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New Hybrid Leucadendrons

A report for the Rural Industries Research and Development Corporation

By Guijun Yan, Ben Croxford and Ralph Sedgley

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Researcher Contact Details
Dr Guijun Yan
Plant Sciences
Faculty of Agriculture
The University of Western Australia
Nedlands, WA 6009
Phone*:* (08) 9380 1240
Fax*:* (08) 9380 1108
Email*:* gyan@agric.uwa.edu.au

RIRDC Contact Details
Rural Industries Research and Development Corporation
Level 1, AMA House
42 Macquarie Street
BARTON ACT 2600
PO Box 4776
KINGSTON ACT 2604

Phone: 02 6272 4539
Fax: 02 6272 5877
Email: rirdc@rirdc.gov.au.
Website: http://www.rirdc.gov.au

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Foreword

There are many outstanding species and varieties in the genus *Leucadendron*, which are important in Australian floriculture industry for its export market. Recent data shows that *Leucadendron*s are a major export group to Japan, comprising 12.8% of Australian imports, third after Waxflowers and Kangaroo Paws. The beautiful flowers of *Leucadendron*s and the muted green leaves, tinted with other soft shades, provide a perfect counterpoint as fillers in flower arrangement. However, lacking of new varieties has become a more and more obvious factor hindering the further development of the industry.

This project is intending to enhance the economic strength of the Australian floriculture industry by assuring the supply of new *Leucadendron* cultivars through interspecific hybridisation and, to facilitate this process, by understanding the reproductive biology underlying the breeding of this genus. The objective of this project is to extend the continuity of supply of *Leucadendron* as cut flowers and to capture a greater share of markets by extending the flowering period to fill in the niches.

Outstanding results have been achieved in this project. Through the development of pollen storage, seed germination and genome investigation techniques, a successful breeding protocol for *Leucadendron* has been developed. Following this protocol, more than 500 different cross combinations from 140 genotypes of 27 species have been attempted. This has resulted in about 20,000 hybrid seeds. Our germination and early selection results so far suggest that most of these seeds are both viable and true hybrids as they share characteristics from both parents. Altogether, 3,122 hybrid plants have been planted on growers' properties and are currently being assessed for their cut flower potential. It is hoped that several new varieties will be selected from these hybrids and will be released to the wildflower industry in the very near future.

This publication considers the breeding process of *Leucadendron* with special emphases on hybridisation, selection, reproductive biology, pollen collection and storage, seed treatment and germination, pollen pistil interaction and chromosomes assessment of *Leucadendron*. We suggest that the information contained in this report can be useful for wildflower growers to develop their own breeding programs.

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Peter Core
Managing Director
Rural Industries Research and Development Corporation

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The contributing *Protea* growers in alphabetical order are:

Abbey Farm – Paul Cook and Cheryl Foster

Amarillo Protea - Ralph and Grace Sedgley

Annie Brook Flower Farm - Wally and Dawn Lewis

Busselton Proteas - John Daykin

Cox Farm - Morris and Miriam Cox

Tan Ridge - Tom and Joan Anthoine

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Abbreviations

BAP	6-benzylaminopurine
°C	degrees Celsius
DNA	deoxyribose nucleic acid
F_1	first generation hybrid
F_2	second generation hybrid
FDA	Fluorescein diacetate
g/L	grams per litre
H_2O_2	hydrogen peroxide
M	molar
mL	millilitre
mm	millimetre
µM	micromolar
MS	mixture of salts as per Murashige and Skoog (1962)
n.a.	not applicable
NaOH	sodium hydroxide
PCB	para-dichlorobenzene
pers. comm.	personal communication
UWA	The University of Western Australia
w/v	weight by volume
x	basic ploidy number
2n	somatic ploidy number
2x	diploid

Contents

Executive Summary

Leucadendrons are widely grown for their spectacular flowering display with many varieties selected or bred from plants "from the wild" and introduced into the worldwide floriculture industry. Australia is a major exporter of high quality *Leucadendron* flowers, as the climate and conditions for them to flourish exists in several places.

Unfortunately for the industry there are several gaps in the year when no commercial varieties are available or when the available varieties have traits limiting their commercial profitability. However massive variation occurs between wild species both in flowering times and other horticultural and floricultural attributes. By crossing the commercial varieties with plants containing specific traits (e.g. flowering time) we hope to produce vigorous new varieties with the desirable traits from both parents.

In this project, more than 500 different cross combinations from 140 genotypes of 27 species have been attempted. This has resulted in about 20 000 hybrid seeds. Our germination and early selection results so far suggest that most of these seeds are both viable and true hybrids as they share characteristics from both parents. Altogether, 3122 hybrid plants have been planted in the field and are currently being assessed for their cut flower potential.

The breeding of this genus has occurred in a systematic way. The first step in this process was to identify the shortcomings of the available varieties and locate parents with desirable traits. 27 different species and several current varieties were selected and breeding began in June 1998. Further species have since been obtained and are currently being raised.

By observing the chromosomes of 25 genotypes from 15 different species we have determined that they all had 26 chromosomes with no obvious morphological differences. This suggests that hybridisation between these species may be more successful than if different chromosome numbers were observed.

Leucadendron plants produce pollen for only a brief period of time and so a protocol involving the drying and freezing of pollen and the viability assessment of stored pollen was developed and implemented. Our results indicate that more than 50% of the viable pollen collected at anthesis is still viable after 1 year in storage when treated in this manner. This allows for crossing to occur between plants regardless of flowering time. The viability of pollen was predicted using the stain fluorescein diacetate that allows the integrity of the pollen cell membrane to be observed. We found that viability predicted by the stain was accurate enough to determine between pollen samples that were or were not suitable for breeding and to measure the rate of decline of stored pollen. We also found this method to be faster and more consistent at predicting pollen viability than *in vitro* germination tests.

As male and female flowers occur on different bushes in *Leucadendron* it was relatively easy to prevent uncontrolled pollination from occurring by bagging and hence isolating the female flowerheads before they become receptive. Once the female flower is receptive, stored pollen from the "father" can be

defrosted and dusted onto the female stigma. The large number of seeds produced from such crossing illustrates the success of this method.

In many cases when more distantly related species were crossed, no viable seed were produced. Some seed appeared to have aborted prior to maturity leaving behind a seed coat containing a dead embryo (post-zygotic abortion). Other crosses resulted in no seed set all. This indicates that pre-zygotic barriers preventing hybridisation between these species exist. A protocol to observe pollen germination and tube growth through the style following controlled crosses was developed. Several scenarios have been observed when using this method with different crossing combinations including; 1) the failure of pollen to germinate, 2) pollen germinates but does not reach the ovary end of the style or 3) the pollen germinates and pollen tubes reach the ovary end of the style. These results were generally consistent with field hybridisation results in that combinations where pollen tubes did not present at the end of the style are the same combinations that failed to set any seed. These results also highlighted examples where crosses could occur in one direction only and variability within a species with regard to pollen tube growth and hence the likelihood of fertilisation. The results from such a test are available within a few days and thus this method could be used to screen parental combinations before committing to field crossing which improves the efficiency of the program.

Most varieties currently grown as cutflowers belong to either the alata or trigona subsections. Plants within a subsection often share many characteristics and are considered more closely related to each other than to species from other subsections. We found that crossing with plants from within a subsection or between these two subsections was relatively successful but crossing between these species and species from other subsections was less successful but in many cases possible. We also found that crossing between hybrid varieties and their parental species and in some cases to other species was successful.

Leucadendrons can be divided into two separate groups (known as sections) based on the characteristics of their seed. These groups pursue a different method of seed storage and differ in their germination requirements. Most of the seeds produced from the breeding were from one section and a low temperature requirement of 11ºC was all that was required for the germination of these seeds. Seeds originating from crosses with the villosa subsection share characteristics of both sections and required a diurnal temperature of 10ºC for 16hrs followed by 20ºC for 8 hrs for maximum germination to occur. Recently seed from the second section has been obtained and early results indicate that germination is slower and at a lower rate than for seed from the first section, however some positive germination responses have been observed following treatment with hydrogen peroxide and exposing the seeds to a diurnal influence.

In 1999 and 2000, seedlings representing almost all of the successful combinations were planted at the farms of participating *Leucadendron* growers. The seedlings were planted and maintained by these growers and the majority of plants survived are growing well and showing a range of characteristics. Only 8.2% of the plants planted in 1999 flowered in 2000 and so detailed assessment of floricultural attributes was not possible but it appears that most will flower between July and December 2001 when

the first detailed assessment of the hybrids will occur. In every year following this third year more plants will flower and hence be available for selection as long as the breeding program continues. It is hoped that some plants will be superior to current varieties. These plants may be PBRed and released in the immediate future with others being selected, cloned and maintained in an elite bank for further trialling and release into the future.

This project has had good success with the development of a breeding protocol which allows Leucadendrons to be crossed regardless of location or flowering time resulting in some 20 000 seeds to date. These crosses have concentrated on species that are currently commercially available and it is likely that many of the offspring will express hybrid vigor and be commercially acceptable. Further refinement of the protocol is likely to focus on overcoming the barriers that have been identified as preventing hybridisation between more distantly related species and species not currently grown. This project has provided the groundwork for a stream of new *Leucadendron* varieties to be released to benefit the Australian industry for years to come.

1. Crossing and Hybrids

1.1 Introduction

The genus *Leucadendron* belongs to Proteaceae and is an important part of the worldwide floriculture industry. In Australia they are a major wildflower export group to Japan, comprising 12.8% of Australian imports, third after waxflowers (*Chamelaucium*) and kangaroo paws (*Anigozanthos*). The worldwide demand for these flowers is significant with Israel alone supplying 37 million stems of one superior *Leucadendron* hybrid (Safari Sunset) to Europe (Ben-Jaacov, Pers. Comm.). *Leucadendrons* grown in Australia however, are mainly exported to Japan and South East Asia (Tranter, RIRDC).

The outstanding features of leucadendrons which make them excellent candidates for export are their robust keeping qualities coupled with the availability of a wide variety of erect plant forms, with colourful bracts, throughout most of the year, and an ability to bear many long straight stems.

There are 79 species of *Leucadendron* (Vogts, 1982) which can vary greatly in many floricultural and horticultural attributes. All the members in the genus are dioecious (with male and female individuals) and therefore there are at least 158 different flower forms as the male and female have different flower displays. The *Leucadendron* floral display often comprises colourful bracts, which surround the central flower head. This flower head is usually yellow (red in some species) for male flower heads prior to anthesis. The female flower head is generally green or silver (sometimes red or brown) with numerous florets partially covered by a fleshly floral bract but leaving the stigma and sterile stamens exposed. It is the bracts and upper leaves that are the showy part generally desired for cut flowers and foliage. The bracts are usually yellow or red, but also white, pink or green and combinations of colours (e.g. green bract with red tips) have been observed depending on the species and season. Some species have more than one colour form e.g. *L. salignum* has either red or yellow bract individuals. Bract colour often intensifies as the flower head develops with some varieties being suitable for harvest long before flowers have reached sexual maturity (Scott, 2001) with other varieties being suitable for harvest at several different stages.

Some *Leucadendron* species have a relatively large flowerhead (30-50mm) surrounded by large brightly coloured bracts (50 -100 mm long) and even bigger in some hybrid varieties which are located terminally on single stems. Some species in this category are *L. laureolum, L. gandogeri, L. strobilinum, L. eucalytifolium, L. discolor, L. salignum L.tinctum* and *L. orientale* with most of the named hybrids having at least one parent from these species. These include Safari Sunset, Red Gem, Inca Gold, Maui Sunset and more recently Rosette. Other *Leucadendrons* have much smaller flower heads (10-20mm) surrounded by smaller but just as colourful bracts. Many such flower heads each on a short stem are attached to each main stem. These stems can be harvested. Species in this category include *L. floridum, L. conicum, L. salicifolium, L. uliginosum* and the named hybrid Pisa. A third flowering product is produced by other species where colorful bracts are either very small or non-existent and the desired product is the neat foliage and the cone following sexual maturity of the female flowerhead. This cone may be silver such as in *L. galpinii* and *L. linifolium* and the hybrid variety Silver Mist or various shades of red such as *L. salicifolium* and *L. coniferum*.

The 79 species have been broadly classified into 2 sections, section A 'Leucadendron' and section B 'Alatosperma' and 14 subsections. Due to the large number of species and the diversity between different species, it is likely that important attributes for floriculture can be combined in superior selections resulting from controlled interspecific hybridisation.

A good example of how breeding could be used to help the Australian industry is with flowering time. *Leucadendron* species flower from March to January, however each species only flowers for a brief period and most of the commercial varieties have finished flowering by November in Australia, leaving a gap of about two months when prices peak in the Japan (RIRDC Research Paper No.

97/41). Species such as *L. uliginosum, L. conicum* and less well known species such a*s L. loeriense, L. album, L. nobile* and *L. radiatum* do flower in this period but may be difficult to grow or are not sought after for their floricultural attributes. Therefore if the floricultural attributes from the present varieties are combined with the flowering time of later species, the *Leucadendron* markets can be extended for another two months and a higher price benefit can be captured.

As well as flower colour, size and time, variation is also reported or observed to exist between species in regard to many commercially important attributes. These include resistance to *phytopthora cinnamoni*, ability to be pruned hard (presence of lignotuber or epicormic buds), limestone (high pH) tolerance, waterlogging tolerance, stem length, incidence of pest and disease and productivity under cultivation. It appears that the shortcomings of a particular species or variety may be overcome by the use of interspecific hybridisation.

However the introgression of desirable traits is only part of the reason for hybridisation. The other major reason is the increase in heterosis (hybrid vigor) that can be expected by crossing two different species. This can result in traits being greater than that found in either parent e.g. larger flower size, longer stem length, increased vigour and yield, increased disease resistance and a longer or different flowering period. Heterosis was a characteristic of the large majority of Proteaceae resulting from interspecific crosses (Brits *et al*, 1983).

The breeding of the Proteaceae as ornamentals started about 30 years ago and some interesting work has been done so far (Littlejohn *et al*., 1995). The *Protea* breeding program in South Africa is aiming at producing cultivars available in each colour and size range that flower successively throughout the year by intraspecific crosses between different ecotypes (Brits *et al*., 1983; Littlejohn *et al*., 1995). The *Banksia* breeding program in Australia has been employing both intraspecific and interspecific crosses and has released some good varieties such as 'Waite Orange' and 'Waite Crimson' (Sedgley, 1995). A *Leucadendron* selection and breeding program started at Fynbos Research Unit, Vegetable and Ornamental Plant Institute, South Africa (VOPI) about 15 years ago (van den Berg & Brits, 1995) and early work involved mainly selection of superior clones. Only recently, a breeding program using interspecific hybridisation techniques was started aiming at development of *Leucadendron* single stem flowers.

From the work carried out at VOPI and Australia on *Protea* and *Banksia*, it can be concluded that most proteaceous plants can be improved by breeding and the South African, American and Australian breeding programs have resulted in many excellent cultivars (Brits *et al*., 1983; Parvin, 1981; Sedgley, 1995). However, *Leucadendron* plants are slightly different from other proteaceous plants. Firstly, the plants are dioecious (Vogts, 1982) which means that self and reciprocal crosses can not be made with any individual plant. Secondly, in contrast to the other genera, very few natural hybrids have been found in *Leucadendron* (Williams, 1972) suggesting difficulty in wide crosses. Thirdly, the *Leucadendron* seeds from most species are reported to take a long time to mature after fruit set (from 7 months to a year). Indeed during the breeding of *Leucadendron* at VOPI, lack of compatibility and low seed set were encountered (van den Berg & Brits, 1995; Littlejohn *et al*., 1995). However, detailed research on the reproductive biology using fluorescent microscopy and advanced technology such as embryo rescue have not yet been exploited in *Leucadendron* breeding. If such new technology can be used, the difficulties can be overcome and the disadvantages can even be changed to advantages.

