STUDIES ON FERTILITY AND CROSSABILITY OF SPECIES IN THE GENUS *LEUCADENDRON*

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Declaration

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

Summary

The exomorphology and size of *Leucadendron* pollen was examined using a scanning electron and light microscope respectively. Pollen was found to have a consistent triangular shape with three apertures. Pollen grain size however, show difference between species, sections and subsections on *Leucadendron.* Pollen of all species examined had a woven appearance like the intertwined threads of a fabric with orbicules present on the surface of *L. chamelaea, L. elimense* subsp. *elimense* and *L. galpinii.* Pollen viability was successfully assessed using a solidified agar medium containing 2g agar and 10g sucrose. Pollen germination for all species was found to be above 55% viability. A diallel layout of crosses has demonstrated conclusively that fecundity differs when crossing between species of the genus *Leucadendron.* Artificial hand pollination was applied successfully on *Leucadendron* and showed repeatedly that seed set following intraspecific crosses between the male and female inflorescence of the same species gave the same high rate of seed set as found in nature. However, seed numbers declined sharply when crossing between species of the section 'Leucadendron'. Seed set following crosses between species of different sections or sub-sections was the lowest and in most cross combinations there was no seed harvested or no seed germination. The diallel layout was useful in identifying incompatible species and for locating possible incompatibility barriers to interspecific seed development. The morphology of the stigma was examined with a scanning electron microscope. Stigma appearance of all species had a consistent round to oval shape, except for *L. rubrum,* which had an elongated shape. Stigma surfaces of all species were densely covered with a large number of unicellular papilar cells on the swollen base. The aniline blue staining technique, together with the fluorescent microscope technique was used to follow the growth of the pollen tube following

compatible and incompatible cross combinations. Pollen on the stigmas of compatible and incompatible species examined showed signs of germination. Pollen tubes grew between the papilla cells in all directions and only the most vigorous ones reached the upper part of the style. From the upper region of the style, yellow green tubes grew cohesively in the middle of the style towards the ovule. In compatible combinations a not more than 4 tubes reached the ovule region, but was difficult to observe when they entered the micropyle for fertilization. In incompatible species a large number of abnormalities occurred beyond the upper region of the style.

Opsomming

Die morfologie en grootte van *Leucadendron* stuifmeel is deur middel van 'n skandeerelektron – en ligmikroskoop bestudeer. Baie klein verskille in stuifmeel morfologie het voorgekom. Diverse verskille in stuifmeelgrootte het wel voorgekom tussen spesies, groepe en subgroepe van *Leucadendron*. Stuifmeelvorm was deurgaans driehoekig en die oppervlakte van die stuifmeelkorrel het die voorkoms van geweefde vesels gehad. Klein, bolvormige struktuurtjies was teen verskillende digthede oor die stuifmeeloppervlak van *L. chamelaea, L. elimense* subsp. *elimense* en *L. galpinii* versprei. Stuifmeelkiemkragtigheid is bepaal deur dit op soliede agar medium te ontkiem en was deurgaans bo 55% kiemkragtig. Onderlings dialleliese kruisings van *Leucadendron* spesies het variasie in saad set getoon. Handbestuiwing is suksesvol uitgevoer en saadset in intraspesie kruisings hoog en soortgelyk aan natuurlike bestuiwing. Saadset en saad ontwikkeling het drasties verswak toe verder vewante spesies as ouers gebruik. As gevolg van hul ondeurdringbare saadhuid is neutagtige sade gewoonlik moeiliker ontkiembaar. Die diallel uitleg was ook nuttig om verenigbare en onverenigbare kruisingskombinasies te identifiseer en om onverenigbaarheidskanse op te spoor. 'n Skandeerelektronmiskoop is gebruik om die morfologie van die stigma te bestudeer. Stigmas was deurgaans rond tot ovaalvormig, behalwe die van *L. rubrum* wat 'n verlengde voorkoms gehad het. Die stigma bestaan uit 'n groot aantal eensellige papilla, wat dig teen mekaar gepak is op 'n geswolle basis. Aniline-blou fluoresserende kleurstof en 'n fluoressensie mikroskoop is gebruik om die pad van die stuifmeelbuis in verenigbare en onverenigbare kruisingskombinasies in *Leucadendron* te volg. Stuifmeelontkieming het in alle kruisingskombinasies geskied. Stuifmeelbuise het in alle rigtings tussen die papilla

gegroei en slegs die mees kiemkragtige stuifmeelbuise het die boonste deel van die styl bereik. In die styl het die buise dig teen mekaar gegroei en was dit moeilik telbaar. 'n Maksimum van vier buise het die vrugbeginsel bereik, maar dit was moeilik om verder te volg nadat hulle die poortjie bereik het. In onverenigbare kruisingskombinasies het stuifmeelbuise abnormale groeipatrone in die boonste gedeelte van die styl getoon.

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Thesis Outline

This thesis puts together some facets of research on hybridization of species of the genus *Leucadendron* as potential sources of commercial cut-flower cultivars undertaken since 1997 at the ARC Fynbos Unit, Elsenburg, Western Cape. Results are presented in three chapters in scientific article format (Chapters 2, 3 and 4), appropriately cross-referenced with respect to materials and methods, but each with its own list of publication references relating to the particular chapter topic. This leads inevitably to some duplication but was preferred to a single alphabetical list covering the entire thesis. Research chapters are preceded by an introductory review and full bibliography to date (Chapter 1). The latter includes publications not referred to in the text and is intended to provide a permanent library data base on the subject. Chapter 5 (Conclusions) is intended to summarize the current state of knowledge on the subject and to indicate avenues for future research. Relevant tables and figures follow the publication reference lists of each chapter separately.

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Literature review and Bibliography

The Fynbos Flower Industry

The South African fynbos flower industry has its origin in the harvesting of blooms from natural resources within the Cape Floral Kingdom (CFK) or *Flora Capensis*. This is the smallest plant kingdom known but reputed to be a major resource of natural floristic diversity (Cowling *et al.*, 1994). Indigenous flora holds an estimated 0.44% share of the world flower market (Coetzee *et al.*, 1999), with an annual foreign earning of R94.91 million (Sappex News, 2002a). Sales are valued at R25 million (Kaiser Associates, 2000; Sappex News, 2002a). Annual growth projections are 5-10% (Sappex News, 2002a). The industry employs some 25 000 workers and has been a major source of income in rural communities of the Western Cape (Sappex News, 2002a).

Commercially utilized flowering families of fynbos are Bruniaceae, Asteraceae, Cyperaceae, Ericaceae, Fabaceae, Haemodoraceae, Rhamnaceae, Retziaceae, Rubiaceae and Proteaceae (Littlejohn and Robyn, 2002). The most important flowering genera are members of Proteaceae, endemic to the southern tip of Africa (Coetzee and Littlejohn, 2001), viz., *Leucospermum* (Criley, 1998) and *Leucadendron*. Genera from Australia are *Grevillea* (Leonhardt and Criley, 1999) and *Banksia* (Sedgley, 1998). Proteaceae used to a lesser extent are the genera *Faurea, Serruria, Mimetes, Aulax, Paranomus, Orothamnus, Spatalla, Diastella, Sorocephalus, Telopea* and *Isopogon* (Coetzee and Littlejohn, 2001).

45% of fynbos woody plant export products are cultivated in their countries of origin. Zimbabwe contributes 30%, Australia 13%, USA 5%, Israel 2% and New

Zealand, Chile, Spain, France and Portugal in total 5% (Coetzee *et al.*, 1999; IPA, 2002). These countries contribute to deliveries to the traditional market, Europe, and recently also to the United States and Japan.

Flower harvesting currently depends primarily on some 89 000 ha of natural resources, mainly in mountainous areas, 2795 ha extensive broadcast seed sown blocks and 950 ha cultivated in monoculture blocks (Coetzee *et al.*, 1999). As a successfully commercialized ornamental crop, Proteaceae has ranked the SA Fynbos industry as a small but important component of the overall agricultural sector in the Western Cape (Coetzee *et al.*, 1999). Although the Western Cape is known as the major production area, increasing quantities are also cultivated and delivered by the Eastern-Cape, Gauteng and the highlands of Kwazulu–Natal (Oberholster, 2003). The international industry also includes Australia, New Zealand, Zimbabwe, Chile, USA, Portugal, Israel, France and Hawaii (Coetzee and Littlejohn, 2001; Leonhardt and Criley, 1999).

Leucadendron is becoming an important product for Dutch producers (Sappex News, 2002b). As it produces the most adaptive, vigorous and easily propagated plants, with long lasting cut stems, species are commercially used as cut flowers and foliage (Coetzee and Middelmann, 1997), landscape plants, potted plants (Brits *et al.*, 1992) and as dried products (Coetzee and Middelmann, 1997). *Leucadendron* offers a wide range of colourful foliage, green, red, orange, gold, silver, bright yellow and soft yellow. In interspecific hybrids red and yellow are combined to display a bicolour appearance during flowering, such as the cultivar 'Disco Date'. The male pom-poms are sought after for their white-silver, red or yellow appearance just before pollen is shed. The extended

vase life and the red, red-orange or brown appearance of the female cones a few weeks after flowering make them more in demand than the male inflorescence.

According to 2001 export figures, thirty million stems of *Leucadendron* foliage were sold via the Dutch auction, a 10% increase above the previous year (Sappex News, 2002b). This figure exceeded the 2.5 million protea stems sold via the auction. Although South Africa has the advantage of being home to all species of *Leucadendron*, Israel, who flooded the export market with cultivar *Leucadendron* 'Safari Sunset', delivered the bulk (80%) of the consignment. Supplementing the variety of shapes, sizes and colours are SA, Zimbabwe, Kenya and the newcomer Portugal (Sappex News, 2002b). 'Safari Sunset' was one of the first *Leucadendron* cultivars, developed by New Zealand where the aesthetic and decorative value of this flowering genus was first realized, long before South Africa (Bell, 1988). Today, *Leucadendron* is ranked the second largest commercial genus but least studied of the family Proteaceae. Moreover, as in *Protea,* the level of species richness in *Leucadendron* is greater than in all other members (Rebelo, 1995). The product is becoming increasingly important on the Dutch auction for mixed bouquet production due to its long vase life and exotic appearance.

The most widely used cultivars flower during the late winter and early spring (Littlejohn, 1997). As a filler product it is used together with traditional flowers such as roses and lilies between larger blooms to add an exotic look to mixed bouquets. Larger blooms from cultivated stands are useful as focus points in spectacular indigenous floral arrangements. Wild harvested female cones are utilized by the dried flower industry in dried bouquets and decoration displays.

Leucadendron stems for fresh flower bouquets are predominantly harvested from the wild or from broadcast seed sown blocks (Coetzee and Middelmann, 1997). Uniform stems exported as single stem bunches are harvested from organized cultivated blocks and contribute 40% of the cut flowers from South Africa (Littlejohn and Robyn, 2000). Cultivars are usually superior species selections or interspecific hybrids that outperform their wild relatives in vigor, yield and aesthetic appeal (Van den Berg and Brits, 1995). Some of the cultivars are probably chance hybrids discovered in seedling blocks by farmers, but many more are the result of controlled pollinations. Interspecific hybrids selected from seedling populations resulted in the release of a number of cultivars from Australia and New Zealand such as the cultivars 'Safari Sunset', 'Silvan Red', 'Long Tom', 'Inca Gold' and 'Pisa' (Matthews and Carter, 1983). Other hybrid cultivars such as 'Chameleon' (*L. salignum* X *L. eucalyptifolium*) and 'Rosette' (*L. laureolum* X *L. elimense*) (Littlejohn *et al.*, 1998) were released in South Africa.

Although past records of species and hybrids of *Leucadendron* display a wide range of floricultural variation as indicated above, breeding research has been largely limited to sporadic observations and selections based on a "marketable product" approach (Littlejohn *et al.*, 1995), motivated by interests and opportunities in a growing industry rather than by the need to establish an organised ongoing breeding programme, as was the primary motive of the present study at its inception.

The Genus *Leucadendron*, Family Proteaceae

Utilization of plants of the family Proteaceae dates back to the 1600s when trees of the wild almond, *Brabejum stellatifolium* were grown densely as a barrier against cattle

thieves (Vogts, 1989). Proteaceae is reportedly a very useful source of wood for fuel and timber, wild almond seed for food, bark for tanning leather and roots and leaves for treating diarrhea (Rourke, 1980). The most important Proteaceae species utilized to achieve international status as a gourmet dessert nut is the native food plant *Macadamia integrifolia* from Australia. More recent floricultural developments have been summarized by Littlejohn *et al.* (1995) referred to in the foregoing review of the current national and international fynbos industry.

Geographical distribution

Proteaceae is one of the most prominent of the Southern hemisphere flowering plant families. The family has origins dating some 140 million years ago before the break-up of the supercontinent Gwondwana (Johnson and Briggs, 1975). Before the break-up of Gwondwana the family had split into three subfamilies Proteoideae, Persoonoideae and the Grevilliodeae. Proteoideae occur mainly on the southern tip of Africa but also in Australia and New Zealand, whereas the Grevilliodeae are predominantly found in Australia, South America and southwestern Pacific Islands, with only one species found in Africa (Venkato Rao, 1971).

The majority of Proteaceae endemic to Southern Africa are geographically distributed between the towns Nieuwoudtville in the northwest and and Grahamstown in the east, an area botanically classified as the Cape Floral Kingdom (CFK) (Rebelo, 1995). More than two-thirds of the species are confined to the mountainous sandstone area from Cape Alghulas to Ceres, with some species growing in specific mountain ranges. *Leucadendron* comprises 91 different taxa, (Table 1) (Rebelo, 1995; Williams 1972) with some confined to specific habitats, showing no species variation, and others fairly widely

spread over the CFK and displaying considerable species diversity. (Williams, 1972; Rebelo, 1995). Three of the taxa extend the habitat range into Kwazulu-Natal, with one of the new discoveries found in Pondoland (Van Wyk, 1990).

Growth Habit

Proteaceae are perennial, evergreen, flowering and always woody shrubs (Rebelo, 1995). Growth habit is variable and ranges from upright trees, such as the riverside *Brabejum stellatifolium* and *Leucadendron argenteum*, to vertical spreading and ground hugging shrubs, such as *Protea, Leucospermum* and *Leucadendron* are predominantly found in Southern Africa (Rebelo, 1995; Williams, 1972). Flowering stems are upright or drooping and vary in length and diameter. The stems are packed with long-lived leathery leaves, which withstands the windy hot and dry summers of the Western Cape (Rebelo, 1995). The sclerophyllous leaves help to prevent water loss, and the lignified tissue stops them from collapsing when water is scarce. With these drought resistant qualities plants can continue photosynthesis long after ordinary leaves have wilted (Rebelo, 1995).

Fire has been found to be the stimulus for reproduction and regeneration of fynbos plants (Cowling and Richardson, 1995) and seems to have assisted in the evolution of reproductive characteristics of many fynbos plants (Bond and Slingsby, 1983; Bond, 1985). Some Proteaceae species have lignotubers at the basal part of the stem which facilitate survival in these frequent fires (Williams, 1972; Rebelo, 1995). The lignotuber has the ability to resprout from dormant vegetative buds in the basal plant stems, especially when the upper stems and leaves have died or been killed, e.g by fire.

As protea seedlings age they develop proteoid roots on their lateral roots at the soil surface up to 10cm in length. The cluster root system appears to be best developed

under a canopy of old plants. These multicellular rootlets appear to have a vital role in the nutrient and water uptake by the plant (Lamont, 1986).

Inflorescence

The uniqueness of the Proteaceae floral structure lies in the unusual display of flowers. Each of the thirteen different genera of the Proteaceae can be identified by the manner in which the flowers are aggregated in the flower head. The large number of small florets, neatly packed in a flower head, is botanically termed an inflorescence (Rebelo, 1995). The florets can be arranged in spikes, racemes, panicles or capitula, with the latter being the most common arrangement in species with floricultural potential.

Each inflorescence is comprised of a dense cluster of spirally arranged florets, born on a central woody axis. Involucral bracts, which encircle the woody core can either be very conspicuous, as in *Protea*, or inconspicuously reduced as in *Leucospermum,* or variable inconspicuousness as in *Leucadendron* and *Banksia* (Pretorius, 1986; Rebelo, 1995). In *Protea*, the colourful conspicious papery involucral bracts are easily confused with petals (Rebelo, 1995).

All genera of Proteaceae are hermaphroditic excepting the dioecious genera *Aulax* and *Leucadendron,* which carry either terminal male or female capitulums on different plants (Collins and Rebelo, 1987). The seed head of *Leucadendron* is a woody cone packed with spirally arranged floral bracts (Collins and Rebelo, 1987; Rebelo, 1995).

What strike the eye in *Leucadendron* are the upper or involucral leaf structures and colours. The basic leaf colour is green. During the growth phase the normal leaf colour is either green or red, as in *L. salignum* and *L. glaberrimum* (Williams, 1972). In *L. salignum* there are some individual plants with colourful leaves which may occur

among plants with green or yellow leaves and all possible intermediates (Williams, 1972). A colour pigment commonly found in flowers and fruit that gives the deep red tinge is anthocyanins e.g red apple skin. This change to red leaf colour is caused by the addition of sugars to an anthocyanin precursor. The bright red is largely influenced by the accumulation of anthocyanins on bright, cool days. The aesthetic floristic appeal of the inflorescence arises when the leaves encircling the inflorescence convert into bright yellow during flowering time. Other colours occurring during flowering time are overlays to yellow. The leaves of the silver tree, *L.argenteum* are silver. Silver is the result of waxiness and hairiness of the leaves. Basic colour change from green to yellow to green can be influenced by many factors such as light intensity, temperature and the nutritional status of the plant.

The floral bracts of the female inflorescence enlarge into a condensed woody cone. The male inflorescence, however, encompasses a large number of unprotected florets (in *L. globosum* 585 were found in one case) subtended by inconspicuous papery floral bracts (Williams, 1972). The showy male 'pom-poms' are only displayed during a short flowering period of about two weeks after which they wilt and turn black (Littlejohn, 1997). Distinctive of *Leucadendron* is the enlarged and showy upper leaves (involucral leaves) that shield the inflorescence and convert into bright yellow during flowering time. The onset of flowering has been found to be generally preceded by a single colour change during the year, excepting *L. salignum* X *L. discolor* hybrid clones, which undergo several, colour changes during the year (Littlejohn, 1997).

The individual proteaceous flower

The individual florets of Proteaceae are tubular or gullet-shaped (Rourke, 1980; Vogts, 1989). Instead of having distinctive sepals and petals, Proteaceae flowers have a set of four fused segments, called the perianth (Rebelo, 1995). The development of mature inflorescences generally follows a consistent pattern within each group with flowers closest to the periphery opening first. The only exceptions are some species of *Vexatorella, Serruria* and *Sorocephalus* in which flowers open in the reverse order (Vogts, 1989; Collins and Rebelo, 1987).

Hermaphroditic genera *Protea, Leucospermum, Banksia* and *Grevillea* (Collins and Rebelo, 1987) have pollen and seeds borne on the same flower. Pollen grain maturation occurs in the anthers, which are protected inside the four fused perianth segments. Powdery or sticky yellow pollen is deposited just prior to anthesis on the specialized region of the style, the pollen presenter (Rebelo, 1995). At flowering time the perianth segments reflex, separate and curl back to expose the pollen on the pollen presenter. At this time the stigma is not receptive for pollen, a phenomenon known as protandry (Vogts, 1971). Stigmas of *Protea, Leucospermum, Banksia* and *Grevillea* have a smooth appearance with a distinctive stigmatic groove where pollen tubes enter the pistil (Van der Walt and Littlejohn, 1996a; Fuss and Sedgley, 1990)

The genera *Leucadendron* and *Aulax* differ from all other Proteaceae in that they are dioecious. The male and female flowers are structured with the same number and arrangement of floral parts. Female flowers of *Leucadendron* appear in an acropetal sequence and usually open upwards in approximately seven consecutive days until all are displayed. A few days before anthesis, short, yellow stalk like organs protrude between

the bracts of the cone. These are each a flower and the carpel is described as having a simple pistil with a unilocular ovule, enclosed in a tube of four two-lobed perianth segments (Williams, 1972; Rebelo, 1995). At flowering time the yellow perianth segments reflex and display a microscopically small stigmatic area receptive to pollen. Each male flower has the same basic structure as the female flower within species, with a reduction of non-functional parts (Williams, 1972). Each functional style ends in a modified pollen-presenter covered with four perianth segments (Williams, 1972). As in the hermaphroditic flowers, a two-thecous anther is attached to the inside of the perianth from where the mature pollen is deposited on the pollen presenter.

