoxylon* (subgenus *Symphyomyrtus* section *Adnataria*) differs from most eucalypts due to gynodioecy and secondary pollen presentation on the upper style. While hermaphrodite trees can set low levels of autogamous seed in the absence of pollinators, both female and hermaphrodite trees show a high level of outcrossing in open pollinated seed crops.

- Physiological interspecific isolation mechanisms occur in the eucalypt pistil and generally prevent wide hybridisation. Success of the pollen-pistil interaction is related to the taxonomic distance between parental species, with closer crosses being more successful in interspecific pollen tube growth.

- Interspecific hybrids within the *Bisectaria* and between the sections *Bisectaria* and *Adnataria* can be produced by controlled pollination. Some intersectional crosses show hybrid breakdown at the early seedling stage.

The results from this study can be used to interpret ecological observations on gene flow within and between populations, to provide information for the formulation and implementation of breeding programmes for eucalypt improvement through outcrossing and interspecific hybridisation, and to contribute to the clarification of taxonomic relationships.

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APPENDIX: Ellis, M. F., Sedgley, M., and Gardner, J. A. 1991. Interspecific pollen-pistil interaction in *Eucalyptus* L'Hér.(Myrtaceae): The effect of taxonomic distance. *Annals of Botany* **68**, 185-94.

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