It is likely that limitations with current varieties grown in Australia can be improved by careful controlled interspecific hybridisation. New varieties are likely to rejuvenate the Australian industry and improve their competitiveness on the worldwide floriculture market. Research into the reproductive biology underlying the breeding of this genus should ensure a supply of new *Leucadendron* cultivars into the future.

1.2 Objectives

1.2.1 To determine a protocol that would enable the hybridisation of *Leucadendron* species flowering at different times or at different locations.

1.2.2 To investigate the potential for creating hybrids between different taxa within *Leucadendron* and determine barriers that may be preventing hybridisation**.**

1.2.3 To improve the range of *Leucadendron* varieties available to Australian growers by

a) Extending the period of time over which high quality *Leucadendron* flowers can be produced to include November and December.

b) Improving the durability of "fire sensitive" species by hybridisation with species containing a lignotuber or epicormic buds.

c) Improving the colour range to produce a more equal balance of red and yellow flowers throughout the year.

d) Providing *Leucadendron* varieties bred and selected in Australia so that they are adapted for Australian conditions.

e) Producing unique, named varieties with hybrid vigour that will shorten the establishment phase and allow for early production and improve market potential.

1.2.4. Initiating a long term self-sustaining breeding program for the production of several new off-season varieties into the future.

1.3 Materials and Methods

1.3.1 Selection of Parents

Parents with useful traits were identified by researching literature and from discussions with local *Protea* Growers. Parents were selected in an effort to achieve the breeding objectives as described above. Following selection, parents were assigned a 3 letter code (representing species) and a 2 digit number (representing individual genotype). This and further information relating to individual parents was recorded in a database. Included are notes on horticultural attributes (e.g. flower colour or form, presence of lignotuber, vigor etc), gender (male or female) and seed group. Flowering date of each parent was recorded for each season (it is likely to vary according to location, weather or management practices) and information regarding the wild habitat of these species if it was thought to be useful to the breeding program. All plants were from managed plantings under irrigation.

1.3.2 Pollen Collection

Most of the pollen collected (31 genotypes from 16 species) was from plants grown at Amarillo Proteas in Karnup, WA, with other pollen being sent to UWA (as flowering stems) from various flower farms in the Southwest of WA.

Pollen can be collected from male flower heads following anthesis. It is often abundant and easily collected from many species. The method used in this breeding program was as follows;

a) Cut large stems containing many flowers when between half and two thirds of the flowers in each flowering head have presented pollen on to the sterile stigma.

b) Place cut stems at room temperature away from insects and draughts in a standard vase solution. In many species immature flowers will continue to open (present pollen) over several days in these

conditions. This pollen plus any remaining from time of collection can be easily seen as yellow powder on the stigma.

c) Separate pollen from flowering heads by removing the entire head from the flower stem and sieving using a fine mesh sieve above clean paper. Care was taken not to rub or knock pollen from the stigmas when removing flower heads from stems. Flowers without bracts that enclose the head (e.g. *L. galpini*) were cut from stems above the sieve and hence were not touched at all. Flowers enclosed by bracts (e.g. *L. discolor*) were removed by gripping the flower head at the top where immature flowers have not yet shed pollen.
d) Pollen is scraped from paper and transferred to an ependorf tube.

e) The open ependorf tube is then placed in a sealed jar containing freshly dried silica gel at 4 ºC for 48 hours.

f) Ependorf tubes containing pollen are numbered and labeled with the parent plant's code (see above) and the collection date.

g) At this stage a sample is taken to assess viability using the FDA method or in vitro germination method, the rest is stored at either –20ºC (short-term storage) or –80ºC (longer-term storage). See Chapter 3.

1.3.3 Isolation of female flowers

Most of the female parents (86 genotypes from 25 species) used in this project were located at Amarillo Proteas in Karnup, WA, with a further 2 female plants located at Purely Proteas in Mandurah, WA.

On female *Leucadendron* plants each floret on the receptacle has a thick, fleshy floral bract, large enough to cover the perianth tube, but leaving the tiny perianth segments, with their sterile stamens and fertile stigma, free and peeping over the edge (Vogts, 1982). As the stamens are sterile there is no possibility of self-pollination in this species. This makes controlled pollination much easier as physical emasculation is not necessary. Hence isolation of receptive stigmas is all that is required. This was achieved by placing a perforated plastic bag over the flowerhead or flowerheads (depending on plant form) and attaching it securely around the stem with twist wire when stigmas first become visible on the developing receptacle.

1.3.4 Pollination

From 7 – 14 days after first appearing the stigma is white and fleshy and appeared to remain so for about 2 weeks or more (depending on species and weather) even though the sterile stamens turn black and wither.

At this stage, pollination was attempted by removing the bread bag and dusting dry pollen onto the stigma using a small paintbrush. The pollen being used was kept dry (stored in ependorf tube) and cool (packed in ice in an insulated container) whilst in the field and was immediately frozen at minus 20ºC on returning from the field. The bread bag was replaced following pollination to ensure no uncontrolled crossing occurred.

On several plants, one or more bagged flowerheads were left unpollinated with the bag left secured to determine the effectiveness of bagging as a means of preventing uncontrolled pollination

1.3.4 Cone and Seed Collection

Despite literature recommending that *Leucadendron* seeds should not be collected for at least one year following fertilization, we found that for the species we used as parents it was better to collect

the cones much earlier than this. For example we collected mature *L. floridum* seed just over three months after pollination as seed was already being released. We also found that parrots were particularly fond of certain fruits (e.g. *L linifolium*) especially if they are full of seed and thus most cones were collected within 5 months of pollination.

Fruit were collected by breaking the whole cones from the mother plant and placing them in paper bags. These bags were hung in a dry warm location with good ventilation for at least one week.

Mature seeds generally fell freely from the dry cones however it was noticed that many seeds were left remaining in the fruit. Examination of these seeds shows that in almost all cases seed that did not fall from the cone on it's own accord was not viable with a small number of viable seeds sometimes stuck between the woody bracts that have opened to release seed from surrounding florets. This seed was gently recovered using forceps. Sometimes seed that fell freely following drying was also not viable. These were often easy to identify, as they are either flat or soft to touch. Viable seed are firm to touch and are stored in the short term in paper envelopes at room temperature and in the longer term at 4ºC sealed in a desiccator with dry silicon gel.

The number of cones collected and the proportion that had swollen with seed were recorded, as was the number of presumed viable seeds collected from each cross combination.

1.3.5 Seed Germination

Two methods were routinely used to germinate seeds:

The first method involved sowing seeds in Autumn and Winter in trays containing 1 part sand : 1 part perlite in a shadehouse. Immediately prior to sowing all seeds were immersed and agitated in a 1% Sodium hypochlorite solution for 20 minutes. This solution was prepared by adding 25ml of "Whiteking™" bleach to 75ml of deionised water. Germinating seeds and young seedlings received 5 minutes irrigation spray per morning. Soon after germination and preferably before the appearance of true leaves, seedlings were recorded and carefully transferred to individual tubes. At this stage root development is limited to a single taproot (no fine lateral roots) and hence transplanting can occur with little risk of serious root damage to the young seedling.

The potting mix used contained 3 parts commercial potting mix (no added nutrients) to 1 part perlite with 30 g of Osmocote for Australian Native Plants per 10 litres of soil mix. This was then placed into 50mm x 50mm x 175mm square tubes, with 42 pots fitting tightly into a nursery tray and the whole lot was then steam sterilized for 1 hour.

Once the soil had cooled seedlings could be carefully pricked out of the germination media and transferred to the tubes.

An *in vitro* method was also used to germinate seeds. Seeds were surface sterilized by immersion and agitation in 1% sodium hypochlorite or 1% hydrogen Peroxide for 20 minutes. The seeds were removed in front of a laminar flow and aseptically rinsed in sterile water (This step was left out if H_2O_2 was used as sterilising agent). Seeds were sown on a piece of sterile filterpaper moistened with sterile water inside a sterile petri dish. The petridishes were then sealed with parafilm and were put into a cool room at a controlled temperature of 11ºC.

As soon as the radical emerged to a length equal to that of the seed it was removed from the petri dish and planted into a 50mm tube which had been prepared as described above.

When the plants were 10 to 15 cm high, the apical meristem was removed to encourage bud break and hence multiple branching. Any plants showing signs of disease were immediately quarantined to reduce the risk of pathogens spreading. The seedlings were fertilised with a rotation of one-quarter strength fish emulsion and one-quarter strength Thrive™ depending on current growth rates. The

seedlings tended to grow vigorously and no major problems were identified with the nursery care of the seedlings.

1.4 Results

1.4.1 Selection of Parents

Eighteen species and 4 hybrids were selected and made available as pollen-donor parents based on traits observed or reported as shown in table 1.1.

Table 1.1 *Leucadendron* species and hybrids available and selected as pollen donor parents

Species	Section	Sub-section	Number of genotypes	attributes
conicum	B	trigona	1	Late flowering, multi-headed, pink bract
coniferum	B	alata	1	High pH tolerance, vigor, red cone
discolor	B	alata	6	Red flowerheads, good commercial form, single headed
eucalyptifolium	B	alata	2	Phytopthora tolerance, vigor
floridum	B	trigona	1	Can be picked immature, good form, silver foliage, multi-headed
galpinnii	A	villosa	1	High pH tolerance, attractive foliage, silver cone, late flowering
gandogeri	B	alata	7	Large yellow flowers and bracts
laureolum	B	alata	6	Large yellow flowers and bracts, early flowering, high vigor, good stem length, can be sold immature
linifolium	A	villosa	1	attractive foliage, silver cone,
loeriense	B	trigona	1	Late flowering
muirii	B	compressa	2	Late flowering, High pH tolerance
procerum	B	alata	1	Red centred flowerheads, good commercial form
rubrum	A	leucadendron	3	Checkered pattern on cone, neat foliage
salicifolium	B	trigona	1	Vigor, waterlogging tolerance
salignum	B	alata	5	Red bracts, persistent rootstock
spissifolium	B	alata	2	persistent rootstock, red cone
strobilinum	B	alata	1	Large open yellow flowers and bracts
uliginosum	B	trigona	2	Late flowering, long stems, silver foliage
conicum x *floridum*	B	trigona	2	Late flowering, pink bracts, silver foliage
gandogeri x	B	alata	1	Large yellow flowers and bracts
procerum x *discolor*	B	alata	1	Red flowerheads, good commercial form

24 species and 14 hybrids were selected and made available as female parents according to traits observed or reported and are shown in table 1.2.

Table 1.2 *Leucadendron* species and hybrids available and selected as female parents

Species	Section	Sub Section	No. genotypes	attributes
conicum	B	trigona	1	Late flowering, pink bract
coniferum	B	alata	1	High pH tolerance, vigor, red cone
discolor	B	alata	9	Red male flowerheads, good commercial form, can be picked immature
elimense	B	ventricosa	1	Long stem
eucalyptifolium	B	alata	3	Phytopthora tolerance, vigor
floridum	B	trigona	2	Can be picked immature, good form, silver foliage
galpinii	A	villosa	3	High pH tolerance, attractive foliage, silver cone, late flowering
gandogeri	B	alata	2	Large yellow flowers and bracts
laureolum	B	alata	4	Large yellow flowers and bracts, early flowering, high vigor, good stem length, can be sold immature
linifolium	A	villosa	2	attractive foliage, silver cone,
macowanii	B	trigona	1	vigor
muirii	B	compressa	1	Late flowering, High pH tolerance
orientale	A	nucifera	3	Large flowerhead and bracts
platyspermum	B	compressa	1	Attractive cone and foliage
procerum	B	alata	2	Red male flowerheads, good commercial form
rubrum	A	leucadendron	3	Checkered pattern on cone, neat foliage
salignum	B	alata	10	Red or yellow bracts, persistent rootstock
spissifolium	B	alata	2	persistent rootstock, red cone
stelligerum	B	trigona	1	Waterlogging tolerance, bracts give flowerheads a star-like appearance
strobilinum	B	alata	2	Large yellow flowers and bracts
teretifolium	B	compressa	1	Needlelike leaves
tinctum	A	nucifera	1	Large flowerhead and bracts
uliginosum	B	trigona	5	Late flowering, long stems, silver foliage
xanthoconus	A	alata	3	Phytopthora tolerance, vigor
conicum x *floridum*	A	trigona	3	Late flowering, pink bracts, silver foliage
conicum x	A	trigona	2	Red cones, vigor
gandogeri x *strobilinum*	A	alata	1	Large yellow flowers and bracts
salignum x *discolor* (Maui Sunset)	A	alata	1	Commercial variety, persistent rootstock
salignum x *laureolum* (Safari Sunset, Red Gem, Silvan Red, and Inca Gold*)*	A	alata	4	Commercial varieties, Large red or yellow bracts, vigor, long stems
laxum x (Jubilee Crown)	B	cuneata	1	Commercial variety, vigor, good form
floridum x *coniferum* (Pisa*)*	A	trigona	1	Commercial variety, vigor, good form, silver leaves
floridum x	A X B	trigona x	1	Vigor, silver cones

linifolium (Silver Mist)		villosa		

For several species only female plants were available as these are generally more suitable for commercial cutflowers. In an effort to get more male parents and to get other parents with reported useful traits, seed from many species was gathered and the seedlings raised. Most of these have not flowered yet but will be available for breeding into the future. Several of these species are listed in table 1.3.

Table 1.3 *Leucadendron* species propagated to be used as parents in the future

Species	Section	Sub Section	attributes
album	*A*	leucadendron	Late flowering, silver leaves
argenteum	*A*	leucadendron	Silver foliage
barkerae	*A*	nucifera	
chamelaea	*B*	ventricosa	Long, straight stems
concavum	*A*	villosa	Small, oval leaves
daphnoides	*A*	nucifera	Large bracts which change color with time
dubium	*A*	villosa	
flexuosum	*B*	alata	Early flowering
loeriense	*B*	villosa	Late flowering
meridianum	*B*	alata	High pH tolerance, vigor
nervosum	*A*	nervosa	Red male flowerheads
pubescens	*A*	membranaceae	
sessile	*A*	nucifera	Large bracts, tolerance of heavy soils
thymifolium	*A*	villosa	
tinctum	*A*	nucifera	Large flowerhead and bracts

1.4.2 Pollen collection

Pollen collection varied from species to species both in amount collected and viability. The most successful collections came from large headed species e.g. *L. laureolum, L. gandogeri, L. strobilinum, L. discolor* and *L. procerum*. Suspected wind pollinated species *L. salicifolium, L. linifolium* and *L. rubrum* tended to shed pollen at the slightest movement and hence stems were best left for several days in water inside (to allow more pollen to be presented) before shaking the stems above clean paper to release the pollen.

Pollen collected from flowering stems sent to UWA after being packaged in cardboard boxes yielded the lowest quantity of pollen per flower due most likely to physical removal during the packing and transport. Despite this suitable amounts of pollen were obtained from most of the species available.

Pollen viability also varied greatly. The FDA method showed that for most species viability ranged from 15% to 80% and for hybrids as low as 4.5%.