Seed morphology, storage and dispersal

The so-called "seeds" of proteas are all, botanically speaking, fruits. The fruit (hereafter referred to as seed) is a one-seeded achene. The embryo is surrounded by two inner seed coat layers, one of which is woody, and an outer membranous covering (pericarp) (Jordaan, 1945). Within Proteaceae two different seed types occur, namely nutlike achenes and winged or hairy achenes. Proteaceae has special adaptations for storage and dispersal of seeds in the fire-prone and nutrient-poor environment of the fynbos vegetation (Rebelo and Rourke, 1986).

Hard-shelled nutlets, often called the *Leucospermum* fruit, also occur in the *Serruria, Spatalla, Sorocephalus, Orothamnus, Diastella, Vexatorella, Paranomus, Mimetes*, and in 38 species and three subspecies of *Leucadendron* (Vogts, 1989; Bond and Slingsby, 1983; Rebelo and Rourke, 1986). The majority of nutlike seeds are dispersed annually after the flowering season. With the exception of the cone bushes, all Proteaceae producing nutlets are myrmecochorous, i.e., they are dispersed and hoarded

by ants in nests (Bond and Slingsby, 1983) in the immediate vicinity of parental plants. Nutlets of *Leucadendron* have thick, solid or rounded seeds, which are heavy and wingless. Those of *Leucospermum* species are greyish white, smooth, hard and shiny, whereas *Serruria* produce oblong, hard nutlets covered with short matted, woolly hairs. Within the species of *Leucadendron* subsections 'Arid' and 'Sun' Conebushes, the hard-shelled achenes produce large, edible and rounded 'cached' fruit. They are released over a short period of two months after flowering and predated by rodents (Rebelo and Rourke, 1986). All large hard-shelled nutlets are triggered into germination by the same external stimuli as the other Proteaceae nut-like species.

Flat seeds, are equipped with hairs or wings, like parachutes to serve as buoyancy devices in high winds or air currents (Rebelo, 1995; Midgley, 1998). Seeds produced by serotinous species are maintained between the hygroscopic bracts of the seed head for many years. When moisture supply to the flower head terminates due to fire, drought or heat, the floral bracts dry, reflex and release the achenes. Germination then commences rapidly onto the soil surface (Brits, 1980) in a period of about 8 weeks after the winter rains (Van Staden, 1966; Mitchell *et al.*, 1985).

Myrmecochory and serotiny are unusual storage strategies in world vegetation (Bond, 1985). Both assist in the building of seed banks and in the reduction of seed predation (Bond, 1985). *Leucadendron* is the only genus producing both seed types and seed morphology has therefore been useful in taxonomic classification (Rebelo, 1995). In a comprehensive taxonomic treatise, Williams (1972) classified all flat winged species into a Section 'Alatosperma', and the other species producing nutlets are variable between both a Sections 'Leucadendron' and 'Alatosperma'.

Flowering time and Pollination

The natural flowering time of the Proteaceae in the CFK is between autumn and early summer (Vogts, 1971). Flowering periods of different species and sub-species is staggered with no one species flowering throughout the season. Great variation exists in the length of flowering time and the month or months in which they flower (Rebelo, 1995; Littlejohn, 1997). Flowering times of *Leucadendron* are spread over the winter period of the Southern hemisphere from June to September (Littlejohn, 1997). Flowering times of clones of the hybrid *L. salignum* X *L. discolor*, *L. laureolum* and clones of the hybrid *L. salignum* X *L. laureolum* are similar (Littlejohn, 1997).

In most Proteaceae the reproductive organs of male flowers in the compound flower head mature before the reproductive organs of female flowers. Since stigma receptivity follows anthesis in most of the Proteaceae (Collins and Spice, 1986, Sedgley *et al.,* 1985, Fuss and Sedgley, 1991, Brits and Van den Berg, 1990), the mode of pollination of the hermaphrodite species and, obviously, all dioecious species, favours cross-pollination. However, in some *Protea* and *Leucospermum* species self-pollination also occurs (Wright *et al.,* 1991). Pollen is deposited prior to anthesis on the specialized pollen presenter and is collected by flower visitors seeking nutrient-rich nectar prior to stigma receptivity (Johnson and Briggs, 1975; Fuss and Sedgley, 1990). Pollen agents in the CFK have been found to be insects, rodents, birds and beetles (Rebelo, 1995). While larger scarabaied beetles are known to pollinate *Protea*, beetle pollen-vectors in *Leucadendron* are smaller, belonging to the families Nitidulidae, Curculionidae and Alticidae (Hattingh and Gilliomee, 1989). In his revision of *Leucadendron*, Williams (1972) estimated that 89% of species are entomophilous, 6.6% anemophillous, with

species *L. salicifolium*, possibly in transit between insect and wind-pollination, and 3.3% extinct.

There are only ten male species of *Leucadendron* from which pollen is scattered in large quantities into the air and transported by the wind (Rebelo 1995). These anemophilous species are characterized by (i) a reduction or absence of hypogynous scales suppression of nectar secretion, (ii) a larger stigmatic surface, (iii) pollen transported by wind and not adhering to the pollen presenter and (iv) absence of a conspicuous coloured involucre (Williams, 1972). Similar to other dioecious genera, *Leucadendron* is regularly visited by small, unspecialised beetles. The pale inconspicuous, and often smaller flowers, also show similarities to other tropical dioecious species studied by Bawa and Opler (1987). Pollen transfer in *Leucadendron* via pollinators appears to be controlled by a combination of early flowering, conspicuousness, abundance of the male inflorescence, and a high protein content of the pollen.

Plant regeneration

Typical of woody plants, Proteaceae is slow growing and has to complete a vegetative regeneration phase of 3-4 years to produce their first flowers, after which flowering is annual (Brits, 1992). Natural regeneration in Proteaceae is from vegetative buds situated on the lignotuber of some species of *Protea* and *Leucadendron (*Rebelo, 1995; Littlejohn, 1997) and seed (fruit with embryo) as a result of sexual reproduction in all Proteaceae species. Vegetatively produced plants exhibit a true genetic copy of the parental plant. As a result of genetic recombination, the seedlings regenerated from embryos originating from the same seed head display a combination of characteristics of both parental species.

Seed set

Hermaphrodite members of Proteaceae are reported to produce extremely low numbers of viable seed per inflorescence under natural conditions (Vogts, 1960; Horn, 1962; Brits, 1984; Rebelo and Rourke, 1986; Esler and Cowling, 1990). Natural seed set is in the range of 1-30% in *Protea* (Horn, 1962; Vogts, 1971; Van Der Walt and Littlejohn, 1996b) and up to 16% in *Leucospermum* (Horn, 1962; Lamont, 1985).

Dioecious, *Leucadendron* and *Aulax,* however, refutes this commonly held perception of natural low seed set in Proteaceae as 0-80% fruit set has been observed under natural conditions (Jordaan, 1945). Mean fruit set for *Leucadendron* and *Aulax* was found to be 94% and 77%, respectively (Rebelo and Rourke, 1986; Collins and Rebelo, 1987). Fecundity was also found to vary within and between the species of *Leucadendron* (Van den Berg and Brits, 1995).

A common problem in cross compatibility studies within genera of Proteaceae is further reduction in viable seeds per inflorescence (Van den Berg and Brits, 1995). Seed set in *Protea* and *Leucospermum* are even more reduced when hybridizing species within the same genera (Brits, 1992; Van den Berg and Brits, 1995; Van der Walt and Littlejohn, 1996b). Cross-compatibility between species of *Leucadendron* within section 'Alatosperma' produce a much higher progeny of 60% seed set per inflorescence than in *Protea* and *Leucospermum* (Van den Berg and Brits, 1995). However, preliminary results of the crossability of taxonomic sections of *Leucadendron* show that seed set per cone is of the same low order as in natural seed set in *Protea* and *Leucospermum* (Van den Berg and Brits, 1995). Van den Berg and Brits (1995) also noted a decline in seed set, apparently associated with increasing phylogenetic distance.

Interspecific Incompatibility

Research over the years has identified three barriers in flowering plants to prevent either fertilization or the production of fertile progeny (De Nettancourt, 1977). S*terility* relates to disturbances within the cells of the embryo sac, whereas *incompatibility* and *incongruity* disrupt the normal functioning of the intimate relationship between the pollen and pistil. Although the latter two fertilization barriers show similar final results, the mechanisms at work is different (Hogenboom, 1973). Incompatibility functions within a population and species, whereas incongruity operates between populations and species (Frankel and Galun, 1977).

Incompatibility or intraspecific incompatibility acts during the progamic phase, which is the period between landing of the pollen on the stigma and double fertilization. The process is genetically controlled by the inhibiting action of the incompatibility gene controlled by multiple alleles at one or two S-loci (De Nettancourt, 1977).

Interspecific incompatibility is understood as any of the post-pollination processes prevented through an absence of pollen germination or abnormal behaviour of pollen tubes the formation of hybrid zygotes combining the genomes of two different fertile species (De Nettancourt, 1977). The phenomenon is termed by Hogenboom (1973, 1975) as incongruity. According to the latter it is a process completely distinct from the non-functioning of the pollen-pistil relationship in self-incompatibility. Hogenboom (1973) describes the cause of non-functioning as a lack of genetic information in one of the parents concerning structure and or physiology of the other parent (Hogenboom, 1975).

The nature of interspecific barriers

Sexual barriers in interspecific hybridization are recognized in pre-and post- fertilization processes in the pistil (Van Tuyl, 1996). It is essential to describe the nature of the barrier in the mutual relationship to determine applicable breeding methods to circumvent possible breeding hurdles.

In compatible pistil-pollen relationships, pollen tubes seem to appear as slender callose strands which grow cohesively and appressed to the tissue along the length of the pistil (Beharav and Cohen 1995; Van Roggen *et al.,* 1988). Various forms of barriers have been identified in plant breeding, which disrupt sexual compatibility between species. Factors identified in flowering plants to limit interspecific crosses are as follows:

External interspecific barriers in the field

- Failure of pollen to reach the stigma (as in Proteaceae) due to geographical isolation or staggered flowering times (Herscovitch and Martin, 1989).

Pollen – pistil interspecific barriers

- Failure of pollen to germinate and to develop a pollen tube (Callan and Thompson, 1986).
- Pollen tube arrest in the stigma as in *Citrus* (Kahn and De Mason, 1986).
- Branching of tubes in the styles of *Grevillea* (Martin and Herscovitch, 1989).
- Inhibited tubes with swollen tips in *Macadamia* (Sedgley, 1983)
- Winding growth pattern and subsequent arrest of pollen tubes in the style as in *Lilium* (Van Roggen *et al.,* 1988).

- Pollen tube arrest in the lower style or in front of the micropyle as in *Lilium* and *Crocus* (Chichirico, 1996), or pre-fertilization barriers in the lower style as in *Bromeliaceae* (Parton *et al.,* 2001).
- Thick tubes filled with callose, thickened ends and bifurcated tube formation as in *Cucumus* (Beharav and Cohen, 1995).
- Tube growth beyond the egg filling the embryo sac with coil extensions of the tube as in *Rhododendron* (Williams *et al.,* 1986); failure of fertilization (Chichirico, 1996).
- Failure of the zygote to develop into a seed, and the seed into a mature plant as in *Limonium* (Morgan *et al.,* 2001).

Van Tuyl (1996) describes the simplest form of pollen-pistil disruption as it occurs prior to fertilization. Pollen grains of 'foreign' species fail to germinate on the stigma or, in the event of pollen germination, tubes fail to reach the ovule. Sometimes embryos begin to develop, but they abort at an early stage. Disharmonies do occur between the developing embryo and the endosperm or the zygote to develop into a seed. If the growth of the embryo is inhibited by the endosperm, the hybrid can sometimes be saved by dissecting out the young embryo and growing it in nutrient culture (Proctor *et al.*, 1996).

Stigma morphology and pollen tube growth

Stigma surfaces in angiosperms vary widely in morphology and respond differently to pollination (Heslop-Harrison and Shivanna, 1977). Investigations as early as 1964 indicate a relationship between stigma morphology and the type of incompatibility

(Heslop-Harrison and Shivanna, 1977). Research has then focused on features such as the form of the stigma, presence or absence of papillae, naked appearance, wet or dry stigmas and the presence of a distinct stylar canal or a solid style. A comprehensive survey undertaken by Heslop-Harrison and Shivanna (1977) of the stigma surfaces of some 1000 species and 900 genera and 250 families classified stigmas into two principle types, viz, 'dry' with little or no surface secretions and 'wet' with surface secretions at maturity. According to Elleman *et al.* (1992) stigmas of the 'dry' type have a smaller receptive area than 'wet' stigmas. Based on morphological features, each stigma type is further subdivided taking into account characteristics such as the presence or absence of papillae, branching and localization of papillae (Heslop-Harrison and Shivanna, 1977). The study classifies Proteaceae, particularly genera *Embothrium* and *Grevillae* as 'dry' stigma type with unicellular papillae on the surface (Heslop-Harrison and Shivanna, 1977). In *Protea* (Van der Walt and Littlejohn, 1996b), *Banksia* (Fuss and Sedgley, 1991 and *Macadamia* (Sedgley *et al.*, 1985) the pollen receptive area is specialized with papillae enclosed in a small but distinct groove. Pollination in these genera is dependent on the widening of the groove and a build-up of extracellular stigmatic exudate up to peak receptivity. Pollination in Proteaceae can only be effective if the pollen lies in the papillated stigmatic groove when it is fully open, which can take up to six days following anthesis in Protea (Van der Walt and Littlejohn, 1996a).

Pollen tube penetration in angiosperm occurs in a number of ways following pollination responses in the stigma. In 'dry' stigmas it has been reported that tubes can grow between cell layers along the length of the papilla, penetrate all the layers of the stigmatic cells and grow between the plasma membrane and inner face of the wall. The

tubes can then enter the stigmatic papilla or grow extracellular on the surface (Elleman *et al.,* 1992). De Wet *et al.* (1990) describes three types of style structures identified in plants through which the pollen tube can grow. Open styles are usually characteristic of monocotyledonous plants with a mucilage-filled stylar canal, solid styles having transmitting tissue to guide the pollen tube to the ovary are usually found in dicotyledonous plants and semi-solid styles exhibit intermediate features as described in avocado (Sedgley and Buttrose, 1978).

Techniques examining stigma morphology and the path of the pollen tube down the style

In all morphological studies concerning stigmas, scanning electron microscopy (SEM) was used to obtain a three-dimensional view since the structure can be examined in detail due to higher resolving power and wider depth of focus than with light or stereomicroscopy (Wetzstein, 1989; Van der Walt and Littlejohn, 1996a). The use of SEM and aniline-blue fluorescence to follow the path and behaviour of pollen tubes after pollination have been applied to many species crosses to study the nature of the crossing barrier. Although SEM shows a three-dimensional natural image of the tube, researchers favour the aniline-blue fluorescence technique (Martin, 1959) as it is more cost-effective when screening large numbers of pistils and it is technically easier and faster to apply in any species having variable stem thickness (Van Roggen *et al.,* 1988).

Pollen viability

The germination and normal growth of the pollen tube, containing the two male gametes is vital as a prelude to fertilization in Angiosperm. Therefore the need to determine

pollen quality in horticultural species arises when assessing the fertility of parent plants and hybrids in breeding and genomic research, and in monitoring pollen state during storage. Several methods are available for evaluating pollen quality in flowering plants that include *in vivo, in vitro* and histochemical approaches. In his thesis Van der Walt, (1994) summarize the practicality of various available approaches to assaying pollen quality in flowering plants without any definitive recommendations. On a purely theoretical basis he ranked methods in the order: *in vivo* approach > *in vitro* approach > fluorochromatic procedure > enzyme testing > stainability testing.

The *in vivo* approach may not be practical because of the long time elapsing between pollination and the development of viable seed. Any post-pollination disturbance in the pollen-pistil relationship may prevent seed production even though the pollen may germinate normally (Heslop-Harrison *et al.*, 1984). These methods are time-consuming and impractical for testing large numbers of samples.

In vitro and histochemical tests have been developed which rapidly determine pollen tube development before fertilization (Stanley and Linskens, 1974). These tests require a small pollen sample, a germination medium and a light microscope for observations and pollen counts (Stanley and Linskens, 1974). *In vitro* assays require optimum growth conditions to established and maintain normal tube development. However, optimum conditions are not always established in *in vitro* medium as most of the pollen tested undergoes tube arrest before reaching the normal length (Visser, 1955). Methods regularly used are the hanging drop (Van der Walt and Littlejohn, 1996c), the spot test, well, agar and membrane assay (Stanley and Linskens, 1974). The method most frequently used, easy to handle, and providing the possibility of permanent

mounting is the agar assay (Stanley and Linskens, 1974). It is also the most practical method for screening large numbers of pollen samples simultaneously. Agar provides moisture at a constant relative humidity and various nutrient and other pollen stimulants can be incorporated. Rates of pollen-tube emergence and extension vary considerably among species (Heslop-Harrison, 1987). Pollen behaviour in artificial medium varies widely with regard to the rate and amount of moisture taken up. Van Teighem (1869) reports 30 genera of, which the pollen germinates in water, whereas others report pollen to be sensitive to an ample amount of water. The stigma provides a suitable chemical milieu for pollen function. Heslop-Harisson and Shivanna (1977) made a distinction between species with 'dry', having no free flowing secretion, and those with 'wet ' stigmas in terms of pollen hydration. In species with 'wet' stigmas, pollen is received immediately into a fluid matrix and hydration is likely to begin immediately. Therefore, pollen from plants with 'wet' stigmas is found to germinate readily in a liquid medium whereas that from 'dry' stigma species often requires special conditions to establish an environment near a natural hydration rate (Bar-Shalom and Mattson, 1977).

Many factors related to the pollen germination medium such as the effects of carbohydrates, boron, calcium, micronutrients, pollen density, pH and temperature, have been discussed in pollen viability studies as they influence pollen viability *in vitro* (Van der Walt, 1994).

Carbohydrates control the rate at which water is taken up by the pollen grain. They function mainly as an osmoticum (Vasil, 1964; Leduc *et al.,* 1990), and regulate water supply to the pollen grain, thereby controlling bursting of pollen (Visser, 1955; Vasil, 1964; De Bruyn, 1966). Carbohydrate sources thus far used in pollen germination

mediums are sucrose, fructose, galactose, rafinose, mannitol and sorbitol. However, the use of these single exogenous carhohydrate sources resulted in hardly any *in vitro* germination of *Audouinia capitata* (De Lange and Boucher, 1993). Instead, a composite medium of sucrose, fructose and glycerol resulted in good germination, the latter being the most effective carbohydrate. The most generally used source of carbohydrate in *in vitro* viability assays in tomatoes is sucrose (Abdul-Baki, 1992).

High germinabilities of more than 90% have been found in *Protea* (Van der Walt and Littlejohn 1996c) when applying the *in vitro* hanging-drop method. The following germination medium was proposed as optimal for Protea pollen: 300mg.l^{-1} $Ca(NO_3)_2$. $4H_2O$; 200 mg.$l^{-1}$$MgSO_4$. $7H_2O$; 100mg.$l^{-1}$$H_3BO_3$; 0.5 M sucrose; pH 7.0 and an incubation temperature of 25°C (Van der Walt, 1994).