Table 1.4 Viability of fresh *Leucadendron* pollen

Species	**% viability (FDA)***
conicum	67.4
coniferum	73.1
discolor 1	82.8
discolor 2	56.3
discolor 3	53.5
eucalyptifolium	65.5
floridum	30.2
galpinii	64.3
gandogeri	49.4
laureolum	31.6
linifolium	15
muirii	54.4
procerum	70.6
rubrum	20
salicifolium	75.5
salignum	64.5
spissifolium	20
strobilinum	71.1
uliginosum	74.7
conicum x floridum	9.5
Gandogeri hybrid	4.5
procerum x *discolor*	79.6

* % Viability for each genotype was determined by averaging the viability results for the first 3 vials of pollen per genotype that were collected. The viability of each vial was determined by counting and scoring at least 200 pollen grains that were prepared using the FDA method.

Pollen germination results tended to be similar but lower than FDA results. Germination rates tended to vary even with the same sample and sometimes no germination would occur. Due to this variability no results have been presented on in vitro germination. For many samples germination was higher when boric acid and nutrients were added to the sugar solution than when compared to germination in straight sugar solution.

1.4.3 Isolation of female flowers

Some bagged flowerheads were intentionally left unpollinated to test the efficiency of bagging as a means of preventing uncontrolled crossing. In most cases cones failed to develop and it was clear that bagging was preventing uncontrolled pollination. In 2 cases (*L. discolor* and *L. muirii*) some partial development of the cone occurred and a few small hollow seeds developed.

Preliminary assessment of hybrid offspring at seedling stage or in the field suggest that on at least 3 female Leucadendrons (*L. floridum*, *L. discolor* and *L. procerum*) used in the breeding program, bagging may have not prevented some uncontrolled pollination as well.

1.4.4 Cone and Seed Collection

Most female flowerheads developed into cones following fertilisation. In many cases however the cones were small and had not developed properly and did not contain any viable seed. This generally occurred when there was a large phylogenetic distance between parents e.g. when *L. orientale, L. elimense* or *L. rubrum* were crossed with species in the alata or trigona subsections. More rarely it occurred between closely related species e.g. when *L. salignum* female was crossed with some

members of it's own subsection e.g. *L. laureolum* no cone development occurred. When there was no cone development there was no seed set.

Some cones had already began to release their seeds at the time of collection e.g. *L. floridum* and *L. xanthoconus*. The number of months between crossing and collection was between 3 and 6 depending on species. Non-serotinous species e.g. *L. orientale* should be collected first.

Some fruit had been badly damaged and in some cases completely removed by parrots. Cones most affected by this were on *L discolor, L. salignum, L. linifolium* and *L. floridum.*

In many cases cone development was good and following desiccation viable seeds readily fell from the fruit. The total number of seeds produced per year is shown in Table 1.5

Table 1.5. The number of seeds produced from controlled crosses within *Leucadendron*

	Number of Apparently viable seed	
Year	**intraspecific**	**interspecific**
1998	3271	12855
1999	332	1894
Total	3603	14749

Once again, phylogenetic distance was closely related to seed set. All intraspecific crosses were successful. Crosses within a subsection were generally successful and in many cases set as many seed as intraspecific crosses. Crosses between subsections within their section often set seed while crosses between sections had the lowest rate of seed set. Seed set per cone for some of the combinations attempted are shown in Table 1.6.

Table 1.6 Effect of phylogenetic distance on average seed set following controlled crosses with in Leucadendron

	Mean number of seeds produced per cone collected for each combination			
Female Species	intraspecific	Interspecific within a subsection	Interspecific between subsections within a section	Interspecific between sections
conicum	25.7	20.6	12.4	0.2
coniferum	22	8.5	1.6	n.a.
elimense	N.A.	N.A.	0	0
eucalyptifolium	12.9	20.2	1	n.a.
floridum	n.a.	8.2	4.0	n.a.
galpinii	18.9	7.6	2.5	1.5
gandogeri	21.7	26.1	2.8	2
laureolum	35.5	9	0	n.a.
linifolium	n.a.	n.a.	4.5	0.2
orientale	n.a.	n.a.	0.6	0
rubrum	n.a.	n.a.	n.a.	0
salignum	24.5	14.5	2.3	n.a.
spissifolium	n.a.	11.4	5.0	n.a.
strobilinum	32.6	11.9	3.5	n.a.
tinctum	n.a.	n.a.	0	0.1
uliginosum	n.a.	12.3	8.5	n.a.

Two female parents (*L. elimense* and *L. platyspermum*) failed to set any seed from all combinations attempted. L. *tinctum* and *L. orientale* produced only 1 and 2 seeds respectively from all combinations attempted. In general species from Section A failed to set many seeds except when crossed within their own species.

All female hybrids that we attempted to cross set seed when crossed with at least one of its parental species and in many cases to a third species as shown in table 1.7.

Table 1.7. Seed set of Female *Leucadendron* hybrids

	Mean number of seeds produced per cone collected for each combination			
Female hybrid	Backcross to first parental species	Backcross to second parental species	Cross with other species within the parental subsections	Cross with other species in a different subsection
Pisa	1.1	6.1	6.7	n.a.
Jubilee crown	n.a.	n.a.	n.a.	0.1
Red Gem	8.5	6.5	n.a.	n.a.
Silvan Red	4	1.8	n.a.	n.a.
Safari Sunset	10	2.8	5.4	n.a.
Inca Gold	13.3	6.2	7.4	n.a.
Maui sunset	21.5	11	3.0	n.a.

In many cases cross success as measured by seed set was different depending on which parent was the pollen donor. *L. discolor* and *L. salignum* were two examples of this. Crosses within their species or with each other resulted in numerous seed being set. When pollen from other species was used with either of these 2 as the female parent, seed set was very low or unsuccessful at all. However when *L. salignum* or *L. discolor* pollen was used on other female plants from other species, seed set was often very good.

Table 1.8 Maternal effect of some *Leucadendrons* on mean number of seeds set per cone

Female parent	Male parent			
	discolor	*salignum*	*laureolum*	*floridum*
discolor	**60**	4.8	0	0
salignum	14.5	**23.7**	0	0
laureolum	34.5	8.3	**15**	3.25
floridum	4.5	4.3	4	**8**

1.4.5 Seed Germination

In 1999, 72.4% of the seed that was sown in the shadehouse in a sand/perlite mix germinated and 77.4% seeds germinated that were sown *in vitro* at 11ºC. The germination rates were similar regardless of which parents were used but seeds resulting from crosses involving a parent from the alata subsection (especially as seed parent) germinated at a slightly higher percentage (see table 1.9). Seeds from crosses when the female was from the trigona or villosa germinated at a slightly lower rate however it was noticed that seeds that didn't germinate *in vitro* were often found to have an aborted embryo or embryo infected by bacteria or fungi. As some seeds were not viable it is difficult to determine trends in seed germination. Therefore, in the following years seed that didn't germinate was inspected for viability.

Table 1.9 1999 *Leucadendron* seed germination results

Subsection of parent					
female	male	Number of cross combinations	Total Number of Seed Sown	Number of seed germinated	Seed germination (%)
trigona	trigona	10	178	113	63.5
trigona	alata	27	618	407	65.9
alata	alata	96	1322	1059	80.1
alata	trigona	14	155	110	71.0
villosa	alata	9	68	22	32.3
villosa	trigona	6	53	28	52.8
others		8	80	53	66.2
Totals		170	2474	1792	66.2

In 2000, seed came from crosses done either in 1998 or 1999 and the results are presented separately. Seed was germinated both *in vitro* and in the shadehouse but there were no significant differences in germination percentage and so the results have been combined.

There was very little difference in the germination rate between seeds collected in 1998 and 1999 with only a slightly higher germination result from the younger seeds (Tables 1.10 and 1.11). Regardless of year, seed germination was very good where the female parent was from the alata subsection. However when the female parent was from the trigona subsection and the male from the alata the germination results were much lower (<50%). Seeds from the villosa or other subsections also tended to germinate at a fairly low rate.

Not only did viable seeds with the female parent from alata germinate better than seeds from other subsections they also had the highest percentage of viable seeds when compared to seeds produced from crosses with the female parent from one of the other subsections.

Table 1.10 *Leucadendron* seed germination of seeds collected in 1998

Subsection of parent						
female	male	Number of cross combinations	Total Number of Seed Sown	Number of viable seeds	Number of seed germinated	Seed germination (%)
trigona	trigona	1	60	31	31	100
trigona	alata	12	230	111	50	45.1
alata	alata	29	714	588	574	97.6
alata	trigona	1	7	2	2	100
villosa	trigona	3	28	0	0	
villosa	alata	1	1	5	4	80.0
Totals		47	1040	737	661	89.7

Table 1.11 *Leucadendron* seed germination of seeds collected in 1999

Subsection of parent						
female	male	Number of cross combinations	Total Number of Seed Sown	Number of viable seeds	Number of seed germinated	Seed germination (%)
trigona	trigona	4	18	14	14	100
trigona	alata	2	24	13	6	42.9
alata	alata	62	1396	1262	1238	98.1
alata	trigona	3	26	18	18	100
other		4	26	10	6	60.0
Totals		75	1490	1317	1282	97.3

1.5 Discussion

1.5.1 *Leucadendron* Breeding methodology

Efficiency of breeding protocols

The protocol used in this breeding program has worked well. The protocol determined for the collection, storage and viability testing of pollen allowed crosses to occur regardless of flowering time and location. In many cases, large amounts of seed were produced that were relatively easy to extract and germinate.

Pollen collection

Pollen collection for the species available was relatively easy due mainly to the copious amounts of pollen produced. Pollen from species that are insect pollinated could be harvested by sieving the entire flowerhead to remove pollen. This mimics the action of the insects that remove the pollen in nature. This pollen often stuck together to form larger clumps of pollen which were easily handled.

Wind pollinated species tended to lose their pollen prematurely and thus were more difficult to harvest. Pollen was harvested by simply shaking a male flowering branch above clean paper. This pollen is more difficult to handle as it blows away at the slightest air movement but due to its abundance, sufficient amounts could still be collected. It is important that no other pollen sources are exposed when handling such pollen, as the potential for contamination is high.

Pollen storage

Pollen from most species survived being stored at sub zero temperatures (providing it had been desiccated) for at least one year and in many cases pollen collected in 1998 was still viable and has produced viable seed when used in 2000. Although the viability of pollen was shown to decrease with time (see chapter 3) the large amounts available and the relatively large size of the stigma meant that many pollen grains could be applied to each stigma. There are also several stigmas on each flowerhead and thus a low pollen viability rate still has a large chance of resulting in some successful fertilization. This is also important when hybrid pollen is used as it generally has a lower viability.

Some crosses from 1998 resulted in offspring that were very similar to the female parent species. These plants were often easy to identify at the seedling stage if compared to a seedling of the same age from the female species. This suggests that bagging of the female flowerheads did not prevent all uncontrolled pollinantions. The most likely reasons for this is either we were too late in bagging the flower or that insects were able to gain entry or were already on the plant before bagging. The appearance of these seedlings was often very different from seeds resulting from the intended cross

and in many cases they were identified and held back from field trialling. We intend leaving at least one bagged flowerhead unpollinated per plant to monitor the effectiveness of bagging in the future.

Seed collection

Cone collection and seed extraction was straightforward but in many cases cones must be monitored carefully and harvested before the seeds are released or birds destroy them. Some seed harvested within a few months of crossing has germinated well and grown into vigorous plants. Care must be taken when sorting seeds as many aborted seeds have a well formed seed coat and are difficult to distinguish without damaging the seed.

Germination

Seeds from species within the alata and trigona subsections were identified as having a cold temperature requirement for germination (see Chapter Four). This was achieved and good germination rates resulted especially if the female parent was from the alata. Seed from the trigona germinated well in the first year and in experiments (see Chapter Four), but germinated poorly in the 2000. Most of the latter were from 1998 and this may indicate that trigona seeds do not keep well in storage. Trigona (and alata) seeds are canopy stored and once released from the plant (usually after fire) they must germinate during the first rainy season or they are likely to be predated. This is consistent with the poor germination rate of the stored seeds.

Seeds from some species from the subsections leucadendron, nucifera, nervosa and membranaceae were only recently obtained but have shown some positive response to both diurnal temperature and treatment with hydrogen peroxide. The results however are not as high or as fast as seed from the alata and trigona. This is probably due to a different fire survival and germination strategy to the serotiny shown by the alata and trigona subsections (see Chapter Four). These seeds fall from the cone at maturity to the ground where ants collect the seed and store it underground in their nest. The ants do not damage the seed, which germinates only following a complex set of environmental stimuli (normally in autumn after a fire or clearing), which is not fully understood. Temperature treatments, oxygenation treatment e.g. soaking in hydrogen peroxide, scarification or treatment with other water soluble germination stimuli such as smoke water or gibberelic acid will be attempted in the future to improve the germination rate of these species and any resulting hybrids.

Seedlings raised over winter and spring grew well and losses of plants at this stage were restricted to plants with serious deformities (see Chapter Two). As there was a limit on the number of plants that could be trialled, the biggest, healthiest and most promising plants were selected first leaving behind small, deformed or plants clearly not from the intended cross.

Seed that did not germinate in 2000 was cut open to observe the embryo. In many cases the embryo was small and shriveled and it is assumed that post zygotic abortion due to genetic incompatibility was responsible. This was observed across all cross combinations with wider combinations leading to more abortions. In table 1.10 and 1.11 it can be seen that large numbers of seeds with the female parent from the trigona and villosa were not viable while most seeds with the female parent from the alata were viable. Only seed that appeared viable were sown and so the difference reflects the relative ease of identifying non viable seeds when the female parent was from alata. The significance of the female parent, both in predicting viability and in germination is because the seed coat in completely made of tissue from the female and not both parents. This helps to explain some of our observations.

1.5.2 Selection of parents and their ability to form hybrids

Plants from several different species were made available but there was a bias towards species from the alata or trigona subsections. There was also a strong bias towards female plants as these are in many cases more suitable as cutflowers than the male form. This allowed for good information to be gathered in terms of the potential for creating hybrids within or between these subsections but it was more difficult to assess the potential for hybridisation between and within other subsections due to a lack of genotypes, especially male genotypes. Nevertheless, information regarding the potential for creating hybrids between the different taxa of *Leucadendron* has been achieved. Crossing within and between the alata and trigona subsections is relatively successful while crossing between these sections and other subsections is much less successful but in some cases possible.

The success of many cross combinations depended on which species was the female parent. This is illustrated by *L. salignum* which, when used as a female parent set very few or often 0 seed when crossed with other species except it's own and the closely related *L. discolor*. However when *L. salignum* pollen was used on female plants from other species, many viable seed were often produced.

Studies involving the observation of pollen pistil interaction from the above crosses show that pollen from other species is prevented from growing through the style of *L. salignum* and hence do not reach the ovary and thus fertilisation does not occur. This appears to be fairly consistent across 9 of the female genotypes of *L. salignum* we have used for crossing. One genotype however has a lower propensity to block the progress of pollen tubes within the style and thus some viable seeds have been produced from crosses with this plant.

There are 2 important implications for what we can say of the ability for hybrids to be produced between different species where only a few female plants were available i.e. from subsections other than alata and trigona. By default most crosses between these subsections and species from the other subsections involved using pollen from the alata or trigona. It is therefore unknown what the outcome would be if the parents were reversed. The second important implication from the *L. salignum* example was the variability within a species to form hybrids. Our results indicate that different genotypes will vary in their ability to hybridise with other species. Further evidence of this can be seen from hybridisation results from South Africa which report success for some combinations which failed to set seed in our experiments and failure (0% seed set) for combinations in which we achieved numerous seeds (Robyn and Littlejohn, 2001). The most likely explanation is variability within the species.

Another reason why crosses may have been unsuccessful when alata or trigona pollen was used on other subspecies especially within the section Leucadendron (A) may be the relative length of the styles. Styles from *L. orientale* or *L. rubrum* for example are several times longer than the styles of members of the alata and trigona subsections and hence pollen from these subsections would be required to grow a pollen tube several times longer than it would normally. Pollen pistil interaction studies have shown that these tubes often do not reach the ovary end of the style and hence fertilisation does not occur. However the reverse cross is more likely to produce pollen tubes able to reach the ovary.

For all these reasons it was decided to obtain seed from many species outside of the alata and trigona subsections which are currently being raised with the aim of performing further crosses in the future. The species chosen also have a range of other useful characteristics as described in table 1.3

We found the fertility of hybrid plants to be relatively high and this has at least 2 important implications for further breeding; 1) it allows the introgression of traits from more than 2 species into the one variety and 2) the ability to backcross to one of the parental species so that a recessive trait that is lost in the F_1 generation may be expressed in the F_2 generation.

1.5.3 Improving the range of Leucadendron varieties

Extending Leucadendron flowering period

Leucadendron parents flowering from June to December have been collected for the breeding of *Leucadendron*s. We were able to produce hybrids between species regardless of flowering time by using stored pollen and thus we expect to see a range of flowering times in the resulting offspring.

We are hoping to incorporate the attributes of current varieties with the flowering times of selected parents to produce new varieties that flower either earlier or later than current varieties. Out of season varieties are likely to attract premium prices. We expect detailed information regarding flowering time of the hybrids to be collected and assessed this year.

Improving Leucadendron durability

Many *Leucadendron* varieties used as cut flowers should not be pruned below the current season's growth and hence plants become progressively taller and more difficult to manage after each season. However some species (e.g. *L. spissifolium* and *L. salignum*) have the ability to produce fresh growth from old wood (epicormic buds) and hence can be pruned in a manner which allows for optimal management practices. This trait is possessed by many of the current commercial varieties (e.g. Safari Sunset, Maui Sunset, Red Gem, Inca Gold) resulting from interspecific hybridisation with *L. salignum*.

All 15 female species from the alata and trigona subsections, many hybrids and some species from other subsections set seed when crossed with *L. salignum*. Several of these hybrids have displayed evidence that they possess this epicormic trait although pruning following this season's flowering will be required to confirm this ability. Many crosses have also been performed with *L. spissifolium* and several of the hybrids produced are also currently being assessed for this trait.

Introducing more red Leucadendron varieties

Most species of *Leucadendron* with prominent bracts are generally yellow, silver or green, however most *L. salignum* plants have very attractive red bracts. This trait has been incorporated into some of the commercial varieties (e.g. Safari Sunset and Red Gem). Hybrids were produced between *L. salignum* and almost all of the other species attempted and several of these will flower for the first time this year. Red pigmentation is already clearly visible in the bracts and upper leaves of many of these hybrids and thus it appears that we have been successful with introducing the red color into a large range of plants. Following flowering this season, detailed information regarding the inheritance of bract colour will be gathered and assessed.

Most male *Leucadendron* flowerheads are yellow prior to anthesis however 2 very closely related species (*L. discolor* and *L. procerum*) commonly produce male flowerheads which are a stunning red colour. We have attempted to introduce this feature into several other forms and varieties by using pollen from *L. discolor* and *L. procerum* on the other species used. Most combinations within the trigona and alata subsections were successful and several hybrids were produced and are being trialled. Most of these crosses will flower for the first time this year when detailed information regarding the inheritance of color will be gathered and assessed. One male hybrid (*L. floridum* x *L. discolor*) did flower last year and the red coloring was present in the flowerhead.

1.5.4 Initiation of a long term *Leucadendron* breeding program.

A protocol has been developed allowing the hybridisation of *Leucadendron* varieties separated by distance and flowering time involving the collection and long term storage of pollen. This method has been very successful within and between the species in the alata and trigona subsections, resulting in more than 20 000 hybrid seed to date. Seed have also been produced from crosses with species from outside these subsections but due to difficulty obtaining parents (especially male parents) the potential for these crosses has not been fully explored.

This first stage of a long term *Leucadendron* breeding program has laid the groundwork for the production of numerous potential new varieties. By careful selection of parents and continuous improvement of the breeding protocol, hybrids will be produced that will continue to improve the competitiveness of Australian *Leucadendron* growers in the world floriculture market.

To take advantage of this opportunity, careful and strict selection criteria need to be applied to the resulting hybrids to minimise the cost of trialling such large numbers. The second stage of this program aims to address this issue using early selection methods. We also plan to develop techniques that allow for the rapid propagation and distribution of selected superior varieties.

1.6 Reference

Robyn, A. and Littlejohn, G. M. (2001) Hybridizing *Leucadendron* Acta Hort 545, 73-76

Brits GJ et al. (1983) Domestication of Fynbos Proteaceae as a floricultural crop. Bothalia 14: 641-646.

Littlejohn GM et al. (1995) 'Marketable product' approach to breeding Proteaceae in South Africa. Acta Horticulturae, 387: 171-175.

Parvin P (1981) Hybridisation of Proteas in Hawaii. First International Conference of Protea Growers: 65-67.

Scott C (2001) South African Proteaceae, What Does and Dose Not Sell in Sydney? FLOWERSWEST magazine 7:6

Sedgley M (1995) Cultivar development of ornamental members of the Proteaceae. Acta Horticulturae, 387: 163-169.

Tranter, D Protea, *Leucadendron* and waratah. RIRDC Report from http://www.rirdc.gov.au/pub/handbook/protea.html

van den Berg GC, GJ Brits (1995) Development of *Leucadendron* single stem cut flowers. Acta Horticulturae, 387:191-198.

Vogts M (1982) South Africa Proteaceae, know them and grow them. Struik, Cape Town, pp 156-159

Williams IJM (1972) A revision of the genus *Leucadendron* (Proteaceae). Contributions from the Bolus Herbarium No. 3. Published by the University of Cape Town, Rondebosch, South Africa.

2. Assessment of Hybrids

2.1 Introduction

Species within the genus *Leucadendron* are widely exploited as a major wildflower export group. Some superior *Leucadendron* hybrids have been bred or selected from seedlings resulting from uncontrolled cross-pollination and have been released as named varieties. Hybrids such as Safari Sunset, Inca Gold, Maui Sunset, Silvan Red, Pisa and Jubilee Crown tend to be very vigorous, producing a good harvest of attractive flowers and foliage on long straight stems. These varieties tend to flower in a limited time of the year leaving gaps in the production line when prices peak in the market.

However, there are other *Leucadendron* species flowering in every month of the year from March (*L. cryptocephalum*) to December (*L. dregei*) and into early January (*L. loriense*). Crossing between species flowering at different times of the year will increase the availability of high quality *Leucadendron* flowers and foliage. Apart from flowering time, several other important floricultural and horticultural attributes can be incorporated into superior varieties produced by interspecific hybridisation.

In 1998, a *Leucadendron* hybridisation program aimed at producing superior varieties for floriculture was initiated. At the completion of the first season's crossing and seed collection there were 12,855 seeds resulting form 164 different interspecific cross combinations and 3,271 seeds resulting from 24 different intraspecific cross combinations. However the greater the number of hybrids to choose from the more stringent the selection process needs to be (Littlejohn, 1997).

Some offspring may be selected out at the seedling stage if they show deformed or excessively slow growth or high susceptibility to disease but currently there are no molecular or morphological markers based on seedling characteristics to help with the early selection of superior *Leucadendrons* for floriculture. Thus the hybrids must be evaluated by growing the plants under standard commercial conditions for *Leucadendron*.

2.2 Objectives

1) To assess and attempt to categorize and record the traits of the offspring produced from controlled crosses within *Leucadendron*. Simple inheritance of qualitative traits such as bract color, presence of lignotuber and sex will be studied as will the inheritance of quantitative traits such as stem length and vigor.

2) Select superior forms of *Leucadendron* for floriculture. Plants must be very vigorous, produce numerous long straight stems of export quality and have major advantages over other varieties currently available especially with regards to flowering time.

2.3 Materials and Methods

Seedling Selection

Resources to plant and assess about 1,700 hybrids were secured in 1999 following a successful crossing season in 1998. Representatives from the 188 different cross combinations that produced seed were selected. We decided to sow 20 seeds or all the seeds if less than 20 from each interspecific combination and 10 seeds from some of the intraspecific combinations (at least one combination per species used). Altogether, 2,474 seeds from 174 different combinations were sown in autumn and winter 1999.

The most vigorous, largest and healthy of these plants were selected and planted in the field in November 1999. A second selection and planting in December 1999 brought the number of hybrids in the ground to 1638. Of the remaining plants, some were potted up into large pots for chromosome counting and some were discarded.

1999 field trial

Selected seedlings were planted out on 6 commercial *Protea* farms in South west of WA in November and December 1999. Plants were arranged in single rows at 600mm intervals (which is approximately half of the spacing for a commercial *Leucadendron* crop) with between 33 and 200 plants per row and 3-5 meters between rows. All management practices including planting, weed, pest and disease control, the amount and frequency of irrigation and fertigation, and staking and pruning have been carried out as uniformly as possible between farms. All seedlings were protected by a fibre glass growth cone, which protected them from extremes of weather and grazing by rabbits and kangaroos. This guard was removed once the plants had become established.

2000 Field Trial

This whole process was repeated in 2000 with seeds produced from both the 1998 and 1999 crossing seasons. In all, 2,530 seeds were sown in autumn 2000 resulting in 1,943 seedlings. Once again the most vigorous, healthy and promising (i.e. interspecific) seedlings were selected by summer to fill the 1,484 new spaces made available for hybrid assessment in 2000. Seedlings that were small, deformed or clearly not from the intended cross were discarded as were many plants from intraspecific control crosses or crosses where many seedlings were available to choose from. Planting and management of the seedlings was the same as the previous year.

First Preliminary Assessment

The first preliminary assessment was conducted in late autumn 2000 and involved the growers filling out a preliminary assessment record which allowed them to tick the boxes they felt best described the form, vigor and likeness to parents for each of the plants on their farm. They were also encouraged to make any comments that they thought were useful. These results were used in part to help decide which of the seeds remaining from the 1998 crossing season should be planted and where our efforts should be focused in further breeding and selection

Second Preliminary Assessment

A second preliminary assessment occurred in October 2000. Information that was gathered in the first preliminary assessment was reassessed as was further information regarding flowering date, survival, sex, bract colour, flower colour and flowering form (i.e. single stem or multi-stem).

2.4 Results

Seedling assessment

Altogether, 1,792 seeds germinated and were raised in a shadehouse over winter and spring of 1999 (Chapter one) however several deformities were noticed amongst some families including albinism and failure of the radical to develop which lead to the death of affected plants. By the end of spring of 1999, 1638 plants were available for field trialling. Another 1,484 seedlings were also planted in year 2000.

A relatively large number of plants could be selected against due to genetic deformities. Albinism was common amongst offspring in which *L. muirii* was the female parent and lead to the death of seedlings at the cotyledon stage. Another deformity with fatal results affected the developing radical.

The radical would emerge from the seed coat and the hypocotyl and cotyledons would appear to develop normally, however the radical does not continue to grow from the base of the hypocotyl. The hypocotyl and cotyledons are green and can survive for several months without the radical but do not form true leaves and all affected plants had died by summer. In the first year, this effect was restricted to crosses between *L. conicum* (female parent) crossed with *L. salignum* (8/18 deformed), *L. discolor* (14/20 deformed) and *L. procerum* (10/17 deformed).

This same effect was observed the following season when *L. conicum* as the female was crossed with *L. salignum* (11/20 deformed) and *L. discolor* (12/24) but also when *L. eucalyptifolium* was used as the female to cross with *L. salignum* 15/15 seeds that germinated had the deformity. Seedlings from all other cross combinations from these parents have developed normally and those seedlings without the deformity from the affected crosses have developed into robust plants with no further complications.

Failure of the radical to develop normally was also observed in seeds that had not germinated several months after being sown. When the testa had been removed and the embryo established in MS media under *in vitro* conditions, cotyledons and a hypocotyl would sometimes form without a radical. This could be maintained and induced to form shoots when 0.1-0.3µM BAP was included in the media.

Further genetic deformity was observed in hybrids from some wide crosses. Examples of this include hybrids with *L. linifolium* as the female parent. Five abnormal seedlings resulting from the cross with *L. salignum* grew very slowly - 2 never grew any true leaves (cotyledons, hypocotyl and radical only) and died within months of germinating while the remaining 3 seedlings were so small at time of planting of first hybrids they still had only 3-5 true leaves in a red sick looking colour. Of these plants, 1 survived nearly 2 years in the shadehouse after which time it was still only about 60mm high before it too died. Similar excessively slow growth rates were observed in some offspring resulting from the crosses *L. linifolium* x *L. salicifolium* and *L. linifolium* x *L. eucalyptifolium.*

In some cases, individuals within a family have shown significantly poorer growth relative to their siblings and these have also been held back from field-testing. A slow growth rate has also been observed in some plants from combinations involving a hybrid. For example, of the 3 seedlings resulting from the cross of Inca Gold x *L. gandogeri* only one was developed enough to plant in the field with the other 2 still being very small (<80mm high) a year later.

First Preliminary Assessment

The preliminary assessment record sent to growers and completed by them in autumn 2000 revealed interesting information despite the plants being so immature. The results are summarised in Table 2.1

Table 2.1 Summary of preliminary assessment of *Leucadendron* hybrids planted in November 1999 and assessed in May and June 2000*

Farm	Number hybrids trialled	% surviving autumn 2000	Plant form (%)			Vigor (%)		
			erect	Semi erect	spreading	High	Medium	low
Abbey Farm	201	86.6	77.6	11.5	10.9	11.5	27.6	60.9
Amarillo Proteas	303	97.4	35.6	33.0	31.4			
Annie Brook	299	94.7	96.8	1.1	2.1	29.0	55.5	15.6
Busselton Proteas	462	98.9	50.8	27.7	21.5	23.4	45.2	31.4
M&M Cox	164	99.4	10.4	40.5	49.1	12.3	67.5	20.3
Tan Ridge	209	84.2	77.3	5.7	17.1	59.1	34.1	6.8

*Due to the immaturity of the plants and because many of the parents used to produce these hybrids are not currently being grown commercially, information on hybrid phenotype relative to that of the parents was difficult for the growers to assess and has not been presented here.

Several growers recorded important comments for particular individuals relating mainly to foliage and form characteristics and several had identified potentially superior selections amongst their plot.

Second Preliminary Assessment

This assessment focussed on plants that had flowered or were flowering. In total 135 plants had or were flowering in October 2000. This represents 8.2% of the hybrids planted. Plants that flowered tended to have one or both parents in the subsection trigona as shown in table 2. There were slightly more male plants than female plants. With the exception of the 2 males with both parents from the alata subsection, no hybrids with parents from other subsections had flowered.

Table 2.2 The influence of parental subsection on the number of hybrids flowering in the first season in the field

Subsection of parents		Number of plants in the Field	Number of flowering plants			Percentage of flowering
Female parent	Male parent		males	females	Total	
trigona	trigona	143	53	43	96	67.1
trigona	alata	311	19	15	34	10.9
alata	trigona	111	2	1	3	2.7
alata	alata	1027	2	0	2	0.2
Total		1592	76	59	135	8.5

At this stage most of the hybrids were still small and those that flowered produced only a few flowers and little information other than sex and colour could be determined at this stage. It was clear in most cases that the flowers we observed were only a fraction of what could be expected the following year and thus important information regarding stem length, productivity, flower size etc could not be assessed with any accuracy.