Heslop-Harrison *et al.* (1984) identified three methods in the histochemical approach, viz, the stainabiltiy test, enzyme test and the fluorocromatic reaction (FCR). All require less time, thus making them suitable for routine screening of large numbers of samples. Although it is a very practical approach Van der Walt (1994) concluded that none of the methods takes pollen vigour into account.

Cultivar breeding strategies

Mass selection

Seed heads of species exhibiting favourable floricultural features are harvested from species populations in the CFK. Single plants are selected from the seedling populations, and the seed collected and grown on to a new population. This cycle can be repeated until visual uniformity is obtained in flowering traits of the seedling plants. In this

procedure it should also be possible to select single plants to be vegetatively propagated as clonal selections at any stage thereby halting the selection cycles. This method of cultivar development can easily be used by farmers and is suitable for species in which little variation is generated through seed. A cultivar selected by this method is the *Protea* cultivar 'Guerna'. Use of the method is limited in dioecious *Leucadendron*. When this type of cultivation is practiced by the farmer, its value in *Leucadendron* is limited. In each generation from seed, usually only the male plants are of economic value , therefore approximately 50% of planted area will be covered by "useless male plants". In many species the male plants can only be identified once it is mature enough to flower, representing an economic loss over time for the farmer. *L. platyspermum* is an exception in that both male and female plants are of value in the floriculture trade.

Single plant clonal selection

Superior single plants are selected from wild populations or cultivated seedling populations for clonal propagation. However, the single plant selections will vary minimally from the species population from which selected. New combinations of floriculturally important traits are seldom obtained through single plant selections from species populations, unless natural mutations occur in the species populations. It is a useful method of selecting for horticultural traits, such as plant vigour, adaptability to different soil types and stem length. However, the disadvantage is that no new types can be selected as the clones will exhibit the exact combination of characteristics of the parental species (Littlejohn personal communication).

Chance interspecific hybrid selection

Chance interspecific hybrids are mostly found in cultivated blocks of *Protea* species. The parental species usually flower at the same time and use the same pollen vectors. Seedling populations selected from cultivated blocks of mixed species may then contain hybrid seeds. One disadvantage for further breeding is that only the seed parent is known and the hybrids are often infertile. Most of the *Protea* chance hybrids in the International Protea Register (Sadie, 2000) were found by farmers cultivating a variety of species and new variations in floral traits are usually a combination of traits both parental species.

Controlled intra- and interspecific hybridization

The use of controlled intraspecific crosses between *P. cynaroides* varieties produced excellent combinations of traits in the cultivars 'Madiba' (Littlejohn, 1999a) and 'Clare' (Littlejohn, 2000a)

Controlled interspecific hybridization in *Leucadendron* has delivered unique combinations of quality floral and horticultural traits in cultivars 'Rosette' (Littlejohn, 1999b), 'Disco Date' (Littlejohn, 2000b), 'Laurel Yellow', 'Chameleon' (Littlejohn, 1999c) and 'Flash' (Littlejohn, 1999d). Single seedling selection follows cross-pollination and superior selections are multiplied by means of vegetative cuttings to maintain uniformity. Hybrid selections are eligible for registration as cultivars with plant breeder's rights as they are unique, stable and reproducible. This method of cultivar development presents great challenges and many economic benefits if the end results are favourable. It has however been noticed that hybrid infertility as observed in 'Rosette' can limit further breeding attempts (Littllejohn, personal communication). Many important horticultural traits, such as upright growth, long stems and late flowering times

are floristically inferior as cut foliage products. Thus, the focus of a breeding program should perhaps be extended to making further crosses between interspecific hybrids thereby increasing the amount of variation from which new selections can be made.

Limitations in breeding Proteaceae

As woody shrubs, breeding progress is limited by the long life cycle of the plants. The plants have a 3 to 4 year vegetative juvenile period after which the plants flower annually. The seed has to mature on the plant and can take up to 9 months in *Protea.* Obligate cross-pollination, as found in *Leucadendron* also inhibits the selection process and no knowledge on the heritabilities of important traits to give guidance as to breeding and selection strategies is yet available. Furthermore, natural flowering times are genetically determined and often inflexible and environmental factors can also influence controlled pollination resulting in poor numbers of seeds.

General aspects of interspecific incompatability and hybridization in breeding

Results of interspecific crossing are of interest from ecological, taxonomic and economic points of view. However, the main objective of interspecific crossing in plant breeding is to combine favourable characteristics of different species within the scope of current knowledge that interspecific crossing can result in hybrid vigour (heterosis), but may also result in inviable or sterile progeny.

Interspecific crossing in Proteaceae has been recorded as having repeatedly resulted in improvement of horticultural and floricultural characteristics such as vigour, travel-durability and vase life, and extension of the flowering period (Littlejohn *et al.*, 1998). According to Williams (1972), crossing of Proteaceae species whose geographical ranges do not overlap should not be a problem. However, a low incidence of interspecific

crossing has also been reported in natural and cultivated populations (Rourke, 1980; Lewis and Bell, 1981; Williams, 1972) and hybridization has generally been found to occur between taxonomically related taxa. An estimated 95% of all natural hybrid cultivars grown locally have been ascribed by Brits (1992) to selection of plants originating from accidental chance cross pollination.

Conventional hand-pollination techniques, which mimic natural modes of pollination, have been developed for cut flower production of *Protea, Leucospermum*, *Leucadendron* (Brits and Van den Berg, 1990) and *Banksia* (Fuss and Sedgley, 1991). Controlled artificial crossing has resulted in several novel plant forms, flower colours and increased yield of marketable stems of up to 100-150% (Brits, 1985; Blomerus *et al.*, 1998; Littlejohn *et al.,* 1998) and many single plant hybrid selections have resulted in the release of new and superior cultivars available on the international floriculture market.

Several breeding research reports indicate that Proteaceae is difficult to hybridize (Brits, 1983), with *Protea* the most difficult, *Leucospermum* intermediate and *Leucadendron* the easiest (Van den Berg and Brits, 1995). Fecundity in *Leucadendron* was found to differ both within and between species (Van den Berg and Brits, 1995).

Controlled interspecific crosses between hermaphrodic species within the *Protea* (Brits, 1983), and *Banksia* (Goldingay *et al*, 1991) result in a further decline or absence of viable seed per inflorescence. Artificial hybridization results of *Leucadendron* indicate that intraspecific crosses produce the highest number of viable seeds (Brits and Van den Berg, 1990). However, interspecific crosses between different subsections produced the lowest number of viable seeds per cone of the same order as in *Protea* and *Leucospermum* (Brits and Van den Berg, 1990). The decline in seed numbers following

artificial cross-pollination is ascribed to self-incompatibility and interspecies cross incompatibility (Brits and Van den Berg, 1990).

Although hybridization in Proteaceae appears to be potentially promising for the production of new cultivars by breeding, low compatibility between species and extremely reduced hybrid seed set appear to be problems yet to be addressed (Brits, 1983). The incompatibility within *Protea* appears to act as a pre-fertilization barrier (Van der Walt, 1994) and hybridization appears to be further barred by variation in flowering times of species, pollinators and possibly other incompatibility reactions (Van der Walt, 1994).

Chromosome numbers within Proteaceae genera are constant with *Protea, Mimetes, Paranomus,* and *Serruria*having x = 11, and *Leucospermum* and *Leucadendron* x = 12 (De Vos, 1943; Stace *et al.*, 1998), suggesting that the species isolation mechanism is of ecological or physiological nature which is probably easier to overcome from the point of view of practical breeding by crossing than the more complicated chromosomal isolation mechanisms (Brits, 1992). Investigations into interspecific incompatibility barriers in Proteaceae are therefore encouraging from the point of view of practical plant breeding.

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Table 1. The scientific or botanical taxonomic classification of *Leucadendron* according to Williams (1972) with common names from Rebelo (1995).

Sub-section	Species
	Section:
	'Alatosperma'
Delta-seed Conebushes	*L. uliginosum* R. Br. subsp. *uliginosum*, *L. uliginosum* subsp. *glabratum* I. Williams, *L. loeriense* I. Williams, *L. rourkei* I. Williams, *L. radiatum* E. Phillips & Hutch, *L. conicum* (Lam.) I. Williams, *L. salicifolium* (Salisb.) I. Williams, *L. pondoense* A.E van Wyk, *L. macowanii* E. Phillips, *L. floridum* R. Br.
Oilbract Conebushes	*L. microcephalum* (Gand.) Gand. & Schinz
Clay Conebushes	*L. lanigerum* H. Buek ex Meisn. var. *lanigerum*, *L. lanigerum* var. *laevigatum* Meisn, *L. modestum* I. Williams, *L. stelligerum* I. Williams
Sunshine Conebushes	*L. diemontianum* I. Williams, *L. flexuosum* I. Williams, *L. salignum* P.J. Bergius, *L. foedum* I. Williams, *L. procerum* (Salisb. Ex Knight), *L. discolor* E. Phillips & Hutch, *L. eucalyptifolium* H. Buek ex Meisn., *L. coniferum* (L.) Meisn., *L. meridianum* I. Williams , *L. xanthoconus* (Kuntze) K. Schum., *L. laureolum* (Lam.) Fourc., *L. cryptocephalum* L.Guthrie, *L. gandogeri* Schinz ex Gand., *L. strobilinum* (L.) Druce, *L. spissifolium* (Salisb. Ex Knight) I. Williams subsp. *spissifolium*, *L. spissifolium* subsp. *fragrans* I. Williams, *L. spissifolium* subsp. *phillipsii* (Hutch.) I. Williams,*L. spissifolium* subsp. *natalense* (Thode & Gilg) I. Williams, *L. spissifolium* subsp. *oribinum* I. Williams
Needleleaf Conebushes	*L. teretifolium* (Andrews) I. Williams, *L. spirale* (Salisb. Ex Knight) I. Williams, *L. nobile* I. Williams, *L. muirii* E. Phillips *L. comosum* (Thunb.) R. Br. subsp. *comosum*, *L. comosum* subsp. *homaeophyllum* (Meisn.) I. Williams, *L. platyspermum* R. Br.

Table 1. (continued)

Sub-section	Species
	Section
	'Leucadendron'
Arid Conebushes	*L. remotum* I. Williams, *L. bonum* I. Williams, *L. pubescens* R.Br., *L. arcuatum* (Lam.) I. Williams
Sandveld Conebushes	*L. coriaceum* E.Phillips & Hutch, *L. brunioides* Meins. var. *brunoides*, *L. brunioides* Meins. var. *flumenlupinum* I. Williams, *L. stellare* (Sims) Sweet, *L. thymifolium* (Salisb. Ex Knight) I.Williams, *L. levisanus* P.J. Bergius, *L. concavum* I.Williams, *L. dubium* H.Buek ex E. Phillips & Hutch, *L. cinereum* (Sol. Ex Aiton) R.Br, *L. galpinii* E. Phillips & Hutch, *L. linifolium* (Jacq.) R. Br.
Ridge-seed Conebushes	*L. sericeum* (Thunb.) R. Br., *L. nitidum* H. Buek ex Meisn
Pauciflor Conebushes	*L. olens* I. Williams, *L. ericifolium* R.Br.
Kouga Conebushes	*L. singulare* I. Williams, *L. sorocephalodes* E.Phillips & Hutch
Jonaskop Silver-Conebush	*L. nervosum* E. Phillips & Hutch
Silver Conebush	*L. dregei* E. Mey. Ex Meisn., *L. album* (Thunb.) Fourc, *L. rubrum* Berm. f., *L. argenteum* (L.) R. Br.
Fuse-bract Conebushes	*L. verticillatum* (Thunb.) Meisn, *L. corymbosum* P.J. Bergius, *L. laxum* I. Williams
Sun Conebushes	*L. barkerae* I. Williams, *L. burchellii* I. Williams, *L. tradouwense* I. Williams, *L. orientale* I. Williams, *L. pubibracteolatum* I. Williams, *L. tinctum* I. Williams, *L. cordatum* E. Phillips, *L. cadens* I. Williams, *L. gydoense* I. Williams, *L. sessile* R. Br., *L. daphnoides* (Thunb.) Meisn, *L. sheilae* I. Williams, *L. meyerianum* H. Buek ex E. Phillips & Hutch, *L. roodii* E. Phillips, *L. glaberrimum* (Schltr.) Compton subsp *glaberrimum*, *L. glaberrimum* subsp. *Erubescens* I. Williams, *L. loranthifolium* (Salisb. Ex Knight) I. Williams
Crown Conebushes	*L. grandiflorum* (Salisb.) R.Br, *L. globosum* (Kennedy ex Andrews) I. Williams, *L. chamelaea* (Lam.) I. Williams, *L. elimense* E. Phillips subsp. *elimense*, *L. elimense* subsp. *Salteri* I. Williams, *L.elimense* subsp. *Vyeboomense* I. Williams

Pollen morphology and viability in *Leucadendron*

Abstract

Pollen germinability in species of *Leucadendron* was studied to determine the viability of different pollen parents. At first the most suitable method for germinating *Leucadendron* pollen was determined using the hanging drop method and the solid agar medium. Counting of germinating pollen grains proved to be more practical and accurate when done *in vitro* using the solid medium technique as pollen tubes burst soon after protrusion in an aqueous solution. The study showed that solid agar at 2% and a solution of 10% sucrose as basal medium was found to reduce the occurrence of bursting and improved germination. Pollen viability of *Leucadendron* species and sub-sections tested in the present study was above 50%. A detailed analysis of germination in six commercial species selected for future use in a diallel crossing programme shows no significant differences between these species. The morphology and grain size of *Leucadendron* pollen was studied using a scanning electron microscope. A high level of consistency was apparent in the shape, structure and the surface of grains, however spherical, wart-like elements (orbicules) of different sizes, appeared to be spread irregularly over the pollen grains of species belong to the section 'Leucadendron'. Summary statistics and the basic ANOVA of pollen grain size measurement showed highly significant differences between species.

Introduction

In Proteaceae studied thus far the stigma is in the form of a microscopic stigmatic groove (Vogts, 1971; Rebelo and Rourke, 1986). Initially it was postulated that only pollen lodged within the groove could germinate (Vogts, 1971). However, Van der Walt (1994), produced SEM images showing pollen close to the groove germinating and the pollen tube growing towards the stigmatic groove, in contrast to *Banksia* where for germination to occur the pollen grains have to be located in the groove itself (Fuss and Sedgley, 1991). In plants with stigmatic grooves the fertility of pollen is a major factor in the success of pollination, as only 1-3 tubes enter the stylar canal through the stigmatic groove. If inviable pollen lies close to or in the stigmatic groove pollination will not be successful.

One of the reasons for poor productivity following self- and cross-pollination in Angiosperma is the failure of the pollen grain to deliver the male gamete to the embryo sac for successful fertilization. Focusing on the pollen parent during preliminary controlled interspecific pollinations it became evident that failure of crosses could be ascribed to failure of the pollen to germinate and develop a pollen tube, or to species-specific structural differences between pollen grains. Working with the widest possible range of species available should then lead to a better understanding of pollen quality and provide a better basis for selection of pollen parents.

The triangular pollen grain is the most common type found so far in the various tribes of Proteaceae (Venkato Rao, 1971). Garside (1946) described *L. argenteum* as having the largest mature pollen grain (45-61 μm diameter) of South African Proteaceae. Williams (1972) in his study on *Leucadendron* taxonomy found that pollen size is

variable, but the variability was particularly noticeable in *L. arcuatum, L. loranthifolium* and *L. pubescens*. However, there were no indications of fundamental differences in pollen grain sizes between species.

Several standard tests of pollen quality and variability, such as germination *in vivo* (Stern and Gazit, 1998), *in vitro* (Stanley and Linskens, 1974; Van der Walt and Littlejohn, 1996a) and histochemical staining (Stanley and Linskens, 1974) have been described. In comparative studies, the most reliable test for pollen viability has been obtained by germinating pollen (Heslop-Harrison *et al.*, 1984).

Pollen viability has been tested *in vitro* in *Protea* by Van der Walt and Littlejohn (1996a). The highest germination rate of all genera tested was obtained on a modification of Taylor's medium in a hanging drop. *In vitro* tests that have been successfully applied in other crops have included the use of agar or gelatin on a plate, the well-test, spot-test and the use of a collodion membrane or dialyzing tube kept in a moist atmosphere (Stanley and Linskens, 1974).

Poor hybrid seed set in *Leucadendron* as reported by Van den Berg and Brits (1995) has prompted the examination of pollen morphology and viability as possible limiting factors in interspecific cultivar development. As a first step in the study of the sequence of events from pollen landing to ovule fertilization this study then focused on exomorphological observations, *in vitro* pollen germination and pollen grain size.

Material and methods

Pollen source

The source of pollen was the *Leucadendron* species collection of the Fynbos Genebank of the Agricultural Research Council at Elsenburg. The collection is maintained by standard cultivation practices as described by Coetzee and Littlejohn (2001), classified according to the nomenclature of Rebelo (1995). At the time of sampling the collection consisted of six species used commercially and 15 other species having potentially valuable floricultural characteristics for future breeding as summarized in Table 2.1. The six commercial species were those used in the diallel crossing programme to be described in Chapter 3.

Pollen harvesting

Pollen of cloned male plants of the different species was used as it became available. 50 inflorescences were harvested between 14h00 and 16h00 on a sunny day. A maximum of 50 male flowering stems were taken to the laboratory and placed in water. At the same time, the remaining pollen on the pollen presenter was brushed off with a soft paintbrush to prevent mixing of old and freshly harvested pollen. Stems were left in the vase until the next morning when newly opened pollen presenters exposed an abundance of fresh pollen. Pollen was funneled into a micro centrifuge tube using a flat point tweezer and camel hair paintbrush and mixed. Samples were scanned for small insects under a stereoscopic microscope and removed before further investigation.

Pollen germination tests

Two basic techniques for evaluation of pollen germination were tested as follows:

1. The *in vivo* technique

Stigmas were harvested 6 days after controlled artificial pollination within species to test pollen germination under natural conditions. Three cones were pollinated and 10 stigmas harvested per cone and treated with aniline-blue stain for observation under a fluorescence microscope. Testing was restricted to the six commercially cultivated species as identified in Table 2.1.

2. *In vitro* techniques including the hanging-drop technique and a solid-medium technique.

Samples of *L. uliginosum* subsp. *uliginosum* were used in preliminary studies as sufficient pollen was available over time to be practically possible to repeat experiments within the same breeding season. *L. uliginosum* results on variable media were used to identify the best combination to test all 18 species on the same medium. It is not practically possible to do all 18 on variable media as pollen of each species is only available for 2-4 weeks and all species used in the diallel study flowers over in the same period of about 2-3 months.

Samples of powdery pollen of *L. uliginosum* subsp. *uliginosum* were collected to establish the quickest and easiest method of viewing and counting viable pollen. *L. uliginosum* was chosen for this study because it is the first flowering species in the collection to produce ample amount of powdery pollen. Germination was then assayed on the full collection of species, barring three species for which too little pollen was

available at the time of investigation, by using the most suitable germination technique and germination medium.

The hanging-drop technique

The hanging-drop technique was applied as described by Stanley and Linsken (1974) using the germination medium formulated for *Protea* pollen by Van der Walt and Littlejohn (1996a), a version modified from Brewbaker and Kwack (1963). A sample of freshly harvested pollen was collected from the pollen mix and scattered over a drop of germination solution placed on a cover slip. Pollen was released from the paintbrush by moving a fine point needle through the hairs. Germination mediums were distilled water and the medium formulated for *Protea*. Sticky pollen collected from insect pollinated species was mixed with a small amount of the germination medium to get an even distribution of pollen collected from different clones of the same species. After pollen had been placed on a drop of germination solution the area was sealed by fitting a glass ring glued to a microscope slide on a ring of petroleum jelly encircling the germination drop. The germination chamber was then inverted and incubated on a warm plate at 23-25°C for 4hrs.