Despite this some of the flowering plants were identified as having desirable characteristics including a male *L. floridum* x *L. procerum* which had red flowerheads and yellow bracts and a female from the cross *L. conicum* x *L. salicifolium* which developed a red cone.

Apparent hybrid vigour was a characteristic of several of the plants including many that had not flowered at this stage as observed by productivity and long straight stems when compared to control intraspecific offspring.

2.4 Discussion

At this stage only preliminary field assessment of the hybrids has been attempted with the first major assessment to occur from June to December 2001. It is only then that the information regarding the inheritance of desirable traits can be obtained and that selections of superior forms can be made.

However, early selections at UWA based on vigor, health and form prior to field testing reduced the number of plants that required field assessment. The plants that were selected against at the seedling stage usually had serious deformities, very slow growth rates or had resulted from an uncontrolled cross. These plants were maintained in the shadehouse and in almost all cases plants that were selected against at the time of first planting were still unsuitable for field trialling the following year. This suggests that such poor vigor expressed as a seedling will continue to be expressed throughout the life of the plant. Therefore selection against seedlings with poor vigor is justified, as it is essential that any new commercial variety is vigorous.

Likewise the selection and exclusion of offspring not from the intended cross at a seedling stage is desirable. This allows more 'true' hybrids from known parents to be trialled. This is important as parents have been specifically chosen for a particular reason and knowledge of the parents allows for assessment of the inheritance of certain traits. In many cases hybrid offspring were clearly distinguishable from those resulting from uncontrolled intraspecific crosses and thus selection can be made with a high degree of certainty.

The first preliminary field assessment provided some valuable information. This was a good opportunity to get some feedback from the growers that managed each assessment block. It is important that good communication occurs between the growers and the plant breeders at all stages of the breeding program but especially in the assessment stage. These growers have first hand knowledge of what does and does not make a good variety. Information received from these growers is usually by personal communication however by completing the first preliminary assessment for the plants on their property growers were able to record and express their thoughts for each individual genotype.

The first preliminary assessment revealed that most of the hybrids had survived the first summer, had an erect growth habit and were showing high or medium vigor.

The differences in recorded forms and vigor between different farms is likely to be due mainly to the fact that in 1999 hybrids from different species were planted on different farms with families and plants resulting from similar species being more likely to be planted on the same farm. Therefore plants on one farm are more likely to be similar to each other than to plants on other farms. This would explain some of the large differences observed in table 2.1. The results are further complicated by the subjective nature of the assessment being carried out by different individuals.

Most of the hybrids were recorded as having an erect growth habit that is important in the production of high quality flower stems. This was a characteristic of most of the parents and hence this result was expected. An exception was some offspring resulting from the very erect parent *L. discolor*. In many cases these offspring had more of a semi-erect or spreading habit at the time this assessment was made. This is probably due to a juvenile growth phase as some of these plants have since produced long upright stems. A juvenile growth phase is also suspected to be responsible for some of the plants recorded as having low vigor e.g. *L. discolor*. If a juvenile phase is identified that is

significantly different to the mature plant in such important characteristics as form and vigor it becomes more difficult to select seedlings at an early stage. However only *L. discolor, L. muirii* and perhaps *L. strobilinum* have shown any evidence of this so far and so there is still a good possibility that early seedling selection is useful for most hybrids.

In 2000, more individuals from fewer families were grown and this allowed for offspring from a single cross combination to be planted in more than one assessment block. This will make assessment of similar forms more difficult if they are on different farms however if adverse conditions affect one farm other members of that family will be safe at another site.

The second preliminary assessment focused on plants that had flowered or were flowering. The vast majority of plants that had flowered had come from crosses between species within the subsection trigona (96/135). Species from trigona tend to have smaller flowers and fruit than those from alata and this may be a reason why they have a higher propensity to flower in their first year. This propensity was transferred to some hybrid offspring.

It is possible that more plants would have flowered in this first season in the ground if all seeds had been sown early in autumn (seeds were actually sown right through autumn and winter) and seedlings planted in the field in spring (not summer as occurred in this case).

There was a slightly higher percentage of male plants flowering and this may reflect the relatively low cost of producing pollen as opposed to producing ovules and nurturing seed and fruit. This may be why only male plants from hybrids within the alata flowered in this first season.

Plants that did flower often produced a display that was inferior to what is expected in subsequent years. Hence no selection could be carried out based on this first year flowering display. It is expected that these plants and plants that have not yet flowered will produce a good display in this second year after planting out. Already many hybrids with parents from the alata can be seen to be initiating flowers on several stems so these should give a good indication of cutflower performance this season.

It is therefore the second year after field planting (third year after crossing) that hybrids are likely to be assessed with any confidence. This assessment will have to be ongoing for the entire potential flowering season (June to December) to ensure hybrids are assessed at peak flowering stage. This may present some problems with male plants as their window of commercial viability is very small (often only a few days) and hybrid offspring are being assessed over 6 different sites with large distances between some sites. Some varieties are also likely to be suitable for harvest at more than one stage e.g. Safari Sunset and these need to be assessed at each commercial stage.

The cost required to evaluate hybrids is estimated to be at least $5.00 per year per plant, including irrigation, planting and maintenance, pest, disease and weed control, fertilising etc. This cost plus the opportunity cost of the land was paid for completely by growers as in kind support additional to the large cash contribution they had already made. Despite this, the contributing farms generously agreed to plant and evaluate a total of approximately 1700 hybrids in the first year.

Another major challenge when assessing potential new varieties is the time taken for the assessment to occur. It is hoped that for some hybrids, seed that is sown early in autumn and seedlings planted in the field in spring will flower by the following season (June to November). However it is likely that some hybrids may not flower until their second or even third year after planting out. Also it is likely that only very limited information regarding attributes as a potential new variety would be available for plants which flower in their first year after planting.

For these reasons it is likely to take at least 3 years from when the cross is made to the first detailed assessment of offspring. That is crossing is done June to November 1998, the seeds are collected late 1998 and early in 1999, germinated in autumn 1999. Seedlings are planted in spring and early

summer 1999, some may flower in June to December 2000 with the first detailed assessment occurring between June and December 2001.

Fortunately once a breeding program has been initiated a new batch of hybrids will become available for selection each year following the third year thus assuring a continual supply of potential new varieties.

Finally, stringent selection criteria need to be developed based on breeding goals. These criteria need to be based on information gathered from both growers and exporters within the industry.

It is hoped that several plants will show superior qualities by this stage (second flowering season) which can then be cloned and maintained in a genetic bank of elite varieties for assessment and release into the future.

2.5 References

Littlejohn, G. M., (1997) Evaluation of *Leucadendron* Selections as Single Stem Cut Flowers Acta Horticulturae 453:53-57

3. Pollen Storage

3.1 Introduction

Collecting and storing pollen is the easiest method of allowing crosses to occur between plants flowering at different times or at different locations. With male *Leucadendron* plants, the pollen is shed at anthesis and adheres to the topmost portion of the style known as the pollen presenter (Vogts, 1982). Several of these flowers are arranged on a central core forming a flowerhead, with many flowerheads being produced by each plant. Flowers present pollen in succession from the bottom of each flowerhead with large amounts of pollen being produced over a very short period of time, often only 1 to 2 weeks from when the first pollen is presented to when the last is presented.

Following presentation, insects quickly remove the pollen of most *Leucadendrons* although wind is thought to be responsible for the pollination of some species e.g. *L. salicifolium, L. rubrum* and *L. linifolium*.

As there is such a brief period of only 1 to 2 weeks in which fresh pollen is available in quantity for each genotype it is important that a protocol for collecting and storing the pollen is developed. To be useful for breeding, stored pollen must remain viable for at least one year, which is until the following flowering season so that all the possible crosses can be attempted.

Fresh pollen is likely to be viable for only a short period of time under natural conditions but two techniques (drying and freezing) are widely used to prolong the life of stored pollen.

Cooling or even freezing the pollen has resulted in pollen from many different taxa remaining viable for long periods of time including the members of the Proteaceae, *Banksia* (Maguire and Sedgley, 1997) (Shchori et al, 1992) and *Protea* (van der Walt and Littlejohn, 1997). Desiccation of the pollen can also significantly improve the survival rate of stored pollen, especially if the pollen is to be stored at sub zero temperatures for long periods of time.

An experiment was designed to determine the effect of desiccation and storage temperature on the survival rate of *Leucadendron* pollen over the period of 1 year.

Four temperatures (20°C, 4°C, -20°C and -80°C) each with or without desiccation treatments prior to storage were tested.

A method for estimating the viability of pollen following storage and immediately prior to use is desirable so that the sample with most viable pollen can be used to enable maximum chance of successful fertilization.

Viability of stored pollen is often assessed by *in vitro* germination in conditions designed to imitate a receptive stigma. Pollen grains that germinate are viable while those that don't are not viable. Several different methods of *in vitro* germination have been reported with varying results for many different species. Germination tests are often considered more accurate than staining tests however they generally are more complicated (larger potential for error or environmental effects) and take a longer period of time, as it can be several hours between sowing the pollen and germination.

A quicker method involves the use of a dye fluorescein diacetate (FDA). FDA is a non-fluorescent ester of fluorescein that can readily penetrate the pollen membrane. A pollen grain that produces esterase will cleave the FDA to release fluorescein which is less able to transverse the membrane (providing it is intact) and thus accumulates in pollen grains with intact membranes. When viewed under a fluorescent microscope, these pollen grains are clearly distinguishable by their fluorescence from those without an intact membrane. This technique is much quicker than germination taking only a matter of minutes. It is thought that state of the membrane is one of the principal determinates

of germination capacity (Heslop-Harrison, 1979). Deterioration of this membrane is a likely reason for a loss of viability during storage.

Preliminary tests indicated that both methods were useful in determining the viability of pollen. Germination tests were in all cases estimating a lower viable percentage than FDA although it showed similar trends. However germination was affected by the media and species used and in some cases results were highly variable even between samples from the same vial. For these reasons FDA was used to estimate pollen viability and these results are presented below.

3.2 Objectives

3.2.1 To determine a protocol for the long term storage of *Leucadendron* pollen

3.2.2 To determine a fast and reliable method to estimate the viability of *Leucadendron* pollen

3.3 Materials and methods

Pollen Collection

The species used in this experiment were 2 genotypes of *Leucadendron discolor* and 1 genotype from each of *L. salicifolium, L. floridum, L. eucalyptifolium* and *L. conicum.*

Pollen was collected by harvesting large stems containing many flowers when between half and two thirds of the flowers in each flowering head had presented pollen onto the sterile stigma.

The bases of the stems were immediately placed in water (keeping the flowers dry) and brought back to UWA being careful not to knock pollen off or allow pollen from different genotypes to contaminate the flowerheads. The pollen was separated from flowering heads by removing the entire head from the stem and sieving using a fine mesh sieve above clean paper.

Pollen that falls onto the paper was easily seen and handled due to its abundance and a small amount of each genotype was transferred into 8 ependorf tubes.

Desiccation

For each of the genotypes involved 4 of these tubes were immediately closed and placed at 4ºC for 48 hours (non-desiccated controls). The other 4 tubes for each genotype were left with the lid open and placed in a sealed jar containing freshly dried silica gel and placed at 4ºC for 48 hours (desiccation treatment). Following this all tubes had their lids closed securely and were exposed to the appropriate temperature treatment.

Storage temperature

For each genotype one desiccated tube of pollen and one non-desiccated tube of pollen was stored at 20°C, 4°C, -20°C and -80°C.

Viability testing - FDA Test

Three samples of pollen from each treatment were assessed for viability using the FDA method after 1, 7, 14, 30, 90, 180 and 365 days in storage.

An FDA solution was prepared by dissolving 2 mg of FDA in 100ml acetone. One drop was placed on a microscope slide and allowed to evaporate.

The tip of a dissecting needle was used to transfer a small sample of pollen from each of the treatments to a clean ependorf tube containing 200 µL (0.2 ml) of 20% sucrose solution. The suspension was thoroughly mixed using a pipette.

One drop of the resulting suspension is placed on the slide on top of the dried FDA. It is important that all the acetone has evaporated as any remaining may affect the viability of the pollen.

A coverslip was applied and the slides were left for at least 5 minutes before being observed.

The samples were observed under a fluorescent microscope. Pollen grains that fluoresced brightly and appeared swollen were scored as viable, while shriveled yellow-green pollen was not considered viable. At least 200 pollen grains from at least 10 different fields of view from each sample were counted.

Pollen Viability - Germination Test

A method for the *in vitro* germination of pollen was also used to assess viability.

The method involved stapling 5 pieces of filterpaper together on one edge. The filterpaper was then placed in a petridish and 8 small square plastic film segments (approximately 1cm²) were inserted under the first sheet in a 4x2 grid pattern.

The papers were saturated with a 20% sucrose solution and left for ten minutes, then the excess solution was poured away and the top piece of filterpaper removed leaving the plastic squares on top of the other 4 pieces of filterpaper.

One sample of pollen was sown on each plastic square and recorded using the staple as a reference point.

The petri dish was covered and sealed with parafilm and placed at 23ºC. Four hours later each plastic square was removed and mounted on a microscope slide, stained with a drop of analine blue and then observed under a fluorescent microscope.

The percentage of germination was estimated by counting at least 200 pollen grains from 10 different fields of view. A pollen grain was classified as germinating when the pollen tube was equal to or longer than the pollen grain.

3.4 Results

Both desiccation and lowering of the storage temperature were shown to significantly improve the survival of stored pollen (Table 3.1 and Figure 3.1).

Pollen from all species, except *L. salicifolium* that was not desiccated and stored at 20ºC (room temperature) had their viability reduced to between 0 and 2.16% by day 7 and 0% by day 30. Pollen from *L. salicifolium* kept relatively well for 7 days at 20°C without desiccation (34.4%) but viability was very low by 14days (1.29%) and none was surviving by 90 days.

The treatments giving the highest survival rates after 1 year for all species (29.4% to 55.26%) were either stored at -20°C or -80°C following desiccation and there was generally no significant difference between each treatment for each species.

Desiccation clearly improved the survival rate for each temperature treatment and in some cases the difference was significant e.g. after 7 days at 20°C *L. conicum* pollen that was desiccated had maintained a viability of 57.2% while non-desiccated pollen had lost all viability.

Reducing the storage temperature of non-desiccated pollen generally improved the survival rate as some non-desiccated pollen was still viable after 90 days at -20°C or -80°C but there was no non-desiccated pollen remaining viable after 90 days at 20°C or 4°C. The exception is non-desiccated pollen of *L. salicifolium* in which viability fell to 1.8% after 7 days when stored at -80°C.

Desiccation and storage at 20°C was also found to be unsuitable for long term storage with viability falling to 0% for all species with in 90 days. Desiccation and storage at 4°C enabled the pollen to retain a higher viability for longer but this viability had fallen to 0% by 365 days.

Desiccation and storage at -20°C or -80°C is a suitable method for storage of *Leucadendron* pollen for at least 1 year after which time as much as 55% of the pollen can still be viable.

Table 3.1 Pollen viability during storage with different treatments as estimated by the FDA method

LDC 03*	**Day 1**	**Day 7**	**Day 14**	**Day 30**	**Day 90**	**Day 180**	**Day 365**
20°C, -D	72.7	1.2	0.0	0.0	0.0	0.0	0.0
20°C, +D	72.7	0.7	0.0	0.0	0.0	0.0	0.0
4°C, -D	72.7	60.6	58.9	23.7	0.0	0.0	0.0
4°C, +D	72.7	53.0	53.9	54.9	1.7	0.0	0.0
neg 20°C, -D	72.7	53.5	60.5	46.4	24.3	7	0.0
neg 20°C,+D	72.7	53.3	66.6	70.9	63.6	18.4	16.9
neg 80°C, -D	72.7	45.3	59.5	61.2	25.2	15.1	33.2
neg 80°C,+D	72.7	60.4	68.4	71.4	60.1	40.3	40.1
LEC 01	**Day 1**	**Day 7**	**Day 14**	**Day 30**	**Day 90**	**Day 180**	**Day 365**
20°C, -D	51.2	2.2	2.8	0.0	0.0	0.0	0.0
20°C, +D	51.2	25.0	6.7	3.4	0.0	0.0	0.0
4°C, -D	51.2	26.7	3.6	1.9	0.0	0.0	0.0
4°C, +D	51.2	33.3	30.4	42.7	30.5	8.3	0.0
neg 20°C, -D	51.2	19.7	7.5	6.7	0.0	0.0	0.0
neg 20°C,+D	51.2	30.9	27.8	37.9	43.9	38.3	29.4
neg 80°C, -D	51.2	11.8	13.0	15.1	8.8	9.8	3.0
neg 80°C,+D	51.2	42.0	43.6	47.1	47.5	40.8	23.8
LDC 10	**Day 1**	**Day 7**	**Day 14**	**Day 30**	**Day 90**	**Day 180**	**Day 365**
20°C, -D	72.0	0.8	0.0	0.0	0.0	0.0	0.0
20°C, +D	72.0	37.1	30.3	0.0	0.0	0.0	0.0
4°C, -D	72.0	18.3	56.1	0.0	0.0	0.0	0.0
4°C, +D	72.0	75.1	56.8	55.3	0.0	0.0	0.0
neg 20°C, -D	72.0	35.1	27.9	23.7	0.0	0.0	0.0
neg 20°C,+D	72.0	72.2	59.4	68.1	68.6	70.9	37.9
neg 80°C, -D	72.0	2.3	2.3	0.0	0.0	0.0	0.0
neg 80°C,+D	72.0	0.0	67.2	66.2	71.5	72.3	41.1
LCF 01	**Day 1**	**Day 7**	**Day 14**	**Day 30**	**Day 90**	**Day 180**	**Day 365**
20°C, -D	68.7	0.0	0.0	0.0	0.0	0.0	0.0

20°C, +D	68.7	57.2	27.6	0.0	0.0	0.0	0.0
4°C, -D	68.7	8.0	4.0	0.8	0.0	0.0	0.0
4°C, +D	68.7	55.0	58.7	45.0	55.2	0.0	0.0
neg 20°C, -D	68.7	5.3	30.1	1.8	0.0	0.0	0.0
neg 20°C,+D	68.7	62.4	64.2	64.6	71.8	65.3	29.5
neg 80°C, -D	68.7	12.2	3.7	3.0	0.0	2.8	0.0
neg 80°C,+D	68.7	62.5	68.0	68.2	67.8	68.7	39.4
LFB 02	**Day 1**	**Day 7**	**Day 14**	**Day 30**	**Day 90**	**Day 180**	**Day 365**
20°C, -D	67.9	0.0	0.0	0.0	0.0	0.0	0.0
20°C, +D	67.9	22.8	28.5	0.0	0.0	0.0	0.0
4°C, -D	67.9	33.7	28.7	15.1	0.0	0.0	0.0
4°C, +D	67.9	67.1	49.9	35.8	41.2	2.8	0.0
neg 20°C, -D	67.9	37.2	2.3	23.9	0.0	12.4	0.0
neg 20°C,+D	67.9	66.2	68.9	52.9	55.9	59.5	45.3
neg 80°C, -D	67.9	58.1	32.9	24.9	36.5	35.0	28.8
neg 80°C,+D	67.9	59.3	61.8	55.5	70.6	63.0	52.3
LSA O1	**Day 1**	**Day 7**	**Day 14**	**Day 30**	**Day 90**	**Day 180**	**Day 365**
20°C, -D	72.3	34.4	1.3	4.9	0.0	0.0	0.0
20°C, +D	72.3	48.1	34.1	33.9	0.0	0.0	0.0
4°C, -D	72.3	75.6	55.4	6.7	0.0	0.0	0.0
4°C, +D	72.3	74.3	73.8	57.3	61.0	8.5	0.0
neg 20°C, -D	72.3	84.1	65.3	40.3	0.0	0.0	0.0
neg 20°C,+D	72.3	71.6	81.2	78.6	80.1	75.1	46.3
neg 80°C, -D	72.3	1.8	0.9	0.0	0.0	0.0	0.0
neg 80°C,+D	72.3	1.3	86.3	81.4	80.1	78.6	55.3

*Treatments
20°C, -D = storage at 20°C without desiccation
20°C, +D = storage at 20°C with desiccation
4°C, -D = storage at 20°C without desiccation
4°C, +D = storage at 20°C with desiccation
neg 20°C, -D = storage at -20°C without desiccation
neg 20°C, +D = storage at -20°C with desiccation
neg 80°C, -D = storage at -80°C without desiccation
neg 80°C, +D = storage at -80°C with desiccation
Genotypes LDC 03 and LDC 10 = *L. discolor*, LEC 01 = *L. eucalyptifolium*, LCF 01 = *L. conicum*, LFB 02 = *L. floridum*, LSA 01 = *L. salicifolium*.

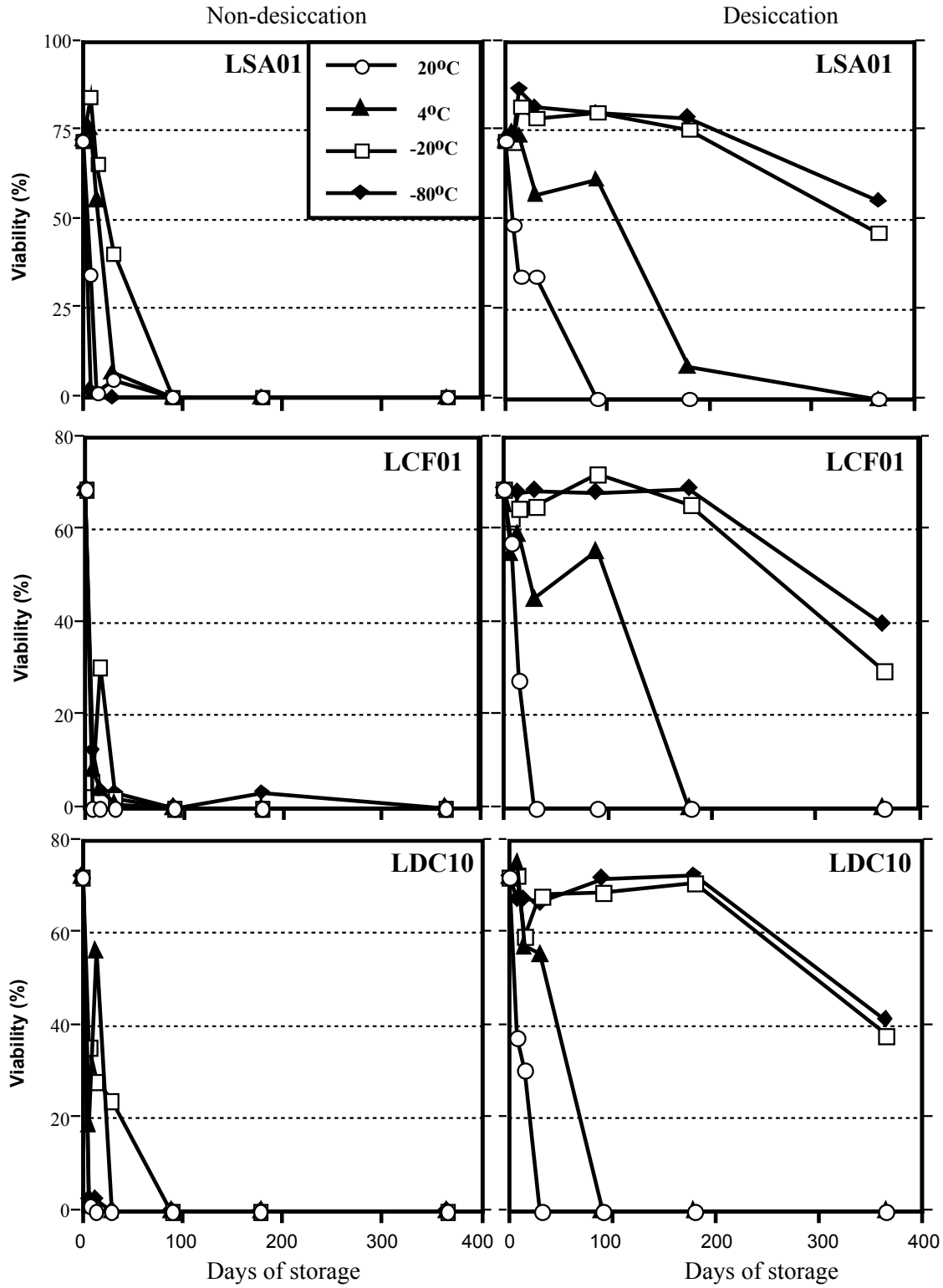

Figure 3.1. Longevity of non-desiccated and desiccated *Leucadendron* pollen stored for one year at four different temperatures, 20°C, 4°C, -20°C and –80°C. LSA = *L. salicifolium*. LCF = *L. conicum*. LDC = *L. discolor*.

3.5 Discussion

These results illustrate how the viability of a sample of *Leucadendron* pollen decreased over time, and how both desiccation and storage temperature influenced this loss of viability. Desiccation of fresh pollen followed by storage at either -80° C or -20°C maintained the highest percentage viability for all species one year after collection (predicted at between 29.4% and 55.3% using the FDA method). This represents more than 50% of the initial viability for each species and this pollen is suitable for crossing. Results from pollen collected in 1998 and used in 1999 and 2000 support the observation that pollen stored using this method is viable and capable of producing seeds when used in crossing after 1 and even 2 years in storage.

These results suggest that it is possible to collect pollen and store it under suitable conditions so that it is available for crossing as it is required i.e. when the desired female parent is receptive.

It was hoped that pollen germination tests could be used to measure the accuracy of the FDA test as germination tests are considered to be more accurate than staining tests. Germination rates followed the same trend as percent viable as measured by the FDA method i.e. germination rates were highest for fresh pollen and decreased to 0% for treatments that were not desiccated and/or stored at 4°C or 20°C. Some pollen from each species was able to germinate using this method after 1 year in storage following desiccation and storage at -20C or -80C but in all cases germination rates were lower than the viability predicted by the FDA method. No pollen germination occurred in samples where the FDA had predicted 0% viability and so it can be seen that the trends predicted by both methods were the same. It is possible that the FDA method is overestimating the viability by including pollen grains that have an intact membrane but are unable to germinate for a different reason. It is also possible that the germination method is underestimating the viability, as some viable pollen may not germinate *in vitro* that may germinate on a receptive stigma.

The FDA method predicts that the viability of fresh pollen used was between 72.7% and 51.2% where as the germination method estimated the viability of fresh pollen to be much lower (between 18% and 5%). It is more likely that fresh pollen has viability closer to what was predicted by the FDA method than by *in vitro* germination.

In vitro germination results often varied between genotypes, time and sometimes even between different samples taken from the same genotype at the same time. *In vitro* germination is also a more difficult method, requiring a longer period of time to set up and measure than the FDA method. FDA results were quick and the results from each of the three samples taken from each species in each treatment were consistent with each other. The method also seems to work equally well for each of the genotypes tested. For all these reasons the FDA method is the method we use routinely for the viability testing of fresh or stored pollen.

Experiments have been carried out in which boron and nutrients are added to the sucrose solution used for *in vitro* germination. This has improved the rate of germination for some species tested but for some other species germination was still low.

The results suggest that pollen that as been adequately desiccated and stored at either -20ºC (conventional domestic freezer) or at -80ºC maintains more than half of its initial viability for at least one year. This means that all cross combinations can be attempted regardless of the flowering period of each parent. Pollen stored at higher temperatures or not adequately dried, generally failed to survive for a whole year and hence would not be available to be used for all potential cross combinations.

The ability to store pollen and check the viability of stored pollen is a crucial part of the *Leucadendron* breeding program. The FDA method of predicting pollen viability is a quick and efficient method that we have used to test all pollen both at the time of collection and immediately prior to use. This allows us to dispose of any pollen that has lost its viability and to only perform crosses with pollen of a measured high viability.

3.6 References

Heslop-Harrison, J. and Y. Heslop-Harrison. 1970. Evaluation of pollen viability by enzymatically induced fluorescence; intracellular hydrolysis of fluorescein diacetate. Stain Technology 45:115-120.

Vogts, M 1982. South Africa Proteaceae, know them and grow them. Struik, Cape Town, South Africa, pp 156-159.

Vogts M (1982) South Africa Proteaceae, know them and grow them. Struik, Cape Town, pp 12.

Maguire, T. L. and Sedgley, M. (1997) Storage Temperature Affects Viability of Banksia menziesii Pollen Hortscience 32(5):916-917.

Shchori, Y., Goren, T. and Ben-Jaacov, J (1992) Polle Germination and Storage in Banksia and some other Proteaceae Plants Acta Horticulturae 316:19-22

Walt, I. D. van der and Littlejohn, G. M. (1995) Preliminary Results on Factors Affecting In Vitro Germination and Storage of Protea Pollen Acta Horticulturae 387 pp199-20

4. Seed Germination

4.1 Introduction

The genus *Leucadendron* can be divided into 2 distinct sections based on the morphology of their seeds (Vogts, 1982). Species in section A 'leucadendron' have round, nutlike fruits that are released 3 to 6 months after flowering. These seeds generally do not have a wing or any other obvious means of dispersal. It is thought that ants will collect these seeds and store them underground in their nests where they eat the fleshy outer pericarp but leave the seed intact and safe from both predators and fire. This strategy is known as myrmecochory. Species from section B 'alatosperma' have flattened, winged seed that remain on the plant (sometimes for several years) protected from predation and fire in a woody cone. Following a fire these seeds are released and fall in to a fire sterilized, nutrient rich ashbed free of competition. This strategy is known as serotiny. Serotinous seeds are reported as germinating more readily than myrmecochorous seeds.

Most of the species of *Leucadendron* used in the first year of crossing belonged to the alata and trigona subsections. These sections display the characteristics of serotiny. Serotinous seeds germinate in the first rainy season following a fire or else they are likely to be predated. Therefore only rain and an appropriate temperature are required for germination to occur. Myrmecochorous seeds are relatively safe underground and although most germination should occur during the first rainy season following a fire, the storage of seeds for several fire periods is possible. Therefore the germination requirements of such species are likely to be complex and germination rates lower and slower. Both seed coat and embryo imposed dormancy have been observed and reported.

The experiment reported in this Chapter was designed to investigate the germination requirements of serotinous species from the subsections trigona, alata and compressa and the requirements of plants from the subsection villosa. Seeds from the villosa subsection display many of the features of myrmecochorous species having round nut like seeds but they also have thinner seed coats more in line with serotinous species (Rebelo and Rourke, 1986). Preliminary experiments suggest that temperature is a major constraint on germination in these species with no germination occurring outside over summer or in a constant temperature room at 25ºC (except for some species in trigona) and good germination occurring outside over autumn and winter and in a controlled temperature room at 11ºC.