The solid medium technique

Different germination mediums were tested, solid medium with 1, 2 or 3g of agar bacteriological, gelrite or gelatine and five sucrose concentrations (0, 5, 10, 15 and 20g) in petri dishes. The dishes were kept in the fridge at 2-7°C and aged for at least 24 hours before use in order to minimize the amount of moisture available for pollen absorption. Powdery and sticky pollen was scattered evenly over the medium using a small paintbrush and a fine point needle to release pollen grains from the hairs of the

paintbrush. Petri dishes were then put into a plastic zip-lock bag and incubated at 23-25°C for 4hrs.

In the *in vitro* germination tests the counting of viable pollen commenced 4hrs after the pollen was sown on the germination medium and continued for about 3 hours. Pollen was judged to be viable only when the length of the pollen tube exceeded the grain diameter. Five samples per species were set up consisting of five petri dishes each divided into four fields of 50 grains per field, i.e. a total of 200 pollen grains. Pollen of 18 species including both 'Alatosperma' and 'Leucadendron' sections was available for this study.

Pollen grain morphology and size

Pollen grain morphology and size studies looked at species from both sections 'Alatosperma' and 'Leucadendron' but were restricted to the six commercial species subsequently used in the diallel crossing programme. Pollen was harvested in a microcentrifuge tube and then sampled for observations using SEM. Pollen of each species was scattered on a separate stub, dried and coated with gold and then transferred to a Jeol JSM6100 SEM operated at 4 kV for selection of the most representative pollen grain in each species sample. Pollen grains were then photographed using Ilford FP4 black and white film. For the measurement of pollen grain size, fresh pollen samples were mounted according to the method of Moore *et al.* (1991) in glycerine jelly and measured under oil immersion at a magnification of X1000 with an Olympus BH-2 light microscope equipped with a calibrated eyepiece. Five samples were used and 10grains

per sample measured. The length of the pollen grain were measured as a index of pollen size.

Results

Pollen availability was generally very poor and sometimes no pollen was available in the morning. Flowering stems were therefore harvested in the afternoon on a sunny day and left to dehisce in the laboratory at room temperature for pollen collection the next morning.

Germination test

Pollen germination commenced within 1-4 hours after pollination and large numbers of pollen grains germinated on the stigmas. Counting pollen grains and tubes proved so difficult that only the presence or absence of tubes could be recorded. However, the presence of tubes was positively identified in all of the six species investigated.

The hanging-drop technique

In initial attempts using *L. uliginosum,* pollen germination failed in both distilled water and on the *Protea* germination medium (Table 2.2.). Within 2 hours after sowing a short protuberance developed that did not exceed the size of the pollen grain. Bursting and tube rupturing then followed after a short while.

The solid medium technique

Pollen germination using *L. uliginosum* failed on the gelatine and gelrite mediums (Table 2.2). In all weak aqueous sugar solutions, and at low agar concentration, the pollen grains tended to burst shortly after the appearance of a protuberance. Where tube

development occurred this was from one of the three apertures only as illustrated in Fig 2.1.

Addition of carbohydrates to the medium showed sucrose to be an effective supplement to promote pollen germination as may seen in Table 2.3. The effect of agar level is highly significant (P = 0.003) and sucrose level marginally so (P = 0.065). However, the best treatment combination recorded was the medium containing 2g bacteriological agar and 10g sucrose and this medium was subsequently used to assay the frequency distribution of pollen germination in the 18 species tested. Results are given in Table 2.4. in terms of % germination where it is evident that germination exceeded 60% in all species tested. This is well within the range acceptable for adequate seed set according to Visser (1955) and Stanley and Linskens (1974).

The detailed analysis of germination in terms of plates and fields within plates was then restricted to the six commercial species selected for the diallel crossing programme (Table 2.5 and Appendix Chapter 2) shows no significant differences between these species.

Pollen grain morphology and size.

Pollen grains viewed under SEM and during pollen germination under a light microscope showed a consistent triangular shape (Fig. 2.2. A-E). Three circular germination pores are located at each corner of the triangle. The surface of the grain has a perforated or woven appearance, like the intertwined threads of a fabric. Although a relatively high level of consistency was apparent in the shape, structure and the surface of grains, some inconsistencies were also observed. (Fig. 2.2. A-C). These spherical, wart-like elements (orbicules) (Fig. 2.2. A) of different sizes, appeared to be spread irregularly over the

pollen grains of *L. chamelaea* and *L. elimense*, both of which belong to the section 'Leucadendron'. Orbicules on the surface on *L. galpinii* were at very low density and were absent in species of the section 'Alatosperma' (Fig. 2.2. D-E).

Summary statistics and the basic ANOVA of pollen grain size measurement show highly significant differences between species ($P < 0.001$, Appendix Chapter 2) permitting a further analysis by orthogonal contrasts of section ('Alatosperma' vs 'Leucadendron') and subsection (Delta-seed vs other conebush types) classifications (Table 2.6.). Both show highly significant differentiation ($P < 0.001$, Table 2.6.).

Discussion

From observations in this study it appears that the exomorphology of the pollen of *Leucadendron* species is similar to that described for *Protea* by Van der Walt and Littlejohn (1996b) and for *Leucadendron argenteum* by Garside (1946). Orbicles or Ubisch bodies as observed in this study have also been reported for Grevillea (Herscovitch and Martin, 1989) and might have a triple role, namely, adhesion to the pollen presenter, adhesion to pollen vectors during transport and to the stigmatic region. Results of the pollen germination tests suggest that pollen of *Leucadendron* is influenced by the osmotic properties of the germination medium. Counting of germinating pollen grains proved to be more practical and accurate when done *in vitro* using the solid medium technique as pollen tubes burst soon after protrusion in an aqueous solution. Despite the fact that harvesting, mixing and sowing of sticky pollen was difficult when using the hanging- drop technique, the counting of pollen tubes was even more difficult when the majority of pollen tubes started to burst before reaching the size of the pollen

grain. Pollen bursting has been ascribed to a high turgor pressure in the tubes due to the uptake of excessive moisture by Visser (1955). After the content has discharged from the tube it was found impossible to count viable pollen as the tubes ruptured and became invisible.

Good germination results in a 20 % sucrose solution have also been found by Sedgley *et al.* (2001). Although pollen bursting occurred on the agar medium, the walls still remained in place for a few hours enabling counting to proceed. Solid agar at 2% and a solution of 10% sucrose as basal medium was found to reduce the occurrence of bursting and improved germination. Agar creates a colloidal nature in the medium and limits the amount of moisture available for pollen hydration (Visser, 1955). On the agar medium, the content of the pollen is only released after tube elongation. Sucrose therefore appears to play an osmoregulatory role in *Leucadendron* pollen, supported by bursting and short tube appearance when sucrose is absent or present at very low concentration in both germination mediums.

Pollen viability of *Leucadendron* species and sub-sections tested in the present study is above 60% which is well above the minimum considered to be desirable for adequate seed set in practical breeding programmes (Visser, 1955). Pollen viability can therefore probably not be regarded as a limiting factor in breeding by crossing different species.

Pollen grain size in this study was found to be within the ranges reported by Garside (1946) and Williams (1972) and have further identified statistically significant diversity between species, sections and sub-sections of *Leucadendron.* The differences in pollen grain size between species indicate that pollen from one species could be rejected

by the stigma of another species. The rejection could be the inhibition of pollen to germinate or pollen tubes to reach the ovule of another species. It seems likely that differences between pollen of different species could contribute to poor interspecific hybrid seed set.

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Table 2.1. Species of the *Leucadendron* Fynbos Genebank of the Agricultural Research Council, Elsenburg . * denotes species currently used commercially and selected for diallel crossing; for accession details see Table 3.1.

Section	Sub-Section	Species (Accession Number)		Provenance
'Alatosperma'	Delta-seed Conebushes	*L. uliginosum* subsp. *uliginosum* (T 94 05 21)	*	Outeniqua Mountains May 1994
		L. conicum (T 94 10 10)	*	Tsitsikamma Oct 1994
		L. floridum (T 94 03 32)		Cape Flats
		L. macowanii (T 94 03 17)		Cape Town
		L. salicifolium (T 94 05 18)		Kleinmond/ Kogelberg
	Oilbract Conebushes	*L. microcephalum* (T 94 03 39)		Kleinmond/ Kogelberg
	Sunshine Conebushes	*L. cryptocephalum* (T 94 03 49)		Potberg
		L. spissifolium subsp. *spissifolium* (T 94 03 70)		Riviersonderend
		L. strobilinum		Cape Peninsula
		L. coniferum (T 94 09 70)		Elim area
		L. discolor (T 94 10 33)	*	Piketberg Oct 1994
		L. procerum (T 94 03 66)		Cederbeg
	Needleleaf Conebushes	*L. nobile* (T 124/94/1)	*	Baviaanskloof Date unknown
'Leucadendron'	Sandveld Crownbushes	*L.galpinii* (T 93 12 05)	*	Albertinia
	Crown Conebushes	*L. elimense* subsp. *vyeboomense* (T 94 09 90)		Elim area
		L. chamelaea (T 94 03 81)	*	Koue Bokkeveld March 1994
		L. globosum (T92 10 01)		Grabouw
	Sun Conebushes	*L. glaberrimum* subsp. *glaberrimum* (T 94 03 42)		Cederberg
		L. daphnoides (T 91 09 02)		Du Toit's Kloof
		L. tinctum (T 93 03 21)		Elim area
	Silver Conebushes	*L. rubrum* (T 94 10 50)		Riviersonderend

Table 2.2. Pollen germination on *in vitro* media in preliminary studies using *L. uliginosum*

Germination medium	Germination
1g agar + 5g sucrose	5%
1g Gelrite + 5g sucrose	0
1g Gelatine + 5g sucrose	0
Hanging drop technique using distilled water or distilled water + 5% sucrose	Some development of protuberances which burst after a short while
1g Agar+ 5-10g glucose	0
1g Agar + 5-10g fructose	0

Fig. 2.1. Pollen tube developing from one aperture.

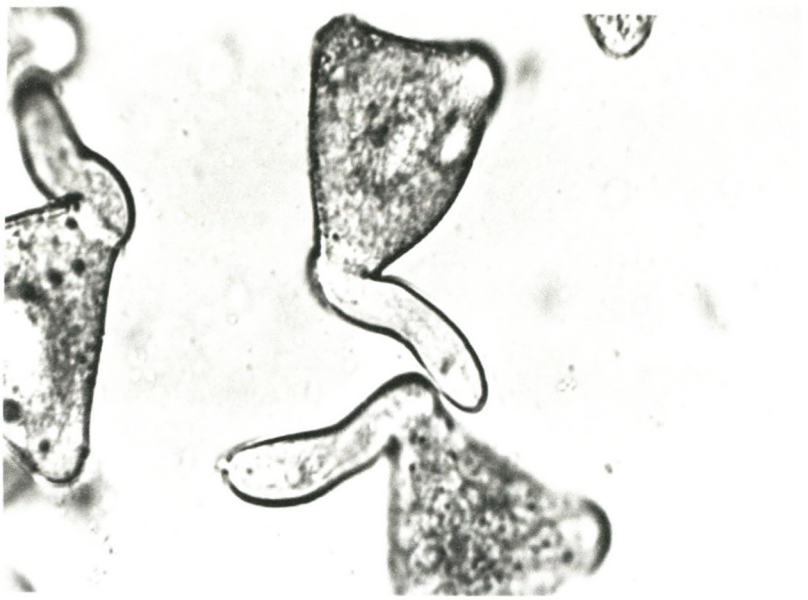


Table 2.3. Pollen germination on different sucrose and agar concentration combinations using *L. uliginosum*, expressed as the proportion germinating out of 1000 grains plated and transformed to radians by the arcsin transformation for analysis of variance.

Proportion germinating	Sucrose (g)	Agar (g)			Mean
		1	2	3	
	0	0.05	0.10	0.00	0.050
	5	0.08	0.45	0.24	0.257
	10	0.25	0.80	0.20	0.417
	15	0.20	0.72	0.10	0.340
	20	0.00	0.70	0.00	0.233
	Mean	0.116	0.554	0.108	0.259

Radians	Sucrose (g)	Agar (g)			Mean	Proportion *
		1	2	3		
	0	0.226	0.322	0.000	0.182	0.033
	5	0.287	0.735	0.512	0.511	0.239
	10	0.524	1.107	0.464	0.698	0.413
	15	0.464	1.013	0.322	0.600	0.318
	20	0.000	0.991	0.000	0.330	0.105
	Mean	0.300	0.834	0.259	0.330	0.105
	Proportion *	0.087	0.548	0.066	0.105	

ANOVA		df	MS	F	P-value
	Sucrose	4	0.1294	3.4247	0.0651
	Agar	2	0.5136	13.5908	0.0027
	Error	8	0.0378		
	Total	14			

* mean radians retransformed

Table 2.4. Frequency distribution of pollen germination in 18 species of the *Leucadendron* Fynbos Genebank of the Agricultural Research Council, Elsenburg. Pollen was germinated on a solid medium of 2g agar + 10g sucrose at 23-25 °C and expressed as a proportion out of 1000 grains plated.

% Germination	Frequency
< 55	0
55–60	1
60-64	4
65-69	7
70-74	2
75-79	2
80-84	0
85-89	1
90-95	1
>95	0

Total	Mean	SD	CV
18	70.78	8.34	0.118

Table 2.5. Means, Coefficients of Variation (CV) and Analysis of Variance (ANOVA) of pollen germination proportions (transformed to radians) out of 50 pollen grains in each of four fields of five replicate plates. Species abbreviations: *L. uliginosum: uli; L. conicum: con; L. discolor: dis; L. nobile: nob; L. galpinii: gal; L. chameleae: cha.* LSD (.01) and LSD (.05) refer to Least Significant Differences at probability levels 1% and 5% respectively.

MEAN radians	t (.01; 24) : 2.797			t (.05; 24) : 2.064				
	uli	*con*	*dis*	*nob*	*gal*	*cha*	LSD (.01)	LSD (0.05)
	1.126	1.079	0.891	1.040	0.978	0.942	0.255	0.188

MEAN proportion *	t (.01; 24) :			t (.05; 24) :				
	uli	*con*	*dis*	*nob*	*gal*	*cha*	LSD (.01)	LSD (0.05)
	0.815	0.777	0.605	0.744	0.688	0.654	0.064	0.035

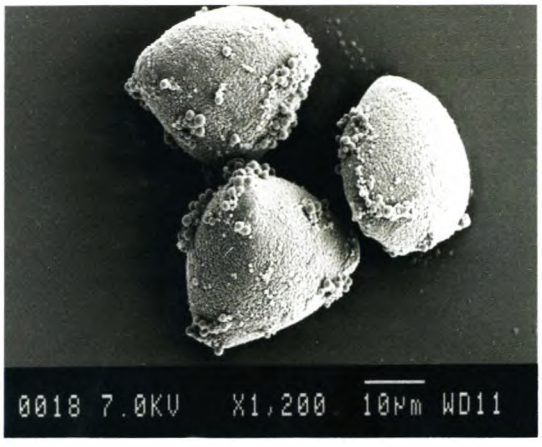
MEAN CV	*uli*	*con*	*dis*	*nob*	*gal*	*cha*
	0.082	0.086	0.211	0.139	0.184	0.187

ANOVA radians	df	MS	F	P
Species	5	0.1550	1.863	0.139
Plates/Species	24	0.0832	3.212	3.318E-05
Fields/Plates/Species	90	0.0259		
Total	119			

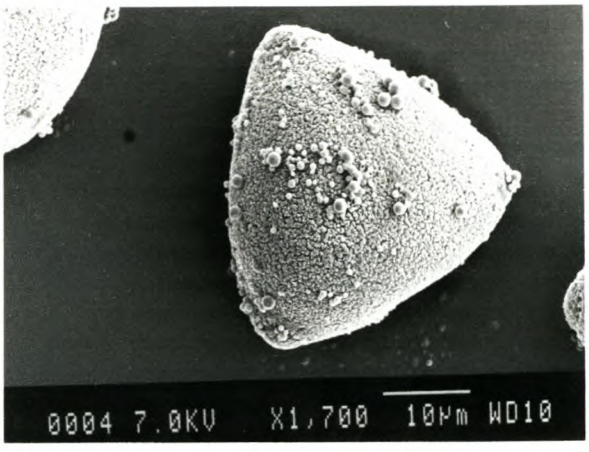
* mean radians back-transformed

Fig. 2.2A-E. The structure of *Leucadendron* pollen.

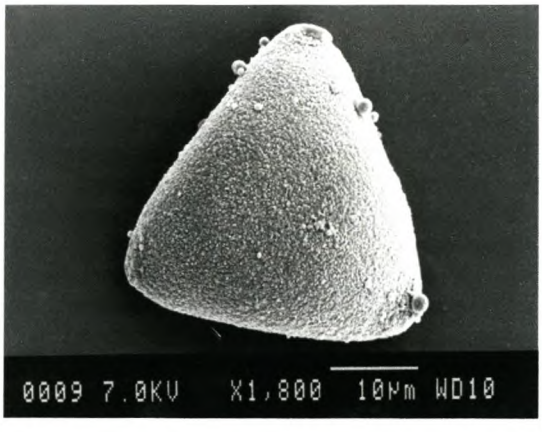
A. *L. chamelaea*



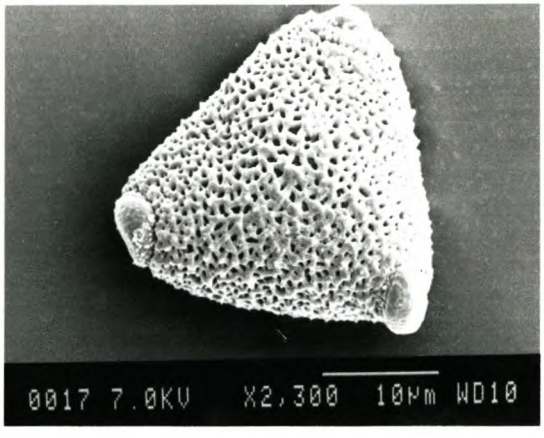
B. *L. elimense*



C. *L. galpinii*



D. *L. uliginosum*



E. *L. discolor*

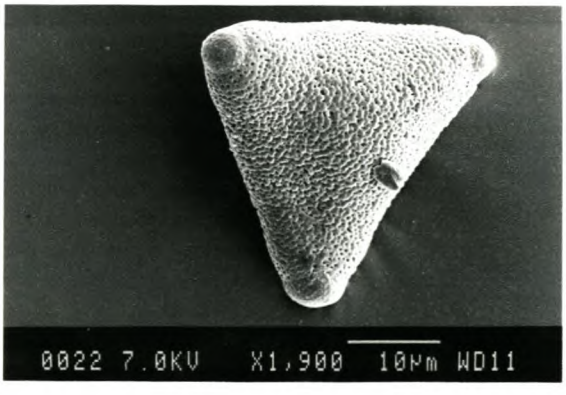


Table 2.6. Means, Coefficients of Variation (CV), Analysis of Variance (ANOVA) and orthogonal contrasts of pollen grain size at X1000 magnification (eyepiece scale unit = 1.1111 μm) of 10 pollen grains in each of five samples per species. See Table 2.5 for LSD and species abbreviations. Alato = section 'Alatosperma'; Leuca = section 'Leucadendron'. Shaded entries highlight cross-checks on the total sums of squares for species in ANOVA and in the two independent sets of orthogonal contrasts.