4.2 Objectives

To investigate the effect of 3 different temperature regimes on the germination of some species of *Leucadendron* used in our current breeding program.

4.3 Materials and Methods

4.3.1 Genetic material

The species used were *L. discolor* and *L. strobilinum* from the alata subsection, *L. conicum* and *L. uliginosum* from the trigona subsection, *L. muirii* from the compressa subsection and *L. galpini* and *L. linifolium* from the villosa subsection. 20 seeds from each genotype except *L. linifolium* were used.

4.3.2 *In vitro* Germination

An *in vitro* method was used to germinate seeds. Seeds were surface sterilized by immersion and agitation in 1% sodium hypochlorite for 20 minutes. This solution was prepared by adding 25ml of

"Whiteking ™ " bleach (4% Sodium hypochlorite) to 75ml of deionised water water. The seeds were removed from the solution under laminar flow and aseptically rinsed in sterile water. For each genotype, 20 seeds were sown on a piece of sterile moist filterpaper inside a sterile petri dish. The filterpaper and water used in these experiments had been autoclaved at 121ºC for 20 minutes prior to use. The petridishes were then sealed with parafilm™. Three petridishes were prepared for each species.

One petridish from each species was placed in the dark at each of the following controlled temperatures: constant 23ºC, constant 11ºC and 20ºC for 8 hours / 10ºC for 16 hours.

As soon as the radical emerged to a length equal to that of the seed it was recorded as germinated. Dishes were observed every 2 days for 58 days.

After 58 days all seeds that had not germinated were dissected and the embryo observed. Any seeds containing an embryo that was white were regarded as viable while seeds containing a shriveled yellow or brown embryo were regarded as nonviable. Germination percentage was determined by dividing the number of seeds that germinated by the number of viable seeds.

4.4 Results

The germination results are presented in table 4.1. A diurnal temperature of 20ºC for 8 hrs followed by 10ºC for 16 hrs resulted in the maximum seed germination for all species in the trial. A constant temperature of 11ºC was just as successful for *L. discolor, L. strobilinum, L. muirii* and *L. conicum* but resulted in lower germination percentages for *L. uliginosum* and *L. linifolium* and completely inhibited *L. galpini* from germinating. Only *L. conicum* and *L. uliginosum* showed any germination at constant 23ºC. The germination percentage was significantly higher at constant 11ºC than at constant 23ºC for *L. conicum* but the difference was insignificant for *L. uliginosum*.

Table 4.1 Germination percentage of *Leucadendron* seeds at constant or diurnally fluctuating temperatures

Species	Section	Subsection	Temperature		
			23ºC	11ºC	20ºC (8hrs)/ 10ºC (16hrs)
L. uliginosum	B	Trigona	61.1	57.9	95.0
L. conicum	B	Trigona	36.8	93.7	94.1
L. strobilinum	B	Alata	0	100	100
L. discolor	B	Alata	0	100	100
L. muirii	B	Compressa	0	100	100
L. linifolium	A	Villosa	0	40	71.4
L. galpini	A	Villosa	0	0	100

4.5 Discussion

This experiment illustrates the importance of temperature in the germination of seeds from these *Leucadendron* species. Species from section B are considered to use serotiny as a seed dispersal mechanism. Seeds from these species are protected during a bush fire inside a woody cone and are released following the subsequent death of the motherplant as a result of the fire. It is important for these seeds to germinate early in the rainy season but not after summer showers. The failure of most of the seeds to germinate at 23ºC is likely to be due to a minimum temperature requirement that was designed to prevent germination following summer rain. *L. conicum* and *L. uliginosum* come from mountainous habitats where rain is more consistent and thus the importance of inhibiting germination until a certain season is less strict. This may explain why some germination occurred at the relatively high temperature.

Leucadendron galpini and *L. linifolium* have seeds that share many characteristics with both myrmecochorous and serotinous seeds. As with serotinous seeds no germination occurred at the relatively high temperature of 23ºC, once again presumably to prevent germination during an unfavorable season (e.g. summer). However seeds from the subsection villosa are reported as not being stored in the canopy (Rebelo and Rourke, 1986) and so if a minimum temperature is all that is required, seeds would germinate when the temperature became low enough (probably in autumn). However the resulting seedlings are likely to be outcompeted by the established vegetation unless they possess a mechanism which allows for seed germination to be inhibited until the first wet season following a fire. It has been speculated that for other myrmecochorous species where a diurnal temperature has been shown to improve germination percentages (e.g. *Leucaspermum cordifolium*) that the diurnal temperature closely reflects the post fire soil temperature in the species natural environment (Brits, 1986). This may explain why the diurnal temperature was an absolute requirement for the germination of *L. galpini* and resulted in maximum germination in the other species as well.

Although good germination percentages were achieved at the diurnal temperature treatment we used in this research, it is possible that for other species of *Leucadendron* a different temperature regime of a lower or higher extreme may be required. For example, *Serruria florida* germinated best at a diurnal temperature of 7ºC night and 20ºC day (Brits, 1986).

Based on the this research, a growth chamber set at a diurnal temperature of 20ºC (8hrs)/

10ºC (16hrs) or shade house condition with fluctuating temperatures in Autumn was used to germinate hybrid seed for planting. A very satisfactory result has achieved (Table 4.2).

Table 4.2 Results of seed germination at different conditions

Conditions	Number of seed sown	Number of seed germinated	Germination rate (%)
20ºC (8hrs)/ 10ºC (16hrs)	1,245	1,107	88.9
Shade house	3,175	2,405	78.9

4.6 Reference

Brits, G. J. 1986. Influence on fluctuating temperature and H_2O_2 treatment on germination of *Leucadendron cordifolium* and *Serruria florida* (Proteaceae) seeds. South African Journal of Botany 52: 286-290.

Rebelo, A. G. and J. P. Rourke. 1986. Seed germination and seed set in southern African Proteaceae: ecological determinants and horticultural problems. Acta Hort. 185: 75-88

Vogts, M 1982. South Africa Proteaceae, know them and grow them. Struik, Cape Town, South Africa, pp 156-159.

5. Pollen /Pistil interactions

5.1 Introduction

There are many naturally occurring barriers preventing hybridisation between species in the wild. Environmental barriers such as geological isolation and different flowering periods have been successfully overcome for the breeding of *Leucadendron* by the development of pollen collection, storage and viability testing protocols (see chapter 3).

From the crossing results obtained in 1998, it became apparent that overcoming these environmental barriers was all that was required in many cases to cross between closely related species (e.g. between species in the same subsection). However wider crosses (e.g. between species in different subsections) and some narrow crosses often resulted in either no seed set or in the post-zygotic abortion of the resulting seeds. This suggests that genetic barriers may also exist in the wide hybridisation of *Leucadendron*.

Post-zygotic abortion may be overcome by early embryo rescue and *in vitro* techniques. However whether an embryo forms or not at all depends on the successful pollen germination on the stigma, pollen tube growth through the style into the ovary and fusion of the gametes to form zygotes.

When no embryo is produced at all, the likely reason would be that the pollen was unable to deliver the gamete to the ovule. It is possible that the pollen was unable to germinate on the stigma, or that the pollen tube was prevented from growing all the way to the ovary. Techniques have been developed to observe the germination and pollen tube growth inside a style (Kho and Baer, 1968). This can be used to determine at which stage the pollen tube is being blocked.

Techniques such as mentor pollen, style truncation and stigma grafting have been used to overcome these barriers in several other species depending at what stage the barrier is occurring (Hermsen, 1977; . Knox et al., 1971). These techniques could be used for the breeding of *Leucadendron* where prezygotic barriers may currently be preventing hybridisation.

5.2 Objectives

5.2.1 To develop a protocol to allow for observation of pollen germination and tube growth on the stigma and within the style for selected cross combinations within *Leucadendron*

5.2.2 To observe and score the ability of pollen to germinate and grow through the style and reach the ovary following selected controlled crosses within and between *Leucadendron* species.

5.2.3 To identify prezygotic hybridistion barriers in the wide hybridisation of *Leucadendron*.

5.3 Materials and methods

Genetic materials

Female parents used to investigate pollen pistil interactions comprised two genotypes of *L. salignum* and one genotype from each of the following species: *L. tinctum, L. orientale, L. rubrum, L. galpini* and *L. muirii.*

Pollen was used from each of the following species; *L. laureolum*, *L. salignum*, *L. discolor, L. procerum, L. floridum, L. gandogeri, L. rubrum, L. salicifolium, L. linifolium, L. conicum* and *L. muirii.*

Crossing

Female flowerheads were covered with a perforated bread bag prior to the stigmas becoming visible. The bag was attached securely to ensure no insects had access to the developing flowerhead. For multiheaded species several flowers on one stem were placed in each bag while single stem *Leucadendrons* had just one or a few flowers per bag.

Once the stigmas were visible and receptive, the whole stem was cut from the plant without removing the bag and the base of each stem was placed in water and brought back to the UWA. Individual flowerheads (each containing several flowers) were removed from large stems so that each flowerhead only had a short stem 15 -25 cm long. These stems were placed into separate jars of water and were maintained at room temperature indoors.

Pollen from only one genotype was used for crossing with the stems in each jar. Pollination was achieved by dusting pollen (collected and stored as part of the breeding program) onto the receptive stigmas using a small paintbrush. All pollen was tested for viability using the FDA method prior to use (see chapter 3) and only pollen with a high viability was used.

Pollen Pistil Assessment

Seventy-two hours after crossing, each pistil was removed from the flowerhead with a pair of forceps by pulling gently at an angle equal to that at which the pistil was protruding. This stage must be done carefully because in many cases most of the style is completely covered by the floral bract associated with each flower, thus the visible part is only a fraction of the entire pistil. This method allowed for many of the pistils to be harvested intact.

Intact pistils were soaked in 8M NaOH for 48 hours to soften the tissues, then rinsed in water and mounted on a microscope slide with a drop of analine blue and squashed with a coverslip. Slides were observed under a fluorescent microscope and compatibility was recorded based on the ability of pollen tubes to grow down the style. Five levels were used to score the compatibility of each style: 0. No pollen germination; 1. Pollen germination but no penetration; 2. Pollen tube penetrate into stigma; 3. Pollen tube penetrate into style and 4. Pollen tube present at the ovary end of the style. Photos were taken using ASA 400 slide film under a Zeiss Axioplan MC 80 microscope. Ten styles were studied for each cross combination and average scores were calculated based on the ten observations.

5.4 Results

Considerable variation in pollen pistil interaction was observed among different cross combinations. The scores ranged from 0 (no pollen germination on the stigma) to 4 (pollen tube presence at the ovary end of the style) (Table 5.1).

When styles pollinated by their own species were observed, many pollen grains could be seen to have germinated with pollen tubes penetrating into the stigma and growing down through the style to emerge at the ovary end of the style. This could also be observed with cross combinations between closely related species, for example, *L. salignum* with *L. discolor*.

For some cross combinations, pollen germination was observed but pollen tubes grew so slowly that they failed to reach the end of the style at 72 hours after pollination, for example, *L. salignum* (LSB11) with *L. laureolum* (LLB05). For some cross combinations, pollen tubes swelled or grew in a zigzag way and never grew into the style properly. For some other cross combinations, pollen did not germinate on the style.

Pollen pistil interaction in leucadendrons studied was generally correlated with the distance between the systematic positions of the species, i.e. the closer the relationship, the higher the

score of the interaction (Table 5.2). Pollen pistil interaction results are consistent with seed set results from field hybridisation. Generally, combinations with high pollen pistil interaction scores also produced seeds in field hybridisation and combinations with low scores (<3.3) produced no seeds.

The length of the styles was found to vary between species and it is expected that pollen from species with short styles might not be able to make it to the end of the style in a species that has longer styles. Some evidence of this can be seen when a relatively long style species *L. orientale* was crossed with species with shorter style e.g. *L. salignum*. Although pollen could germinate and penetrate into the style many did not reach the ovary end and hence could not result in fertilisation.

In some cases genotypes within a species behaved differently. For example *L. laureolum* pollen was unable to penetrate the style of one genotype of *L. salignum* (LSB11) but was able to grow through the style of another selection (LSB10). This result was consistent with our field hybridisation with the first combination producing no seed and the second combination producing viable seed.

Leucadendron murii had no close relatives available to us and we were unable to produce any hybrids with this species from field pollinations. When *L. murii* pollen was used on *L. murii* and the styles observed, pollen germination and growth to the ovary end was clearly seen. Pollen from no other species was able to penetrate and grow to the ovary end of the style and in many cases the pollen was prevented from even germinating.

Table 5.1 Pollen Pistil interactions observed between different species within *Leucadendron*

Genotype				
Pistil	Pollen	Highest pollen tube score	Lowest pollen tube score	Mean Pollen tube score
L. salignum (10)	*L. salignum*	4	4	4
L. salignum (10	*L. laureolum*	4	3	3.3
L. salignum (11)	*L. salignum*	4	4	4
L. salignum (11)	*L. discolor*	4	4	4
L. salignum (11)	*L. procerum*	4	3	3
L. salignum (11)	*L. floridum*	3	1	2
L. salignum (11)	*L. gandogeri*	2	0	1
L. salignum (11)	*L. laureolum*	3	0	1.6
L. tinctum	*L. rubrum*	4	4	4
L. tinctum	*L. salignum*	4	0	2.2
L. tinctum	*L. discolor*	4	3	3.7
L. tinctum	*L. salicifolium*	4	3	3.8
L. orientale	*L. salicifolium*	4	2	3
L. orientale	*L. rubrum*	4	3	3.5
L. orientale	*L. laureolum*	4	2	2.6
L. orientale	*L. salignum*	4	0	1.9
L. orientale	*L. discolor*	4	2	2.7
L. rubrum	*L. discolor*	4	3	3.2
L. rubrum	*L. salicifolium*	4	0	2
L. galpini	*L. conicum*	4	3	3.4
L. galpini	*L. linifolium*	4	3	3.2
L. galpini	*L. salignum*	4	3	2.9

L. galpini	*L. laureolum*	4	0	2.9
L. galpini	*L. rubrum*	0	1	0.3
L. muirii	*L. muirii*	4	3	3.8
L. muirii	*L. conicum*	3	0	1.0
L. muirii	*L. discolor*	1	0	0.1
L. muirii	*L. rubrum*	0	0	0.0
L. muirii	*L. linifolium*	1	0	0.2

Table 5.2. Pollen pistil interactions between *Leucadendron* species

Type of cross	Number. of cross combinations	Score of pollen pistil Interaction*	Seed set (%)
Intraspecific	3	3.82	100
Interspecific (within subsections)	6	2.70	50
Interspecific (between subsections)	20	2.32	5

5.5 Discussion

The above results provide strong evidence that prezygotic hybridisation barriers exist in the wide hybridization of *Leucadendron*. Techniques such as style truncation, mentor pollen or application of plant growth regulators may have to be adopted to overcome the barriers.

The protocol developed for the assessment of pollen pistil interactions in *Leucadendron* appears to work well. When pollen was placed on the stigma of a species it had been shown to hybridise with (from crossing done in 1998), several pollen grains germinated and the tubes grew to the ovary end of the style. This could clearly be seen in intraspecific crosses and crosses between closely related species.