MEAN (μm)			t (.01; 24) : 2.797		t (.05; 24) : 2.064			
	uli	*con*	*dis*	*nob*	*gal*	*cha*	LSD (.01)	LSD (.05)
	25.98	27.93	34.87	28.00	35.31	33.84	6.67	4.92

MEAN CV	*uli*	*con*	*dis*	*nob*	*gal*	*cha*
	0.192	0.194	0.126	0.211	0.225	0.200

ANOVA	df	SS	MS	F	P
Species	5	4262.622	852.524	6.000	9.77E-04
Samples/Sp	24	3409.999	142.083	3.866	2.09E-08
Grains/Sa/Sp	270	9923.013	36.752		
Total	299	17595.634			

CONTRASTS	df	SS	MS	F	P
Alato vs Leuca	1	1931.982	1931.982	13.596	0.001
Within Alato	3	2276.863	758.954	5.342	0.006
Within Leuca	1	53.777	53.777	0.378	0.544
Total	5	4262.622	852.524		
Delta vs "rest"	1	2440.120	2440.120	17.174	3.66E-04
Within Delta	1	95.602	95.602	0.673	0.420
Within "rest"	3	1726.899	575.633	4.051	0.018
Total	5	4262.622	852.524		

Summary Statistics and Analysis of Variance of pollen germination and pollen grain size (JH Louw, personal communication).

APPENDIX Chapter 2

Species name abbreviations: *L. uliginosum: uli; L. conicum: con; L. discolor: dis; L. nobile: nob; L. galpinii: gal; L. chamelaea: cha*

Notes

1. Pollen germination was calculated as the proportion, p, of grains germinating out of 50 grains sampled, transformed to radians, i.e., $\arcsin\sqrt{p}$, as is appropriate for analysis of proportions (Sokal and Rohlf, 1995; Biometrics, 3rd Ed., WH Freeman Co.). Radian means are transformed back to proportions using the standard back-transformation function $\sin(\text{radians})^2$.
2. The more stringent level of statistical significance and LSDs, $P = 0.01$, is advisable for repeated (multiple) comparisons as might be applied to results of this nature.
3. Significant differences between species for pollen grain size in this analysis ($P < 0.01$) and the further classificaion of species given in Table 2.5 leads logically to the further subdivision of sums of squares into meaningful orthogonal contrasts summarized in Table 2.6.
4. Intrinsic variation in metric biological traits, such as pollen and germination counts, commonly exhibit a positive correlation between variance and mean. It is therefore advisable to express the variation in terms of the coefficient of variation (CV) which is free of the scale of measurement of the trait and also allows for free comparison of levels of variation between traits.
5. Highlighted entries in the case of pollen grain size denote cross-checks between alternative calculations of the total variance within samples.

APPENDIX Chapter 2

Pollen germination (radians)

Species	Counts		Total	Mean	Variance w/n Plates	CV
	Plates	Fields				
uliginosum	5	4	22.512	1.126	0.009	0.084
conicum	5	4	21.581	1.079	0.009	0.090
discolor	5	4	17.822	0.891	0.035	0.211
nobile	5	4	20.797	1.040	0.025	0.151
galpinii	5	4	19.560	0.978	0.044	0.214
chamelaea	5	4	18.846	0.942	0.033	0.193
Mean				1.009	0.026	0.157

ANOVA	df	SS	MS	F	P
Species	5	0.775	0.155	1.863	0.139
Plates/Species	24	1.9968	0.083	3.212	0.000
Fields/Plates/Species	90	2.331	0.026		
Total	119	5.103			

Pollen grain size (μm)

Species	Counts		Total	Mean	Variance w/n Samples	CV
	Samples	Grains				
uliginosum	5	10	1298.876	25.978	25.358	0.194
conicum	5	10	1396.653	27.933	29.242	0.194
discolor	5	10	1743.316	34.866	20.140	0.129
nobile	5	10	1399.986	28.000	35.077	0.212
galpinii	5	10	1765.538	35.311	63.667	0.226
chamelaea	5	10	1692.205	33.844	47.036	0.203
Mean				30.989	36.753	0.193

ANOVA	df	SS	MS	F	P
Species	5	4262.622	852.524	6.000	9.77E-04
Samples/Species	24	3409.999	142.083	3.866	2.09E-08
Grains/Samples/Species	270	9923.013	36.753		
Total	299	17595.634			

Seed set and germination in selected breeding clones within species of the genus *Leucadendron* following different pollination methods.

Abstract

Sexual crossability between flowers of male and female plants of the same and different species of *Leucadendron* with potential commercial cut flower features and currently harvested from mountains surrounding the Western Cape was tested to determine the success of offspring following various modes of pollination. Clones of six species with possible breeding value were used in a diallel cross layout, with each dioecious species acting as male and female parents. Data was statistically analysed using Analysis of Variance of seeds produced, seeds germinating and seed germination. Totals and means per cone for seeds produced, seeds germinating and germination shows minor differences between seed set by natural open and self pollination by hand, thus confirming the effectiveness of the artificial hand pollination technique, but a major reduction in seed set by artificial hand occurs in cross pollination between species. Highly significant differences between species are evident for all modes of pollination. Results show significantly higher numbers of seeds germinating and seed germination in crosses within and the 'Alatosperma' group than in crosses within the 'Leucadendron' group and crosses between the groups. Results for seeds set by natural open and self pollination show means of 17.0 and 15.2 percent respectively. Results further indicate that the significant overall reduction in seeds set by cross pollination is also accompanied by increased variance in many crosses.

Introduction

Leucadendron is the major cut foliage genus of the flowering family Proteaceae and is endemic to the southern tip of Africa. Stems are mainly harvested from wild populations or from broadcast seed sown blocks. Some species and hybrid cultivars are produced in cultivated blocks (Coetzee *et al.*, 1999).

Early experimental breeding in Australia, New Zealand (Mathews and Carter, 1983) and South Africa (Littlejohn *et al.*, 1998) have shown that hybridization within and between species, has promising potential for the international Proteaceae industry.

Self and cross incompatibility in natural flowering populations has long been studied over a wide range of families and have identified potential complications in the breeding of new improved cultivars (Van Tuyl *et al.*, 1982). A common problem found in flowering Proteaceae is low seed set relative to the large number of flowers present. Various other studies on Proteaceae (Fuss and Sedgley, 1991; Goldingay *et al.*, 1991; Sedgley *et al.*, 1990), and other flowering families (Birrenkott and Stang, 1989) have been reported.

The 3-4 year juvenile period of *Leucadendron* implies that the development of new hybrid cultivars can take up to thirty years from the time of the initial selection of wild species to the time of flowering of a unique product. This study therefore concentrated on seed set and seed germination following natural and artificial pollination and in crosses between available species in order to provide a basis for selection of species for future breeding purposes.

Material and Methods

Single plants were selected from wild populations at locations in the Cape Floristic Kingdom as summarized in Table 3.1. Clones from vegetative cuttings were rooted and established in a nursery at the Elsenburg experimental station of the SA Agricultural Research Council situated at 33°51'S, 18°50'E; 177m a.s.l. The nursery was managed by routine practices such as drip irrigation in the summer, fertilization, pruning and weed, disease and insect control. Six year-old plants were used in the pollination programmes.

Diallel crosses.

An initial experiment was designed to test the crossability of 15 species of different sections of *Leucadendron*. However, due to unforeseen circumstances including high plant mortality, limited numbers of clones per species and limited numbers of cones per plant, the crossing diallel had to be reduced to 6 parental species and two clones of each. Parental species included male and female accessions of *L. uliginosum* subspecies *uliginosum, L. conicum, L. discolor, L. nobile, L. galpinii, L. chamelaea*. Currently, poor quality cut foliage and cone products of these species are harvested from wild populations and seedling blocks. With exception of *L. nobile*, products of these are sold during and after flowering to fill the shortage of *Leucadendron* products on the market between September and December. Although *L. nobile* has an unpleasant scent it is nevertheless regarded as a useful source of important cut flower characteristics such as late flowering and thin, long and straight stems. With exception of male *L. discolor*, none of these species had previously been used in the development of a hybrid cultivar. The 6 x 6

diallel set of crosses was completed during the 1998 flowering season using various pollination techniques and was repeated in 1999. Two clones of each species were used in each cross combination and two cones per clone for each pollination treatment in each year.

Pollination treatments

The four pollination treatments investigated were

I. Autogamy: cones were covered with glassine bags and left on the plant unpollinated.

II. Open-pollination: cones were left on the plant for natural cross-pollination.

III. Controlled intraspecific hand-pollination: pollen from the male plant transfered to the female plant within the same species. This is equivalent to self pollination in plant breeding and is referred to as such in what follows.

IV. Controlled interspecific hand-pollination: pollen from male plants of a different species.

Controlled hand-pollination

Successful controlled hand-pollination in *Leucadendron* is easily accomplished as no pollen is produced on the female cone and was performed by mimicking natural pollinator activity using a paintbrush as the pollen vector. To test the effectiveness of the pollination technique and to prevent contamination with unwanted pollen, cones were covered before anthesis and left unpollinated. At pollination time the female cone was labeled with information about the pollen parent, the seed parent and the date of pollination. Steps in the pollination procedure were as follows:

Step 1. When one to three florets on the selected female cone (Fig. 3.1. A) showed signs of anthesis the open flowers were removed and the cone covered with a white glasine bag (Fig. 3.1. B) to prevent unwanted pollination by wind and insects.

Step 2. After three to five days depending on the species and on weather conditions the glasine bags were removed and all open flowers pollinated with selected fresh pollen (Fig. 3.1. C). Powdery pollen was collected in small glass vials or eppendorf tubes by using fine-nosed forceps or applied directly from the pollen parent. The microscopically small pollen was applied to the stigmatic area with a fine camelhair brush.

Step 3. Pollinated cones were bagged and pollen application repeated until all the receptive stigmas on the female cone had been pollinated. Cones were left on the plant until seed maturity which could take up to three months for winged seeds and six months for nut-like seeds.

Seed harvesting

Released mature nuts and cones were kept covered to prevent loss of hybrid seeds. Harvesting of cones of serotinous species commenced during the fourth to sixth month after pollination with drying at 30 - 35°C to separate the bracts and discharge the seeds.

Seed germination

Seeds of each cone were kept separately and germinated in petridishes in a growth chamber. Seeds of the two sections were subjected to pre-germination treatments due to their different germination requirements.

Pre-germination treatments

Flat-winged seeds were sterilized with 70% alcohol for 2min. followed by a solution of 15% household Jik with a few drops of tween (a wetting agent) for 15min. Seeds were thoroughly washed with autoclaved distilled water to remove the remaining sterilizing solution. Nut-like seeds with seed-coat-imposed dormancy were subjected to acid scarification and oxygen pre-treatment. Seeds were scarified with H_2SO_4 for 8 min. at 22°C and washed with tap water (Brits and Van Niekerk, 1976). These were then disinfected in distilled water at 50°C for 30min. (Benic, 1986) and incubated in a solution containing 1% H_2O_2 and 200mg/l Promalin® in a dark cupboard for 24hrs. H_2O_2 increases oxygen availability to the embryo while Promalin® increases essential hormone levels which act as promoters for germination (Brits and Van Niekerk, 1976). Seeds were then washed to remove the remaining gelatinous outer pericarp and dried to remove excess water. After seeds of both types had been dried on a paper towel they were further treated with a Thiram concentration of 0.5g/100 seeds to prevent fungal growth in the growth chamber. Seeds were then placed in petridishes on a damp filter paper and incubated in a growth chamber at fluctuating temperatures of 22° - 24°C for 8hrs and 9°-11° for 16hrs. Seed germination was monitored for 4 months.

Data analysis

Analysis of data recorded for seeds produced, seeds germinating and seed germination ratio followed standard procedures to obtain summary statistics, analysis of variance (ANOVA) and significance testing using Microsoft Excel Statistical Functions and Data Analysis facilities. Preliminary data analysis used Statistica Vers. 6. Analysis of data was undertaken by Dr JH Louw.

Results and Discussion

No seeds were observed in flowers that had been protected by glassine bags, thus excluding autogamy as a mechanism of seed production in these species and indicating as well that glassine bag protection successfully prevents contamination in crossing by artificial hand pollination.

Full results of seeds set are presented in Appendix A (Open pollination), Appendix B (Self pollination) and Appendix C (Cross pollination) including summary statistics, ANOVA and the Least Significant Difference (LSD) test statistic, where it should be noted that (JH Louw, personal communication):

(i) Preliminary analysis of seeds produced in cones within clones and clones within species-year combinations detected no significant effects. Seeds of the four cones recorded for each of the two years could then be analyzed as four replicate counts as reflected in the ANOVA tables.

(ii) Year and year-species/cross interactions are not significant with one exception out of nine independent tests ($P = 0.01$ for germination interaction for open pollination). Interaction variance and variance of replicate counts within years could therefore be pooled to obtain the best estimate of Error variance for the calculation of LSDs based on Student's t distribution as in the ANOVA tables.

(iii) Seed germination was calculated as the proportion, p, of seeds germinating, transformed to radians, i.e., $\arcsin\sqrt{p}$, as is appropriate for

analysis of proportions (Sokal and Rohlf, 1995; Biometrics, 3rd Ed., WH Freeman Co.).

(iv) The more stringent level of statistical significance and LSDs, P = 0.01, is advisable for repeated (multiple) comparisons as might be applied to results of this nature.

(v) Intrinsic variation in metric biological traits, such as seed counts, commonly exhibit a positive correlation between variance and mean. It is therefore advisable to express the variation in terms of the coefficient of variation (CV) which is free of the scale of measurement of the trait and permits free comparisons across species and pollination methods.

(vi) Small numbers of seeds produced in some crosses (< 10), leading also to unrealistic or undefined CVs, are highlighted in the relevant tables and were omitted in calculating Mean CVs as given in the ANOVA table for crosses.

Further breakdown of the data in terms of totals and means per cone (Table 3.2) shows minor differences between seed set by natural open and self pollination by hand, thus confirming the effectiveness of the artificial hand pollination technique, but a major reduction in seed set by artificial hand occurs in cross pollination between species. This is at least partly ascribable to practical difficulties and environmental conditions which only became evident during the course of the experiments, and is typical of practical applications of established plant breeding principles (such as diallel crossing) in relatively unexplored crops (JH Louw, personal communication). In this case, pollen and cone

harvesting was found to be difficult as a result of (i) the limited period of time available for pollen and cone harvesting - pollen was only available and stigmas receptive during a two-week flowering period, and the flowering times of male and female flowers both within and between species did not always coincide, (ii) effects of weather conditions - rain, humidity, temperature and possibly other unidentified environmental factors. These factors confound any interpretation of poor crossability due to inherent incompatabity between species which might exist.

Highly significant differences between species in the number of seeds produced, seeds germinating and seed germination are evident for all modes of pollination (open, self and artificial hand cross pollination, Appendices A, B and C) and are summarized in Table 3.1. The results identify *L. nobile* as the most prolific seed producing species under open and self pollination and as having higher than average seed germination. *L. chamalaea* has high seed production but relatively poor seed germination.

Crossability results are further summarized in Table 3.3, providing useful information for future breeding projects. Small numbers of seeds set, including no seeds (0) in some cases, precludes the standard genetic analysis of diallel cross results in terms of combining ability effects (general, specific and reciprocal effects) as was intended in the original design of the experiment, as well as any further analysis in terms of the classification of species according to sub-sections. However, the results show significantly higher numbers of seeds germinating and seed germination in crosses within the 'Alatosperma' group than in crosses within the 'Leucadendron' group and crosses between groups. The reciprocal difference in total seeds set in the cross *L. galpinii* X *L.*

chamelaea is significant but seeds set in this cross with *L. galpinii* as female parent are clearly inviable (0 germination). Differences in seed viability in reciprocal crosses may be ascribed to the cytoplasmic contribution of the female parent, and differences in seed set to endosperm failure as is commonly observed in interspecific crosses (Allard, 1978).

The crossability results in the present study have further identified 15 cases of likely total sterility (no seeds set) and five cases of significantly small numbers (less than 10 seeds) as highlighted in Table 3.3. Seed germination in the cross *L. uliginosum* X *L. discolor* significantly exceeds that of all other crosses, and the means of crosses involving *L. galpinii* and *L. uliginosum* as female parents significantly exceed those of all other species combinations (Table 3.2). Remarkably, *L. discolor* appears to be completely sterile in crosses as female parent (0 seeds set) but highly fertile in both seeds set and seed germination in crosses as male parent.

Results of inherent variability within species and crosses in terms of the coefficient of variation (CV) and mean CV are presented in Table 3.4. Results for seeds set by natural open and self pollination show means of 17.0 and 15.2 percent respectively and is not inconsistent with experience of seed count data in other crops such as Maize and small grain cereals which are typically in a range between 12 and 20 percent in large samples (JH Louw, personal communication). Comparable values for variability in seed germination (14.0 and 8.5 percent) are accountable to the fact that the measurement is a ratio of two correlated variables, in this case, seeds germinating and total seeds set. Results of the further breakdown of the diallel table in terms variability in specific crosses are given in Table 3.5 and shows again the imbalance created by failed cross

pollinations in this programme, as well as totally unrealistic CVs due to small numbers of seeds set as already highlighted in Table 3.3. The results further indicate that the significant overall reduction in seeds set by cross pollination is also accompanied by increased variance in many crosses.

In general, unlike other Proteaceae studied to date (Vogts, 1960; Horn, 1962; Brits, 1984; Rebelo and Rourke, 1986; Esler and Cowling, 1990), *Leucadendron* appears to have relatively high seed set under natural and artificial conditions but also at least some degree of species incompatibility, as has also been found in studies by Williams (1972) and by Van den Berg and Brits (1995). Interspecific incompatibility is termed by Hogenboom (1973) as incongruity, attributable to "a lack of genetic information in one of the partners about the structure and or physiology of the other partner". This is distinctly different from incompatibility observed between plants within a species. Apart from non-genetic factors identified in the present experiments, the poor seed set and failure of seed set in many cases following interspecific crosses appears to indicate interspecific incongruency which cannot be completely ascribed to pollen or ovule fertility as seed set was obtained in at least one of the species cross combinations of the diallel.

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Table 3.1. Classification, accession and provenance of selected *Leucadendron* species in the Fynbos Genebank of the Agricultural Research Council, Elsenburg.

Section	Sub-Section	Species / Accession	Provenance / Date
'Alatosperma' flat, winged seeds	Delta-seed Conebushes	*L. uliginosum* subsp. *uliginosum*	Outeniqua
		female (T 94 05 21)	May 1994
		male (T 94 05 21)	May 1994
		L. conicum	Tsitsikamma
		female (T 94 10 13)	October 1994
		male (T 94 10 10)	October 1994
	Sunshine Conebushes	*L. discolor*	Piketberg
		female (T 95 03 58)	March 1995
		male (T 94 10 33)	October 1994
	Needle leaf Conebushes	*L. nobile*	Baviaanskloof
		female (T124/94/1	Date unknown
		male (T 124/94/1)	Date unknown
'Leucadendron' nut-like seeds	Sandveld Crownbushes	*L.galpinii*	Albertinia
		female (T 93 12 05)	December 1993
		male (T 94 10 56)	October 1994
	Crown Conebushes	*L. chamelaea*	Koue Bokkeveld
		female (T 89 09 18)	September 1989
		male (T 94 03 81)	March 1994

Fig. 3.1A. The stage of the florets when the female cone is prepared for controlled hand-pollination.

Fig. 3.1B. Female cone covered with a glassine bag to prevent contamination with unwanted pollen.

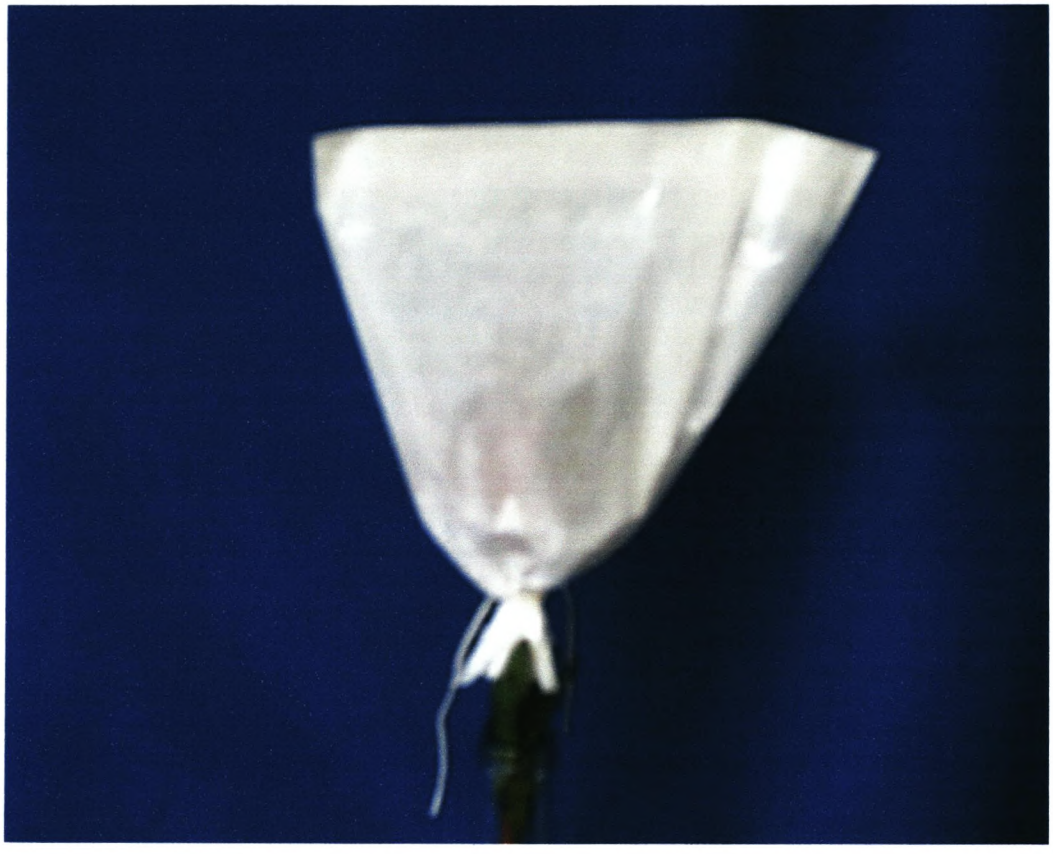

Fig. 3.1C. Artificial controlled hand-pollination with a paintbrush between the male and females inflorescence.

Table 3.2. Totals and means per cone for seeds produced, seeds germinating and germination proportions (transformed to radians) in selected *Leucadendron* species and species crosses as female and male parent. Species abbreviations: *uli: L. uliginosum*; *con: L. conicum*; *dis: L. discolor*; *nob: L. nobile*; *gal: L. galpinii*; *cha: L. chamelaea*. LSD (.01) and LSD (.05) refer to Least Significant Differences at 1% and 5% probability levels respectively.

	Total	*uli*	*con*	*dis*	*nob*	*gal*	*cha*	LSD 0.01	LSD 0.05	Mean	LSD 0.01	LSD 0.05
Seeds												
Open pollination	2804	24.88	20.25	47.25	155.88	30.63	71.63	14.37	10.74	58.42	5.87	4.38
Self pollination	2653	35.75	22.75	39.63	132.25	29.38	71.88	12.62	9.43	55.27	5.15	3.85
Cross as female	1350	11.63	3.45	0	3.50	14.35	0.83	2.36	1.78	5.63	0.96	0.73
Cross as male	1350	5.53	8.00	8.98	2.03	3.88	5.35	2.36	1.78	5.63	0.96	0.73
Seeds germinating												
Open pollination	1996	23.75	16.38	39.25	130.75	15.38	24.00	11.18	8.35	41.58	4.56	3.41
Self pollination	2025	30.50	18.25	38.25	119.88	21.13	25.13	9.14	6.83	42.19	3.73	2.79
Cross as female	435	6.18	2.35		1.93	0.33	0.10	1.25	0.94	1.81	0.51	0.38
Cross as male	435	1.95	2.80	4.98	0.23	0.80	0.10	1.25	0.94	1.81	0.51	0.38
Germination (radians)												
Open pollination		1.409	1.169	1.145	1.182	1.003	0.618	0.209	0.156	1.088	0.085	0.064
Self pollination		1.180	1.121	1.434	1.342	1.022	0.638	0.130	0.097	1.123	0.053	0.040
Cross as female		0.674	0.314		0.167	0.067	0.049	0.119	0.090	0.212	0.049	0.037
Cross as male		0.175	0.170	0.559	0.095	0.235	0.039	0.119	0.090	0.212	0.049	0.037
Germination (proportion *)												
Open pollination										0.784	0.007	0.004
Self pollination										0.812	0.003	0.002
Cross as female										0.044	0.002	0.001
Cross as male										0.044	0.002	0.001

* radians retransformed

Table 3.3. Means per cone for seed produced, seeds germinating and germination proportions (transformed to radians) in the diallel set of crosses between *Leucadendron* species. See Table 3.2 for LSD and species abbreviations. Shaded entries highlight small numbers of seeds produced (< 10).

Seeds

Male \ Female	*uli*	*con*	*dis*	*nob*	*gal*	*cha*
uli	*	0	0	17.50	10.13	0
con	25.38	*	0	0	14.63	0
dis	13.63	14.25	*	0	17.00	0
nob	2.50	0	0	*	7.63	0
gal	12.25	3.00	0	0	*	4.13
cha	4.38	0	0	0	22.38	*

LSD (.05) 3.98
LSD (.01) 5.28

Seeds germinating

Male \ Female	*uli*	*con*	*dis*	*nob*	*gal*	*cha*
uli	*			9.63	0.13	
con	14.00	*			0	
dis	13.13	10.25	*		1.50	
nob	1.13			*	0	
gal	2.13	1.38			*	0.50
cha	0.50				0	*

LSD (.05) 2.10
LSD (.01) 2.78

Germination (radians)

Male \ Female	*uli*	*con*	*dis*	*nob*	*gal*	*cha*
uli	*			0.836	0.038	
con	0.848	*				
dis	1.485	1.010	*		0.298	
nob	0.473			*		
gal	0.371	0.559			*	0.247
cha	0.193					*

LSD (.05) 0.200
LSD (.01) 0.265

* Selfs (see Table 3.2)

Table 3.3. continued

Germination (proportions **)

		Female					
		uli	*con*	*dis*	*nob*	*gal*	*cha*
Male	*uli*	*			0.550	0.001	
	con	0.562	*				
	dis	0.993	0.717	*		0.086	
	nob	0.208			*		
	gal	0.131	0.281			*	0.060
	cha	0.037					*

LSD (.05) 0.039
LSD (.01) 0.069

* Selfs (see Table 3.2)
** Mean radians retransformed

Table 3.4. Coefficients of Variation (CV) for seeds produced, seeds germinating and germination proportions (transformed to radians) in *Lecadendron* species and species crosses as female and male parent. See Table 3.2 for species abbreviations. Shaded entries highlight CVs which are undefined (mean = 0) or unrealistic (due to small numbers).

	uli	*con*	*dis*	*nob*	*gal*	*cha*	Mean
Seeds							
Open pollination	0.190	0.310	0.166	0.140	0.114	0.098	0.170
Self pollination	0.158	0.208	0.088	0.132	0.265	0.062	0.152
Cross as female parent	0.560	0.475		0.406	0.288	0.492	0.446
Cross as male parent	0.293	0.270	0.219	0.848	0.513	0.536	0.446
Seeds germinating							
Open pollination	0.170	0.271	0.230	0.123	0.174	0.189	0.193
Self pollination	0.190	0.246	0.271	0.065	0.176	0.230	0.196
Cross as female parent	0.780	0.539		0.426	1.592	1.069	0.881
Cross as male parent	1.627	0.326	0.295	1.108	0.857	1.512	0.881
Germination (radians)							
Open pollination	0.115	0.190	0.098	0.121	0.221	0.092	0.140
Self pollination	0.053	0.117	0.082	0.032	0.118	0.105	0.085
Cross as female parent	0.630	0.384		0.092	1.491	1.073	0.734
Cross as male parent	1.460	0.192	0.123	0.843	0.732	1.545	0.734
Germination (proportion **)							
Open pollination	0.013	0.036	0.010	0.015	0.048	0.008	0.019
Self pollination	0.003	0.014	0.007	0.001	0.014	0.011	0.007
Cross as female parent	0.347	0.140		0.008	0.994	0.772	0.449
Cross as male parent	0.988	0.036	0.015	0.557	0.447	0.999	0.449

** radians retransformed

Table 3.5. Coefficients of Variation (CV) for seeds produced, seeds germinating and germination proportions (transformed to radians) in the diallel set of crosses between *Leucadendron* species. See Table 3.2 for LSD and species abbreviations. Shaded entries highlight small number cases as in Table 3.3.

Seeds

		Female						
		uli	*con*	*dis*	*nob*	*gal*	*cha*	Mean
Male	*uli*	*			0.406	0.179		0.293
	con	0.240	*			0.299		0.270
	dis	0.260	0.236	*		0.160		0.219
	nob	0.980			*	0.715		0.848
	gal	0.334	0.713			*	0.492	0.513
	cha	0.985				0.086	*	0.536
	Mean	0.560	0.475		0.406	0.288	0.492	0.446

Seeds germinating

		Female						
		uli	*con*	*dis*	*nob*	*gal*	*cha*	Mean
Male	*uli*	*			0.426	2.828		1.627
	con	0.326	*					0.326
	dis	0.221	0.307	*		0.356		0.295
	nob	1.108			*			1.108
	gal	0.731	0.771			*	1.069	0.857
	cha	1.512					*	1.512
	Mean	0.780	0.539		0.426	1.592	1.069	0.881

Germination (radians)

		Female						
		uli	*con*	*dis*	*nob*	*gal*	*cha*	Mean
Male	*uli*	*			0.092	2.828		1.460
	con	0.192	*					0.192
	dis	0.107	0.108	*		0.153		0.123
	nob	0.843			*			0.843
	gal	0.465	0.659			*	1.073	0.732
	cha	1.545					*	1.545
	Mean	0.630	0.384		0.092	1.491	1.073	0.734

Table 3.5. continued

Germination (proportions **)

		Female						
		uli	*con*	*dis*	*nob*	*gal*	*cha*	
Male	*uli*	*			0.008	0.095		0.988
	con	0.036	*					0.036
	dis	0.011	0.012	*		0.023		0.015
	nob	0.557			*			0.557
	gal	0.201	0.375			*	0.772	0.447
	cha	0.999					*	0.999
		0.347	0.140		0.008	0.994	0.772	0.449

* Selfs (see Table 3.2)
** radians retransformed

APPEDICES Chapter 3

Summary Statistics and Analysis of Variance of seeds produced, seeds germinating and seed germination.

APPENDIX A	Open pollination
APPENDIX B	Self pollination (between male and female plants within species)
APPENDIX C	Cross pollination

Species name abbreviations: *uli: L. uliginosum; con: L. conicum; dis: L. discolor; nob: L. nobile; gal: L. galpinii; cha: L. chamelaea*

Open pollination

	Seeds			Seeds germinating			Germination (radians)		
	year1	year2	Total	year1	year2	Total	year1	year2	Total
uliginosum									
Count	4	4	8	4	4	8	4	4	8
Total	98	101	199	93	97	190	5.5098	5.7648	11.2746
Mean	24.50	25.25	24.88	23.25	24.25	23.75	1.3775	1.4412	1.4093
Variance			22.41			16.21			0.0187
CV			0.190			0.170			0.115
conicum									
Count	4	4	8	4	4	8	4	4	8
Total	82	80	162	65	66	131	4.4766	4.8788	9.3554
Mean	20.50	20.00	20.25	16.25	16.50	16.38	1.1192	1.2197	1.1694
Variance			39.36			19.70			0.0424
CV			0.310			0.271			0.190
discolor									
Count	4	4	8	4	4	8	4	4	8
Total	205	173	378	175	139	314	4.7219	4.4382	9.1602
Mean	51.25	43.25	47.25	43.75	34.75	39.25	1.1805	1.1096	1.1450
Variance			61.36			81.36			0.0110
CV			0.166			0.230			0.098
nobile									
Count	4	4	8	4	4	8	4	4	8
Total	620	627	1247	500	546	1046	4.6433	4.8121	9.4554
Mean	155.00	156.75	155.88	125.00	136.50	130.75	1.1608	1.2030	1.1819
Variance			477.27			258.50			0.0173
CV			0.140			0.123			0.121
galpinii									
Count	4	4	8	4	4	8	4	4	8
Total	119	126	245	58	65	123	4.8121	3.2089	8.0210
Mean	29.75	31.50	30.63	14.50	16.25	15.38	1.2030	0.8022	1.0026
Variance			12.27			7.13			0.0489
CV			0.114			0.174			0.221

Open pollination (continued)

	Seeds			Seeds germinating			Germination (radians)		
	year1	year2	Total	year1	year2	Total	year1	year2	Total
chameleae									
Count	4	4	8	4	4	8	4	4	8
Total	274	299	573	91	101	192	2.4534	2.4908	4.9442
Mean	68.50	74.75	71.63	22.75	25.25	24.00	0.6133	0.6227	0.6180
Variance			49.41			20.57			0.0053
CV			0.098			0.189			0.092

ANOVA		Seeds			Seeds germinating			Germination (radians)		
	df	MS	F	P	MS	F	P	MS	F	P
Species	5	21042.23	171.60	4.64E-24	15849.08	240.04	1.38E-26	0.5602	31.4545	3.46E-12
Years	1	1.33	0.01	0.92	21.33	0.32	0.57	0.0218	1.2253	0.28
Interaction	5	43.73	0.36	0.87	85.18	1.29	0.29	0.0683	3.8360	0.01
Within	36	122.63			66.03			0.0178		
Total	47									
Error variance (df = 41)		113.00407			68.363821			0.023969		
Mean CV		0.170			0.193			0.140		
t (.05; 41)		2.021			2.021			2.021		
t (.01; 41)		2.704			2.704			2.704		
LSD (.05)		10.742			8.355			0.156		
LSD (.01)		14.372			11.179			0.209		

Self pollination

	Seeds			Seeds germinating			Germination (radians)		
	year1	year2	Total	year1	year2	Total	year1	year2	Total
uliginosum									
Count	4	4	8	4	4	8	4	4	8
Total	141	145	286	124	120	244	4.8731	4.5703	9.4434
Mean	35.25	36.25	35.75	31.00	30.00	30.50	1.2183	1.1426	1.1804
Variance			39.93			33.43			0.0039
CV			0.158			0.190			0.053
conicum									
Count	4	4	8	4	4	8	4	4	8
Total	83	99	182	70	76	146	4.6302	4.3351	8.9653
Mean	20.75	24.75	22.75	17.50	19.00	18.25	1.1576	1.0838	1.1207
Variance			23.07			20.21			0.0171
CV			0.208			0.246			0.117
discolor									
Count	4	4	8	4	4	8	4	4	8
Total	164	153	317	158	148	306	5.7764	5.6925	11.4689
Mean	41	38.25	39.63	39.50	37.00	38.25	1.4441	1.4231	1.4336
Variance			127.98			107.07			0.0137
CV			0.088			0.271			0.082
nobile									
Count	4	4	8	4	4	8	4	4	8
Total	529	529	1058	498	461	959	5.3294	5.4063	10.7357
Mean	132.25	132.25	132.25	124.50	115.25	119.88	1.3323	1.3516	1.3420
Variance			57.36			60.70			0.0018
CV			0.132			0.065			0.032
galpinii									
Count	4	4	8	4	4	8	4	4	8
Total	110	125	235	83	86	169	4.2033	3.9687	8.1720
Mean	27.50	31.25	29.38	20.75	21.50	21.13	1.0508	0.9922	1.0215
Variance			14.27			13.84			0.0145
CV			0.265			0.176			0.118

Selfs (continued)

	Seeds			Seeds germinating			Germination (radians)		
	year1	year2	Total	year1	year2	Total	year1	year2	Total
chamelaea									
Count	4	4	8	4	4	8	4	4	8
Total	268	307	575	87	114	201	2.4894	2.6131	5.1024
Mean	67.00	76.75	71.88	21.75	28.50	25.13	0.6223	0.6533	0.6378
Variance			259.55			33.27			0.0045
CV			0.062			0.230			0.105

ANOVA	Seeds				Seeds germinating			Germination (radians)		
	df	MS	F	P	MS	F	P	MS	F	P
Species	5	13688.87	145.47	7.90E-23	11992.34	270.29	1.74E-27	0.6295	63.7624	7.09E-17
Years	1	82.69	0.88	0.35	4.69	0.11	0.75	0.0107	1.0813	0.31
Interaction	5	36.94	0.39	0.85	55.54	1.25	0.31	0.0044	0.4474	0.81
Within	36	94.10			44.37			0.0099		
Total	47									
Error variance (df = 41)		87.132622			45.730183			0.009208		
Mean CV		0.152			0.196			0.084		
t (.05; 41)		2.021			2.021			2.021		
t (.01; 41)		2.704			2.704			2.704		
LSD (.05)		9.432			6.833			0.097		
LSD (.01)		12.620			9.143			0.130		

Cross pollination (female/male)

APPENDIX C

	Seeds			Seeds germinating			Germination (radians)		
	year1	year2	Total	year1	year2	Total	year1	year2	Total
1 *uli/con*									
Count	4	4	8	4	4	8	4	4	8
Total	94	109	203	49	63	112	3.3633	3.4209	6.7842
Mean	23.50	27.25	25.38	12.25	15.75	14.00	0.8408	0.8552	0.8480
Variance	19.00	58.25	37.13	10.92	29.58	20.86	0.0563	0.0054	0.0265
CV			0.240			0.326			0.192
2 *uli/dis*									
Count	4	4	8	4	4	8	4	4	8
Total	58	51	109	56	49	105	5.9614	5.9218	11.8833
Mean	14.50	12.75	13.63	14.00	12.25	13.13	1.4904	1.4805	1.4854
Variance	19.67	7.58	12.55	13.33	4.25	8.41	0.0259	0.0326	0.0251
CV			0.260			0.221			0.107
3 *uli/nob*									
Count	4	4	8	4	4	8	4	4	8
Total	14	6	20	7	2	9	2.3852	1.4009	3.7861
Mean	3.50	1.50	2.50	1.75	0.50	1.13	0.5963	0.3502	0.4733
Variance	9.67	1.67	6.00	2.25	0.33	1.55	0.1630	0.1684	0.1593
CV			0.980			1.108			0.843
4 *uli/gal*									
Count	4	4	8	4	4	8	4	4	8
Total	56	42	98	11	6	17	1.7452	1.2191	2.9644
Mean	14.00	10.50	12.25	2.75	1.50	2.13	0.4363	0.3048	0.3705
Variance	13.33	17.67	16.79	2.92	1.67	2.41	0.0092	0.0486	0.0297
CV			0.334			0.731			0.465
5 *uli/cha*									
Count	4	4	8	4	4	8	4	4	8
Total	20	15	35	1	3	4	0.2928	1.2490	1.5419
Mean	5.00	3.75	4.38	0.25	0.75	0.50	0.0732	0.3123	0.1927
Variance	23.33	18.92	18.55	0.25	0.92	0.57	0.0214	0.1473	0.0886
CV			0.985			1.512			1.545

Cross pollination (female/male) continued

	Seeds			Seeds germinating			Germination (radians)		
	year1	year2	Total	year1	year2	Total	year1	year2	Total
6 *con/dis*									
Count	4	4	8	4	4	8	4	4	8
Total	64	50	114	45	37	82	3.9928	4.0834	8.0763
Mean	16.00	12.50	14.25	11.25	9.25	10.25	0.9982	1.0209	1.0095
Variance	8.67	9.67	11.36	4.92	15.58	9.93	0.0065	0.0208	0.0119
CV			0.236			0.307			0.108
7 *con/gal*									
Count	4	4	8	4	4	8	4	4	8
Total	13	11	24	5	6	11	2.0164	2.4569	4.4732
Mean	3.25	2.75	3.00	1.25	1.50	1.38	0.5041	0.6142	0.5592
Variance	6.25	4.25	4.57	0.92	1.67	1.13	0.1194	0.1892	0.1357
CV			0.713			0.771			0.659
8 *nob/uli*									
Count	4	4	8	4	4	8	4	4	8
Total	70	70	140	37	40	77	3.2993	3.3848	6.6841
Mean	17.50	17.50	17.50	9.25	10.00	9.63	0.8248	0.8462	0.8355
Variance	57.67	60.33	50.57	12.92	26.00	16.84	0.0019	0.0116	0.0059
CV			0.406			0.426			0.092
9 *gal/uli*									
Count	4	4	8	4	4	8	4	4	8
Total	39	42	81	1	0	1	0.3063	0	0.3063
Mean	9.75	10.50	10.13	0.25	0	0.13	0.0766	0	0.0383
Variance	3.58	3.67	3.27	0.25	0	0.13	0.0235	0	0.0117
CV			0.179			2.828			2.828
10 *gal/con*									
Count	4	4	8	4	4	8	4	4	8
Total	49	68	117	0	0	0	0	0	0
Mean	12.25	17.00	14.63	0	0	0	0	0	0
Variance	16.92	12.67	19.13	0	0	0	0	0	0
CV			0.299						

Cross pollination (female/male) continued

	Seeds			Seeds germinating			Germination (radians)		
	year1	year2	Total	year1	year2	Total	year1	year2	Total
11 *gal/disc*									
Count	4	4	8	4	4	8	4	4	8
Total	63	73	136	6	6	12	1.2373	1.1448	2.3821
Mean	15.75	18.25	17.00	1.50	1.50	1.50	0.3093	0.2862	0.2978
Variance	8.25	4.92	7.43	0.33	0.33	0.29	0.0022	0.0023	0.0021
CV			0.160			0.356			0.153
12 *gal/nob*									
Count	4	4	8	4	4	8	4	4	8
Total	16	45	61	0	0	0	0	0	0
Mean	4.00	11.25	7.63	0	0	0	0	0	0
Variance	27.33	6.92	29.70	0	0	0	0	0	0
CV			0.715						
13 *gal/cha*									
Count	4	4	8	4	4	8	4	4	8
Total	88	91	179	0	0	0	0	0	0
Mean	22.00	22.75	22.38	0	0	0	0	0	0
Variance	2.00	6.25	3.70	0	0	0	0	0	0
CV			0.086						
14 *cha/gal*									
Count	4	4	8	4	4	8	4	4	8
Total	17	16	33	2	2	4	0.9872	0.9872	1.9745
Mean	4.25	4.00	4.13	0.50	0.50	0.50	0.2468	0.2468	0.2468
Variance	0.92	8.67	4.13	0.33	0.33	0.29	0.0818	0.0818	0.0701
CV			0.492			1.069			1.073

Cross pollination (female/male) continued

ANOVA	Seeds			Seeds germinating			Germination (radians)		
	df	MS	F	P	MS	F	P	MS	F	P
Crosses	13	412.59	26.38	3.02E-24	225.35	48.54	1.96E-33	1.6113	37.01	3.17E-29
Years	1	7.00	0.45	0.51	0.32	0.07	0.79	0.0009	0.02	0.89
Interaction	13	19.46	1.24	0.26	3.57	0.77	0.69	0.0237	0.55	0.89
Within	84	15.64			4.64			0.0435		
Total	111									

Error variance (df = 97)	16.154639		4.499264		0.040886	
Mean CV	0.244588	(crosses = 9)	0.320232	(crosses = 4)	0.124750	(crosses = 4)
Individual crosses	16.154639		4.499264			
t (.05; 97)	1.982		1.982		1.982	
t (.01; 97)	2.625		2.625		2.625	
LSD (.05)	3.983		2.102		0.2004	
LSD (.01)	5.275		2.784		0.2654	
Means of 5 crosses	3.2309278		0.899853		0.008177	
t (.05; 97)	1.982		1.982		1.982	
t (.01; 97)	2.625		2.625		2.625	
LSD (.05)	1.781		0.940		0.090	
LSD (.01)	2.359		1.245		0.119	

Stigma morphology and pollen tube growth in *Leucadendron*

Abstract

The pollen receptive area and the style, as the path of the pollen tube to the ovule, were investigated as possible areas suspected to limit crossability between species of *Leucadendron*. Magnifying the specialized stigma area of species belonging to both sections 'Alatosperma' and 'Leucadendron' by means of a scanning electron microscope (SEM) has shown a difference in the shape, but the structure appears to be the same in all species. The stigmatic surfaces of all species examined were densely packed with a large number of unicellular papilla cells on a swollen base as seen. All stigmas were found to have a round to oval shape, except *L. rubrum* with a deep pseudo-stigmatic groove in the center, which elongates further into the style like stigmas of the hermaphrodite Proteaceae. An application of aniline-blue fluorescence exhibits yellow-green fluorescent callose in the pollen tubes under ultraviolet (UV) light. Pollen germination and penetration commences after about four hours following controlled pollination. In compatible selfs, it is evident that a sharp reduction in tube number occurs in the regions between the upper and lower style. The initial mean tube number on the stigma surface (26.5) is reduced to less than five tubes in the lower style and ovule regions eight days later. In interspecific crosses the initial number of tubes on the stigma surface is significantly reduced (mean = 11.4) in comparison to 26.5 for selfs, and rapidly declines to zero in successive regions of the style. Over all species crosses, no tubes survived beyond the middle style region.

Introduction

Interspecific hybrid cultivars of *Leucadendron* have been found to exhibit improved floricultural and horticultural characteristics (Littlejohn *et al.*, 1998) and their further development holds good prospects for floricultural marketing, both local and international. However, seed production in many cross combinations is poor and therefore justifies further research on pollen tube growth in the pistil following pollination.

Several studies have been made on the reproduction biology of commercial Proteaceae genera, but little is known about *Leucadendron*. Research on reproduction biology of pollen-pistil interactions has shown that the stigma, as the early nurturing area of the pollen, may play a vital role in controlling interspecific hybridization and regulating compatibility relationships between species (Heslop-Harrison and Shivanna, 1977). Pollen tube arrest and disorientation, as well as abnormalities in the growth pattern, have also been identified as major causes of interference in controlled interspecific crossing in breeding programmes. In the family Proteaceae, much work has been devoted to pistil appearance at maturity and pollen tube behaviour following pollination in the hermaphrodite genera *Banksia* (Clifford and Sedgley, 1993), *Macadamia* (Sedgley *et al.*, 1985) and *Protea* (Van der Walt and Littlejohn, 1996a), but again with little attention as yet to the dioecious *Leucadendron.*

The pistils of some Proteaceae have a receptive groove on the tip of the stigma. Only pollen in close proximity of the stigmatic area germinate and produce tube entry into the groove (van der Walt and Littlejohn, 1996b). *Banksia* (Clifford and Sedgley, 1993),

Macadamia (Sedgley *et al.,* 1985) and *Protea* (Van der Walt and Littlejohn, 1996b) secrete an exudate in the pollen receptive region.

Pollen tube studies in Proteaceae (Ito, 1980; Sedgley, 1983; Fuss and Sedgley, 1991; Goldingay *et al.*, 1991; Van der Walt and Littlejohn, 1996a) have shown that it takes some four to seven days for tubes to reach the ovary. Differences in style length of parents as found in species of Lilium can also result in a stylar barrier limiting interspecific crossing (Van Roggen, 1988).

From studies on pollen tube growth in *Macadamia*, it appears that tube growth is arrested in the style due to swollen tips that appeared to discharge their content through a sub-terminal pore (Sedgley, 1983). In *Protea,* tube growth has been found difficult to observe in the woody style region (van der Walt and Littlejohn, 1996a). However, it was assumed that since normal tube development is observable on the stigma surface and in the ovule region, the area of inter-specific inhibition is in the style. In *Grevillea banksia*, no abnormal pollen tubes were observable at first but only a slow rate of growth of tubes (Herscovitch and Martin, 1989)

The objectives of the present study were to describe the appearance of the stigma during anthesis and the behaviour of pollen tubes after pollination within and between species, and to identify as far as possible regions of pollen tube inhibition in the style by pollen tube counts within predefined regions.

Materials and methods

Species used for examining stigma morphology and pollen tube growth of *Leucadendron* were maintained in a field genebank of the Agricultural Research Council based at

Elsenburg experimental farm, South Africa (latitude 33°51'S, longitude 18°50'E; 177m a.s.l.). Plants were maintained by standard Proteaceae cultivation practices (Coetzee and Littlejohn, 2001).

Stigma morphology

Pistils of the different species were excised out of the woody receptacle and fixed in a FAA_{50} solution prior to magnification of the stigma by means of a scanning electron microscope (SEM). Stigmas were mounted on aluminum stubs with silver paste and sputter-coated with gold. Exomorphology was then examined with a Joel JSM 6100 SEM operated at 5kV and photographed.

Pollen tube growth

Preliminary observations on pollen germination and tube growth were made in selfed cones of *L .uliginosum* in order to ascertain the length of time between germination and penetration of the tube into the ovule region. The presence of tubes was recorded daily in regions defined as (1) stigma, (2) upper style, (3) middle style, (4) lower style and (5) ovule.

Subsequent pollen tube studies involved hand-pollinations of the six species of the diallel crossing programme including selfs and crosses. However, cross-pollinations were confined to *L. chamelaea* and *L. discolor* as female parents as being those crosses that failed to produce seed (Chapter 3). Three female cones were prepared in the field as previously described for cross pollination (Chapter 3). Pollinated cones were left on the plant for eight days in accordance with results of the preliminary experiments with *L. uliginosum*. Branches with pollinated cones were harvested and taken to the laboratory where five pistils, each protected in a bract, were excised from the receptacle of each

cone using a stereo-microscope and scalpel. Pistils were fixed in 1:5:5 (FAA) solution for 24hrs, or until the staining procedure commenced. When preparing for staining, pistils were rinsed three times in distilled water and subsequently soaked in 8N NaOH at 75°C for 4hrs in order to soften the tissue for maceration and penetration of the dye. Softened styles were washed three times in distilled water to remove all NaOH and staining was carried out in a 0.1% solution of water-soluble aniline blue dye in 0.1N $K_3PO_4.H_2O$ buffer after 24hrs (Martin, 1959). Pistils were placed on a slide in a drop of glycerine and covered with a cover-slide for examination under UV-light. Fluorescent tubes were examined by means of a Nikon Biophot microscope equipped with an episcopic fluorescence attachment and a UV-2A filter system consisting of a dichroic mirror (430nm), an ultraviolet excitation filter (380-425nm) and a barrier filter (450nm).

Pollen tube growth was examined in the different regions as defined above and the number of tubes was counted in each region as they became visible. Micrograph images were obtained using Agfa XRG ASA 400 colour film.

Results

Stigma morphology

Gross morphology of the stigma is shown in Fig.4.1. The brush-like stigmas varied slightly in shape from oval in *L. discolor* (Fig. 4.1A) to round in *L. salignum* and all other species examined, while *L. rubrum* (Fig. 4.1C), the exception, has an elongated shape with a deep pseudo-stigmatic groove in the center, which elongates further into the style like stigmas of the hermaphrodite Proteaceae. The stigmatic surfaces of all species are densely packed with a large number of unicellular papilla cells on a swollen base as seen

in Fig. 4.1B from the behind. All papillae seem to be of the same length. Some species exhibit an invaginated cleft in the center of the stigma, and at an early stage a small amount of exudate covers the papillae. With the exception of *L. rubrum,* none of the other species has the cleft extending into the style (Fig. 4.1C)

Pollen tube growth

Results of the preliminary experiments with *L. uliginosum* which monitored the presence of pollen tubes in the various pistil regions over a period of seven days are given in Table 4.1 and led to the choice of the eight day delay after pollination before counting tubes in selfs and crosses which followed. Generally, large numbers of pollen grains become visible on the surface of the stigma and pollen germination and penetration commences after about four hours.

Pollen tube penetration occurs between the papillae cells (Fig. 4.2 A) and grows in all directions in the stigma region producing a fan-like appearance (Fig. 4.2 B). Those reaching the base of the stigma appear to grow towards the center of the style, elongating cohesively (Fig. 4.3) towards the unilocular ovule region. Pollen tubes can reach the upper style region within 24 hours and the ovule region after three days. Pollen tubes reaching the ovule region appeared smooth-walled with callose plugs fluorescing at frequent intervals in the style.

The general pattern of species variation and reduction in pollen tube number during successive stages of growth as counted in successive regions is summarized in Table 4.2 and Fig. 4.4. In the case of compatible selfs, it is evident that a sharp reduction in tube number occurs in the regions between the upper and lower style. 0n the average over all species, the initial mean tube number on the stigma surface (26.5) is reduced to

less than five tubes in the lower style and ovule regions eight days later. Observed differences between species in the early stages are statistically significant (Table 4.3) but no further fine analysis of time (region) trends in differences between species is possible because all data stems from single initial samples of cones and pistils repeatedly scored over time thereafter, thereby generating correlated errors, as is common in survival (and growth) data of this nature (JH Louw, personal communication).

In the case of crosses which could be investigated in the present study, the initial number of tubes on the stigma surface is significantly reduced (mean = 11.4 in comparison to 26.5 for selfs, (Table 4.3) and rapidly declines to zero in successive regions of the style. Over all species crosses, no tubes survive beyond the middle style region (Table 4.2, Fig. 4.4). Notably, there was no tube survival beyond the stigma surface in the case of *L. chamelaea* as female parent in crosses with *L. uliginosum*, *L. conicum* and *L. discolor*, and no survival beyond the middle style region in the case of *L. discolor* as female parent in crosses with *L. nobile*, *L. galpinii* and *L. chamelaea*. Differences between species in initial tube numbers on the stigma surface were not statistically significant (Table 4.3).

Another noteworthy observation reflecting pollen-stigma incompatabilty in species crosses in this material is the intrinsic variability of pollen tube number in the initial stigma region within pistils and cones as sampled. While the overall mean number of tubes per cone falls from 26.4 in selfs to 11.4 in crosses, the coefficients of variation are respectively 8.4 and 21.1 percent (Table 4.3).

Discussion

Proteaceae thus far examined have specialized pollen receptive areas on the stigma, containing stigma papillae enclosed within a small stigmatic groove (Van der Walt and Littlejohn, 1996b; Sedgley *et al.,* 1985). Unlike most Proteaceae, the SEM photographs show that *Leucadendron* species have no stigmatic groove, except for the pseudo-stigmatic groove found on the elongated stigma of *L. rubrum.* This study confirms the stigma length of *L. rubrum* to be around 4mm as described by Williams (1972). Stigmas of all species examined are papillated and pollen tubes enter the stigma at any site. Pollen tubes grow in all directions with a large number of tubes directed by transmitting tissue towards the stylar region. Williams (1972) describes the stigmatic surface as being terminal and larger in anemophilous species.

While the pistil structures of *Protea* (Van der Walt, 1994) *Macadamia* (Sedgley *et al.*, 1985) and *Banksia* (Clifford and Sedgley, 1993) are very similar, containing definite or partial stylar canals to direct the route by which pollen tubes grow toward the ovary, *Leucadendron* pollen tubes appear to be directed by transmitting tissue along the entire length of the style. As in other members of Proteaceae (Herscovich and Martin, 1989; Fuss and Sedgley, 1991), compatible intraspecific combinations in *Leucadendron* in the present study show that the number of pollen tubes entering the ovule region is extremely low with rarely more than three tubes entering the ovule region. The reduction of pollen tube growth beyond the stigma region appears to be similar to that in *Protea* (Van der Walt and Littlejohn, 1996a) and *Macadamia* (Sedgley *et al.,* 1985).

Abnormal and highly variable pollen tube growth patterns (Fig. 4.5) and pollen tube growth arrest in interspecific pollinations as recorded in the present study, such as

swollen tube tips, branching of tubes and the discharge of the tube content at sub-terminal pores, has also been described in M*acadamia* (Sedgley, 1983; Sedgley *et al.,* 1985). Pollen tube growth arrest observed in the style beyond the stigma region is clearly another interspecific barrier to fertilization following interspecific pollination although the causes are unknown. According to Shivanna and Shawney (1997) growing pollen tubes utilize stylar nutrients and tube arrest in the style could be due to a lack of suitable nutrients in the style or lack of suitable enzymes in the pollen tube. Van Tuyl (1996) has proposed methods for overcoming interspecific crossing barriers such as pollination of cut styles with growth hormone or stigmatic exudate and the use of mixed sample of pollen or irradiated mentor pollen are worthwhile exploring further from the point of view of breeding new hybrid cultivars.

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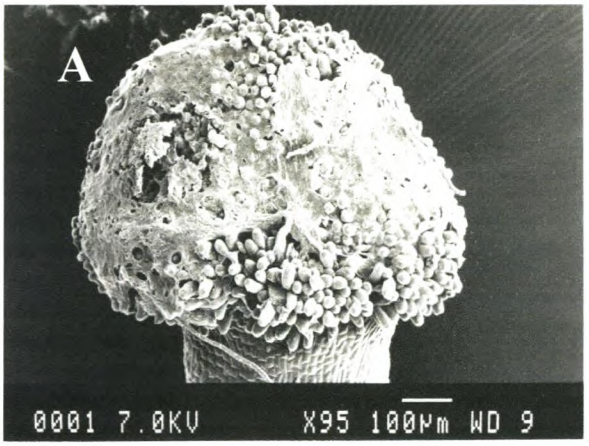
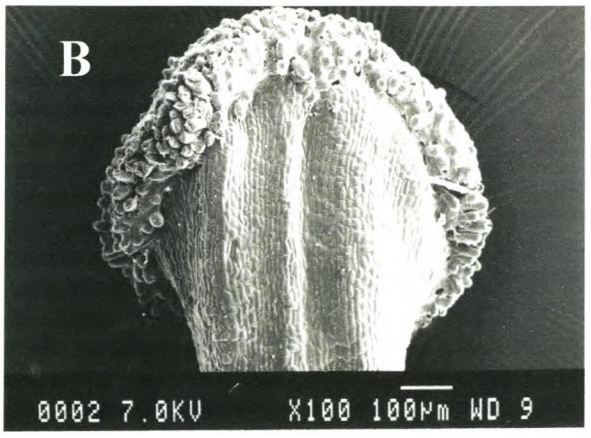
Fig. 4.1A. SEM microcraphs of the front view covered with exudate and **B.** back view of the *L. discolor* stigma. **C.** the elongated stigma of *L. rubrum.*





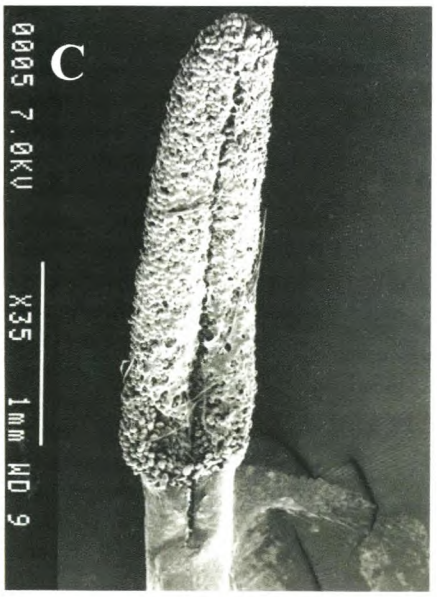


Table 4.1. Incidence of pollen tubes on the stigma surface and in the various regions of the style over time following artificial hand-pollination in the field in *L. uliginosum*. a = tubes absent; p = tubes present.

Days after pollination	Style region				
	Stigma region	Upper	Middle	Lower	Ovule region
0 (4 hrs)	a	a	a	a	a
1	p	p	a	a	a
2	p	p	p	a	a
3	p	p	p	a	a
4	p	p	p	p	p
5	p	p	p	p	p
7	p	p	p	p	p

Fig. 4.2A. Pollen tube penetration through the papillae and **B.** the fanlike appearance of tubes in the stigma region growing in all directions.

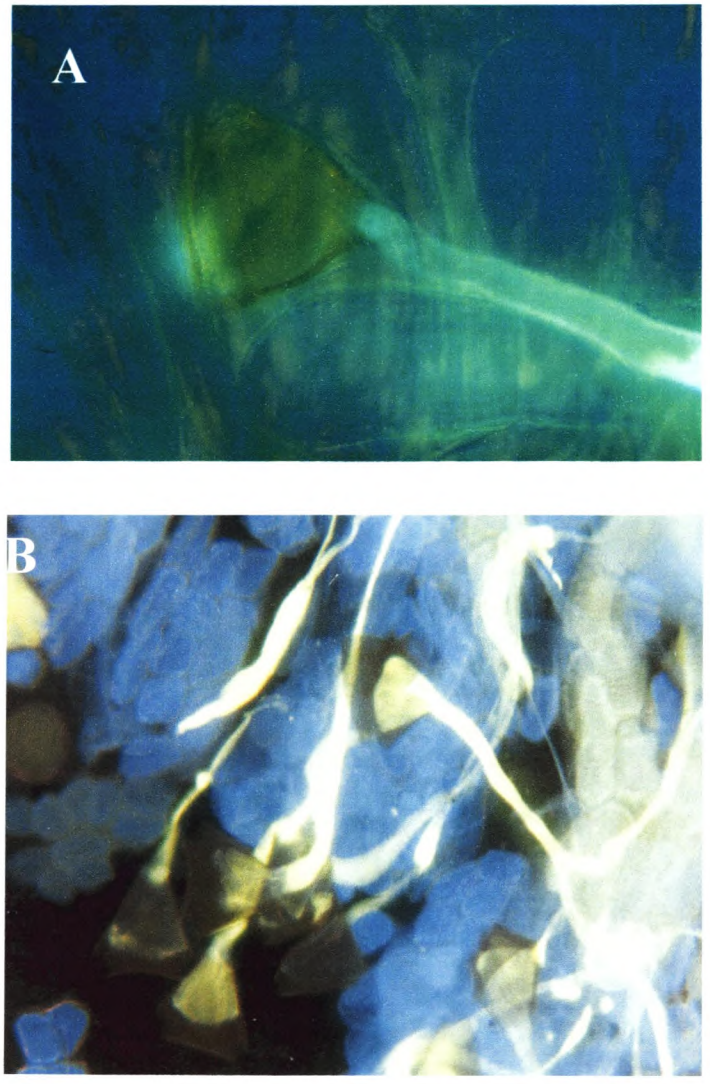


Fig. 4.3. Cohesive growth of normal pollen tubes in the style.

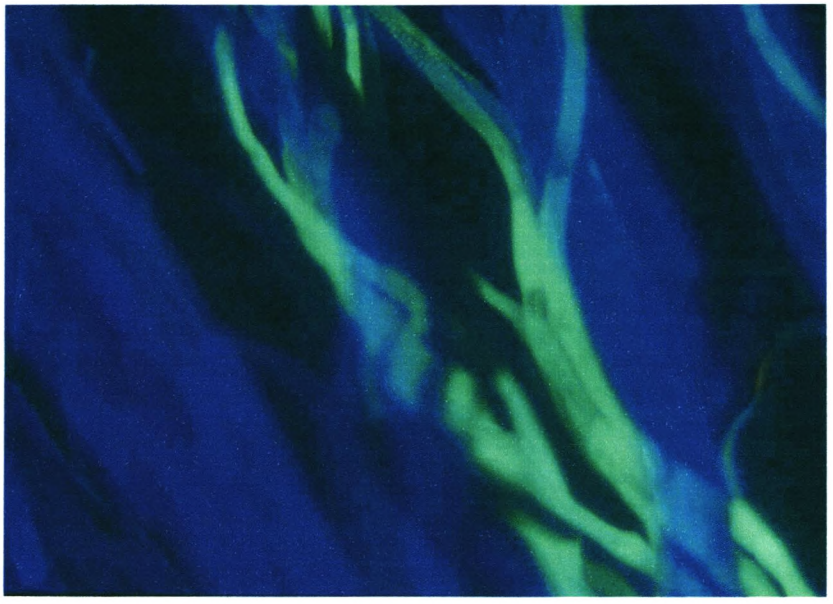


Table 4.2. Mean number of pollen tubes counted in selfs and crosses on the stigma surface and in various regions of the style eight days after artificial hand-pollination in the field. Means based on five pistils of each of three cones harvested. Species abbreviations: *uli*, *L. uliginosum*; *con*, *L. conicum*; *dis*, *L. discolor*; *nob*, *L. nobile*; *gal*, *L. galpinii*; *cha*, *L. chamelaea*.

			Style region				
	Stigma source	Pollen source	Stigma region	Upper	Middle	Lower	Ovule region
(a) Selfs	*uli*	*uli*	31.80	30.20	16.87	2.73	2.53
	con	*con*	23.80	19.93	18.60	2.67	0.60
	dis	*dis*	25.60	20.40	15.73	3.00	2.53
	nob	*nob*	30.47	24.67	15.27	2.13	0.67
	gal	*gal*	24.63	21.67	14.60	1.33	0.73
	cha	*cha*	22.80	17.73	11.60	*	1.40
(b) Crosses	*cha*	*uli*	8.87	0			
	cha	*con*	9.67	0			
	cha	*dis*	10.40	0			
	dis	*nob*	14.53	11.60	4.67	0	
	dis	*gal*	12.40	11.47	0		
	dis	*cha*	14.93	8.67	0.27	0	

* tubes uncountable

Fig. 4.4. Mean number of pollen tubes counted in selfs and crosses on the stigma surface (1) and other defined regions of the style (2 - 5) eight days after artificial hand-pollination in the field. Means based on five pistils of each of three cones harvested. See Table 4.2 for species abbreviations.

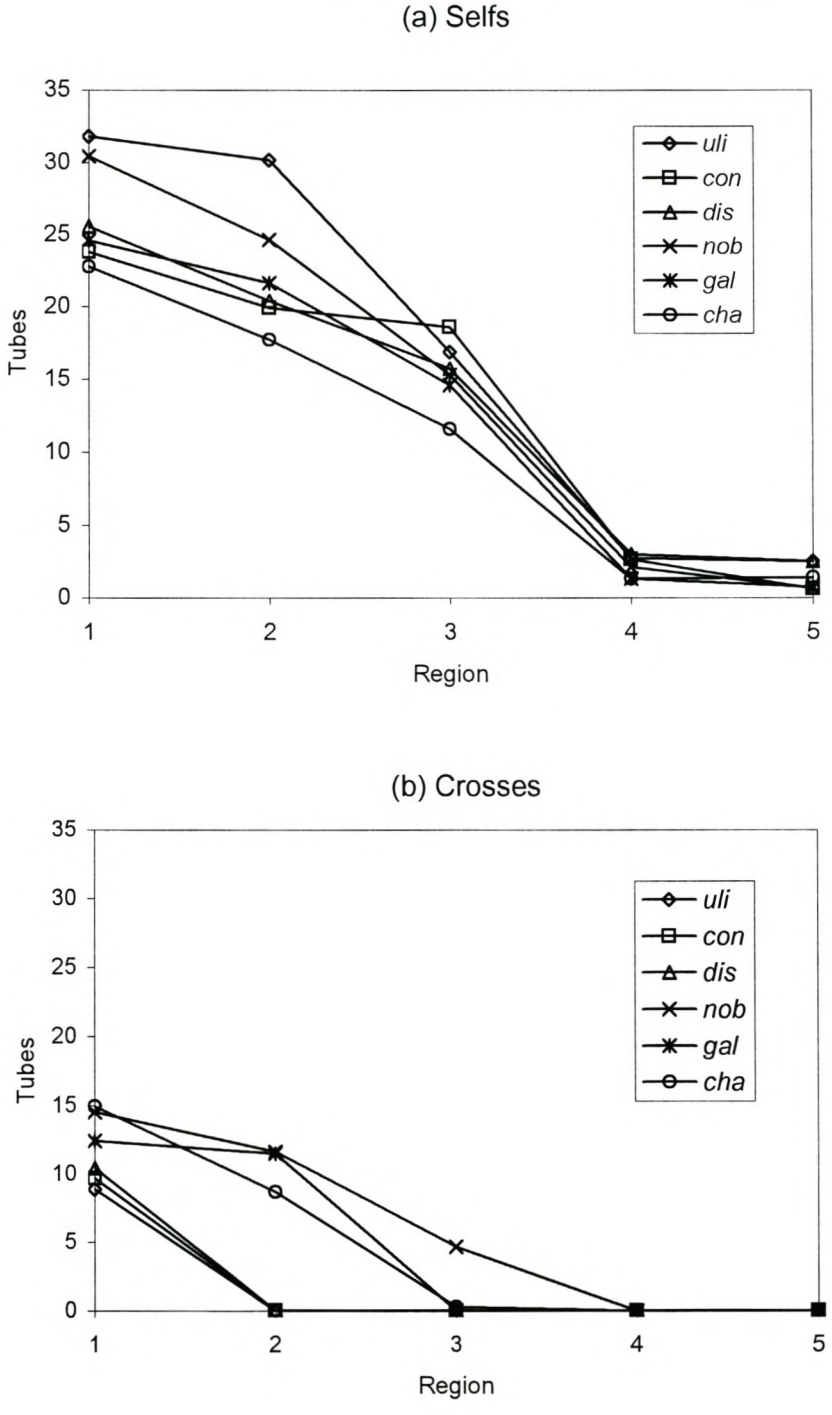


Table 4.3. Summary statistics and ANOVA of mean pollen tube number on the stigma surface of selfs and crosses of *Leucadendron* species. See Table 4.2 for species abbreviations. CV denotes Coefficient of Variation; LSD (0.01), Least Significant Difference at the 1% critical level.

(a) Selfs

Pistil	*uli*	*con*	*dis*	*nob*	*gal*	*cha*
Pollen	*uli*	*con*	*dis*	*nob*	*gal*	*cha*
	32.00	20.00	28.40	31.00	24.20	23.80
	31.20	25.00	22.80	29.00	25.20	19.60
	32.20	26.40	25.60	31.40	23.60	25.00

SUMMARY (means of 5 pistils per cone)

Species	*Cones*	*Sum*	*Mean*		
uli	3	95.40	31.80		
con	3	71.40	23.80		
dis	3	76.80	25.60	Mean	26.47
nob	3	91.40	30.47	CV	0.084
gal	3	73.00	24.33	LSD (.01)	5.56
cha	3	68.40	22.80		

ANOVA (means of 5 pistols per cone)

Source of Variation	*df*	*MS*	*F*	*P-value*
Between Groups	5	42.1813	8.50	0.001
Within Groups	12	4.9644		
Total	17			

(b) Crosses

Pistil	*cha*	*cha*	*cha*	*dis*	*dis*	*dis*
Pollen	*uli*	*dis*	*con*	*cha*	*gal*	*nob*
	7.60	9.40	10.00	14.60	16.20	16.20
	10.20	9.20	11.80	14.20	9.60	9.60
	8.80	12.60	7.20	16.00	11.40	11.40

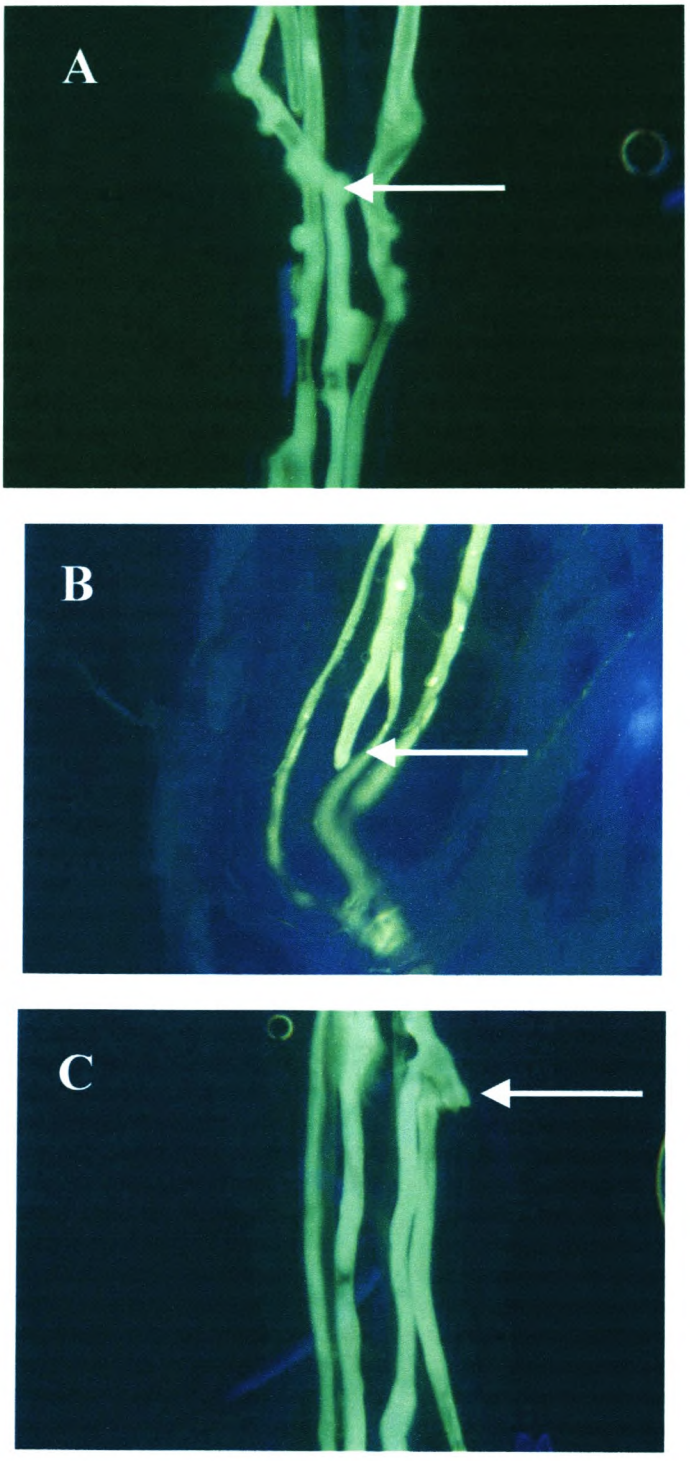
SUMMARY (means of 5 pistils per cone)

Species	*Cones*	*Sum*	*Mean*		
uli	3	26.60	8.87		
dis	3	31.20	10.40		
con	3	29.00	9.67	Mean	11.44
cha	3	44.80	14.93	CV	0.211
gal	3	37.20	12.40	LSD (.01)	6.01
nob	3	37.20	12.40		

ANOVA (means of 5 pistils per cone)

Source of Variation	*df*	*MS*	*F*	*P-value*
Between Groups	5	14.9369	2.57	0.084
Within Groups	12	5.8133		
Total	17			

Fig. 4.5. The → indicate abnormal growth patterns in the style. **A**. sub-terminal pores bursting, **B**. pollen tube arrest and **C**. branching of tubes.



Conclusion

Interspecific hybridization in Proteaceae has over the last decade proved to deliver uniform, quality flowers to the international floriculture industry. The extensive use and ease of cultivation of the hybrid cultivar 'Safari Sunset' as an ornamental cut foliage has ranked *Leucadendron* (conebushes) as one of the leading Proteaceae products on the international floricultural market. Cv.'Safari Sunset' and several other hybrid cultivars were developed in New Zealand from samples of seed of species indigenous to South Africa (Bell, 1988). Of the 91 species and sub-species recorded to date only about 22 are commercially utilized. Currently, opportunities exist to harvest more varied shapes, sizes and colours of the inflorescence, and more marketable products later in the year to fill current gaps in the export market. Although interspecific hybrid cultivars in *Leucadendron* show promising results, seed set following crosses between distantly related species seem to be reduced as in *Protea* (Vogts, 1960; Horn, 1962; Brits, 1984; Rebelo & Rourke, 1986). As has been experienced in other commercial Proteaceae, progress in the development of improved varieties is dependent on breeding research, and should be continued. The large amount of untapped indigenous germplasm, and the need for more variety, has encouraged this study to investigate the crossability of different species and sub-species of *Leucadendron* currently available in South Africa.

In contrast to all commercial hermaphrodite Proteaceae, the dioecious *Leucadendron* displays male and female inflorescences on separate plants. Exomorphological features of *Leucadendron* pollen appear to be similar to the triangular triporate pollen found by Van der Walt (1996) in *Protea*. Pollen availability and harvesting caused an early delay in pollen morphological and viability

studies. To accommodate the slow process of tube development and pollen counting within the same day, flowering stems should be harvested in the afternoon when pollen appears to reach maturity. Light intensity and temperature appear to play major roles in pollen maturation. Powdery pollen of wind-pollinated species is harvested more easily than in insect pollinated species. The latter have sticky pollen adhering to the pollen presenter and is more difficult to collect.

A high frequency of viable pollen can be counted *in vitro* on a solidified medium of 2g agar and 10g sucrose. Pollen fertility in *Leucadendron* is consistently high (>55%) and well with in the range (Visser, 1955) of other species considered to be adequate for production of viable seed for cultivar development. Sucrose is shown to be an effective supplement for promoting pollen tube development. Within one hour after pollination pollen starts to germinate and develop pollen tubes from one of the three apertures of the pollen grain. Germination occurs over the whole surface of the stigmatic region. Pollen grain size differs significantly between species, sections and sub-sections of *Leucadendron*.

Controlled intraspecific pollination results in a frequency of seed set comparable to seeds harvested in natural pollination, and in much higher seed viability than in the hermaphrodite Proteaceae as reported by Vogts (1960), Horn (1962), Brits (1984), Rebelo and Rourke (1986) and Esler and Cowling (1990). However, controlled crosses between more distantly related species from different sub-sections and sections (classified by seed type) repeatedly show a lower numbers of seeds set per cone. Chapter 3 demonstrates conclusively that low seed numbers and non-viable seed results could be ascribed to interspecific incompatible barriers.

Artificial controlled hand-pollination (Brits, 1983), mimicking actions that normally occur during natural pollination has been successfully applied to

Leucadendron. The use of glassine bags during controlled crossing by hand pollination successfully prevents contamination by unwanted pollen. Crossability is higher in the number of seeds germinating and seed germination in crosses within the 'Alatosperma' group than in crosses within the 'Leucadendron' group and between groups. A major reduction in seed set occurs in cross-pollination between species.

Incompatibility studied in Proteaceae (Martin and Hescovitch, 1989) appears to act as a pre-fertilization barrier. Van Tuyl (1996) recognized sexual barriers in interspecific hybridization as pre- or post-fertilization processes in the pistil and he describes the simplest form of pollen-pistil disruption as it occurs prior to fertilization.

Focussing on the pistil as possible barrier to interspecific crossability, magnification by SEM shows that *Leucadendron* stigmas are different to the common structure of the stigmas observed in *Protea* (Van der Walt, 1996), *Grevillea* (Herscovitch and Martin, 1989) and *Macadamia* (Sedgley *et al.,* 1985). The stigmas of *Leucadendron* vary slightly in shape from round to oval in all species excepting *L. rubrum* which is elongated. The pollen receptive stigma consists of a large number of unicellular papillae packed on a swollen base. On the stigmas of species used in the diallel crosses, germination occurs over the entire stigma surface within a few hours following pollination. A large number of pollen tubes enter the pistil and grow between the papillae cells in all directions. In compatible selfs the mean tube number on the stigma surface is around 26 with a further reduction to less than 5 tubes in the style. Species crosses, however, have a reduced number of tubes present on the stigma surface. This declines to zero in the ovule region of the pistil.

Interspecific incompatibility barriers such as abnormal tube growth patterns, tube branching, bursting and leaching of the tube content occur beyond the stigma region of the pistil. Pollen tube abnormalities of this nature have been found in

Grevillea (Martin and Herscovitch, 1989) and *Macadamia* (Sedgley *et al.*, 1985) of Proteaceae. Methods for overcoming interspecific crossing barriers and avenues for future research are pollination of cut styles together treatment with growth hormone or stigmatic exudate and the use of mixed sample of pollen or irradiated mentor pollen as proposed by Van Tuyl (1996).

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