Sometimes pollen was able to germinate but unable to penetrate through the style of a related species e.g. in the same subsection. As the pollen tube does not reach the ovary no fertilisation occurs and hence no seed is set. These results are consistent with field hybridisation results.

Similar results were observed with distantly related species which was again consistent with field hybridisation results. In many cases this occurred when the female species had a relatively long style (e.g. *L.rubrum*, *L. orientale*) when compared to the length of style from the pollen donating species. Although the pollen can germinate and penetrate into the style, most do not reach the ovary end and hence would not result in fertilisation. Some individual tubes reached the end of the style and were recorded as 4's (see results) however the vast majority of pollen tubes had not reached the ovary end after 72 hours). This may be overcome by style truncation, a process where the stigma and most of the style tissue are removed and the pollen is placed on the cut surface. The pollen tubes then have less distance to travel and hence there is a higher probability that they will reach the ovary.

Evidence that different genotypes within a species behave differently was observed with the 2 different genotypes of *L. salignum* when crossed with *L. laureolum*. This suggests that there is still hope for obtaining hybrids between 2 given species even if crossing or pollen pistil interaction results have so far been negative. By investigating pollen pistil interaction several different genotypes within the desired species can be screened at low cost and a result is available after just 72 hours. Field crossing can then be undertaken with compatible genotypes safe in the knowledge that the pollen tube will be able to penetrate to the ovary.

On some occasions, many pollen were able to germinate and penetrate the ovary end of the style for very distant combinations e.g. *L. tinctum* x *L. salicifolium* and *L. tinctum x L. discolor*. No viable seed was obtained from such crosses in the field but the presence of aborted embryos or empty seed coats suggests post zygotic abortion of the hybrid embryo. The results of our pollen pistil interaction studies support the idea that in such crosses the pollen is not inhibited by the stigma or style tissue and is able to reach the ovary. Early embryo rescue and *in vitro* culture may be used to raise the offspring from such wide crosses.

L. muirii supported the poorest germination and pollen tube growth when pollen from different species was applied to the stigma. In many cases the pollen was prevented from even germinating. This may be due to the stigma recognising the foreign pollen and preventing or hindering its germination and growth. Mentor pollen is a process where killed pollen from the female species (in this case *L. muirii*) is mixed with viable pollen from the species desired for hybridisation. Chemicals produced by the mentor pollen may be recognized by the stigma and allow the pollen from the different species to germinate and grow. Another method that may be used in this situation involves cutting the stigma off *L. muirii* and grafting on a stigma from the pollen species. The pollen easily germinates on the stigma from its own species and the resulting pollen tubes may grow down into the ovary of *L. muirii* and fuse with the ovule resulting in a hybrid zygote.

5.6 References

Kho, YO and Baer J (1968) Observing pollen tubes by means of fluorescence. Euphytica, 17: 298-302

Hermsen JGT (1977) General considerations on interspecific hybridisation. Proceedings of 8th Eucarpia Congress, (pp. 299-300) Madrid, Spain

Knox RB, Willing RR and Pryor LD (1971) Interspecific hybrids in Poplars using recognition pollen. Silvae Genetica, 21: 65-69

6. *Leucadendron* cytogenetics

6.1 Introduction

Knowledge of cytogenetics is important in breeding programs. It provides information for classification and the determination of the relatedness of potentially useful species and may provide reasons for the success or failure of crosses. Studies of chromosomes also enable the understanding of the genome relationships, genetic architecture, gene action and nuclear behaviour at meiosis and mitosis (Singh, 1993). Interspecific hybrids are of great value for genetic analysis as well as for the practical use of gene transfer into crops (Sanchez-Monge and Garcia-Olmedo, 1977).

Leucadendron is a major wildflower export group in the Australian floricultural industry with many important species and varieties (Criley and Parvin, 1993; Anonymous, 1991; Anonymous 1997). There are about 80 species in the genus *Leucadendron* which may be grouped into 2 sections comprising 14 subsections (Vogts, 1982).

Because only limited studies of the cytogenics of *Leucadendron* have been carried out, it is not known whether the genome structure of *Leucadendron* is as complex as that of other wild flower genera. The paucity of cytological information (De Vos 1943, examined the chromosome numbers of only two genotypes) of *Leucadendron* made it urgent and essential for our breeding program to undertake a genetic study of the genus.

Other families and genera of wildflowers, such as Myrtaceae (Rye, 1979; Smith-White, 1948; 1950; 1954), and *Boronia* (Smith-White, 1954; Stace, 1992; 1993; Astarini, *et al.* 1999) have attracted more attention from the cytologists. In the genus *Chamelaucium* (family Myrtaceae) different polyploidy levels were found to exist at both interspecific and intraspecific levels (Rye, 1979; Yan et al., unpublished) although without variation of basic chromosome number. In *Boronia* (family Rutaceae) the chromosome number was more diverse with different chromosome numbers and ploidy levels at both interspecific and intraspecific levels (Stace, 1993).

6.2 Objectives

To check chromosome numbers and ploidy levels of the taxa used in our breeding program

To examine whether any aneuploidy chromosome number variation or ploidy level is involved in the evolution of *Leucadendron* genomes.

To provide genetic background for *Leucadendron* breeding prograns.

6.3 Materials and Methods

The genotypes used for cytological examination included 25 genotypes involving 15 species. The species used were *L. conicum, L. dicolor, L. eucalyptifolium, L. floridum, L. galpinii, L. laureolum, L linifolium, L. murii, L. procerum, L. salicifolium, L. salignum* and *L. strobilinum*. The interspecific hybrids used were *L. conicum* x *L. discolor, L. floridum* x *L. coniferum, L. floridum* x *L. discolor, L. floridum* x *L. laureolum, L. floridum* x *L. procerum, L. floridum* x *L. salignum, L. floridum* x *L. strobilinum, L. linifolium* x *L. conicum, L. strobilinum* x *L. discolor, L. uliginosum* x *L. conicum* and *L. xanthoconus* x *L. salicifolium.*

Root tips of the plants were chosen for this purpose. The roots used in the study were taken from potted seedlings growing in a shade house at the University of Western Australia. Collected roots were pretreated with saturated aqueous paradichlorobenzene for 4 hrs at room temperature and then fixed in Cannoy's I fixative (95% ethanol:acetic acid::3:1) for 24 h. After being hydrolysed in 1M HCL at 60°C for 8-10 min, the samples were stained in Feulgen solution for 2 h, and then squashed

in a drop of FLP orcein on microscope slide (Jackson, 1982). After placing the cover slip onto the slide, the slide was heated and then pressed firmly to flatten cells. All observations were made using a ZEISS Axioplan Microscope MC 80 and photographs were taken on Pan F film using a 35 mm camera. Only metaphase stages in which individual chromosomes were clearly distinguishable were used for making counts. At least 10 dividing cells were usually counted for each sample to determine the chromosome number.

6.4 Results

The chromosome number of 2n = 26 was found to be the same for all 25 genotypes from 15 different species as determined in this study (Table 5.1). The chromosomes of these genotypes were small, less than 2 μm when fully condensed, and the accurate count required the use of a 100x oil immersion lens (Figure 5.1). No morphological differences were found on chromosomes between different genotypes.

Table 5.1 Cromosome numbers in *Leucadendron* species and hybrids

Serial No.	Taxa	2n No.
1	*L. eucalyptifolium*	26
2	*L. salignum*	26
3	*L. discolor*-12	26
4	*L. discolor*-7	26
5	*L. floridum*	26
6	*L. galpinii*	26
7	*L. laureolum*	26
8	*L. linifolium*	26
9	*L. muirii*	26
10	*L. procerum*	26
11	*L. salicifolium*	26
12	*L. strobilinum*-01	26
13	*L. strobilinum*-03	26
14	*L. conicum*	26
15	*L. conicum* x *L. discolor*	26
16	*L. floridum* x *L. coniferum*	26
17	*L. floridum* x *L. discolor*	26
18	*L. floridum* x *L. salignum*	26
19	*L. floridum* x *L. strobilinum*	26
20	*L. floridum* x *L. laureolum*	26
21	*L. floridum* x *L. procerum*	26
22	*L. linifolium* x *L. conicum*	26
23	*L. strobilinum* x *L. discolor*	26
24	*L. uliginosum* x *L. conicum*	26
25	*L. xanthoconus* x *L. salicifolium*	26

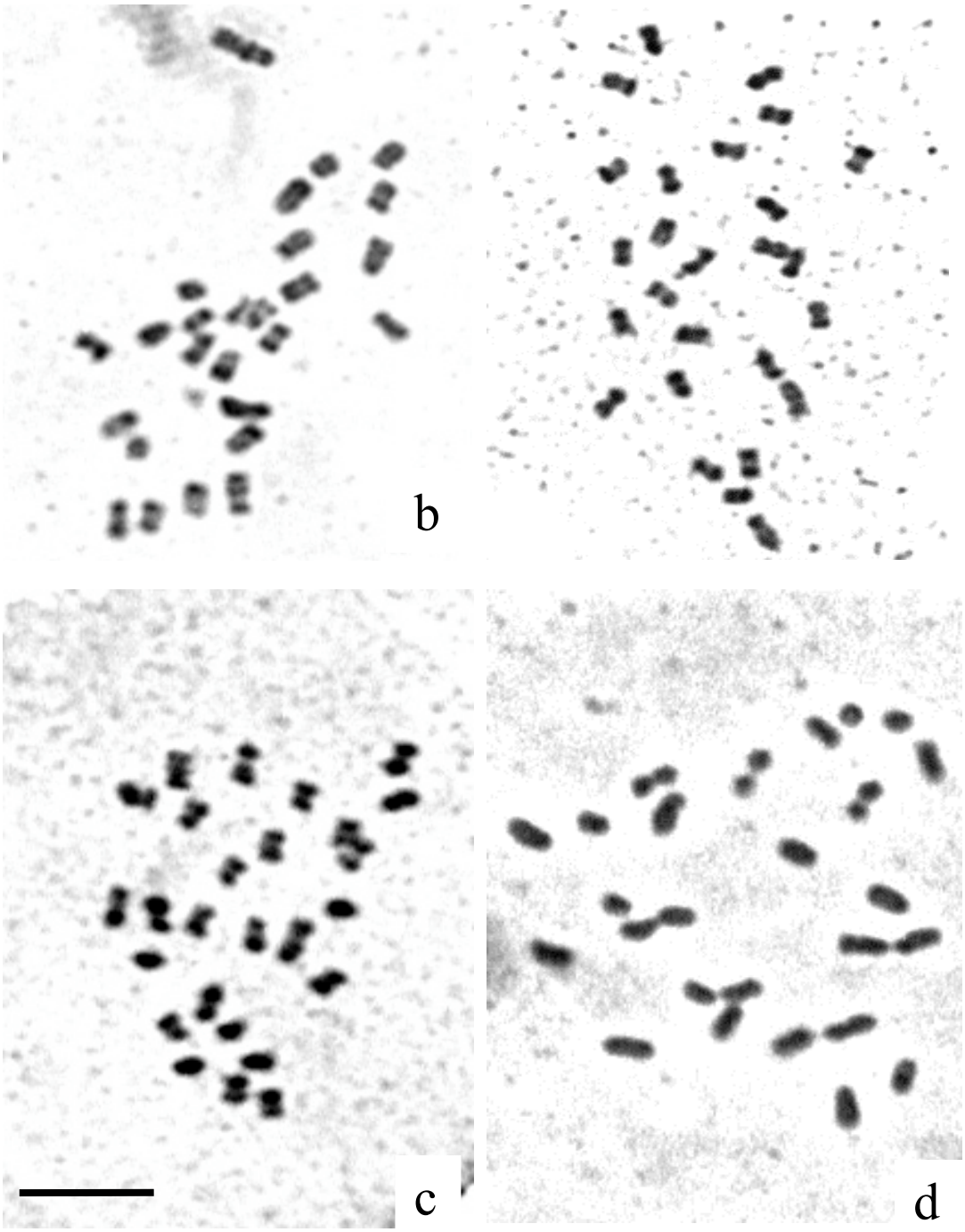

Figure 5.1. Mitotic chromosomes of *Leucadendron* species. (Bar represents 5 μm)

a. Root tip chromosomes of *L. salignum*, 2n = 2x = 26.
b. Root tip chromosomes of *L. galpinii*, 2n = 2x = 26.
c. Root tip chromosomes of a hybrid between *L. linifolium* and *L. conicum*, 2n = 2x = 26.
d. Root tip chromosomes of a hybrid between *L. uliginosum* and *L. conicum*, 2n = 2x = 26.

6.5 Discussion

The observed chromosome number is consistent with de Vos (1943) who examined the chromosome numbers of only two genotypes. However, after examining a reasonably large number of genotypes (25 genotypes of 15 species) no aneuploidy and euploidy chromosome number variation was observed in *Leucadendron*. In the family Proteaceae, there is considerable variation in the chromosome numbers among different genera, however, intrageneric chromosome number variation is uncommon compared with the other Southern hemisphere family Myrtaceae. The chromosome number in genus *Leucadendron* was constant indicating the genome is conservative, at least at the chromosome number level.

The constant chromosome number in *Leucadendron* may partially explain the easy success of the hybridization between *Leucadendron* species (Chapter One) and indicate that with further wider hybridization it may be possible to create more colourful or novel new varieties.

6.6 references

Anonymous, 1991. *Leucadendron* hybrid. Plant Variety Journal – PBR Australia. 4:8-9

Anonymous, 1997. the denomination of 'World Vision'. Plant Variety Journal – PBR Australia. 10:56

Astarini, I.A., G. Yan & J.A. Plummer, 1999. Interspecific hybridization of boronias. Australian Journal of Botany, 47:851-864

Criley, R.A. and P. E. Parvin, 1993. New cut foliages from Australia, New Zealand and South Africa. Acta Hort. 337:95-98

de Vos, M.P., 1943. Cytological studies in genera of the Proteacea. South African Journal of Science 40:113-122

Growns D. J., Chris Newell, John Considine, and Guijun Yan. 1999. Waxflower selection, breeding and development - an overview. IV International Symposium on New Floricultural Crops. Chania - Crete, Greece 22 -27 May, 1999

Jackson, R.C., 1982. Polyploidy and diploidy: new perspectives on chromosome pairing and its evolutionary implications. American Journal of Botany. 69:1512-1523

Rye, B. L., 1979. Chromosome number variation in the Myrtaceae and its taxonomic implications. Australian Journal of Botany. 27:547-573

Sanchez-Monge, E. & Garcia-Olmedo, F., 1977. Interspecific Hybridization in Plant Breeding. Madrid, Spain.

Singh, RJ, 1993. Plant Cytogenetics. CRC Press. USA

Smith-White, S., 1948. Cytological studies in the Myrtaceae II: Chromosome numbers in the Leptospermoideae and Myrtoideae. Proceedings of the Linnean Society of N.S.W. 73:16-36

Smith-White, S., 1950. Cytological studies in the Myrtaceae III: Cytology and phylogeny in the Chamaelaucoideae. Proceedings of the Linnean Society of N.S.W. 75:99-121

Smith-White, S., 1954. Cytological studies in the Myrtaceae IV: The sub-tribe Euchamaelaucinae. Proceedings of the Linnean Society of N.S.W. 79:21-28

Smith-White, S., 1954. Chromosome numbers in the *boronieae* (Rutaceae) and their bearing on the evolutionary development of the tribe in the Australian flora. Australian Journal of Botany, 2:287-303

Stace, H.M. & J.A. Armstrong, 1992. New Chromosome Numbers For Rutaceae. Australian Systematic Botany, 5: 501-505

Stace, H. M. J.A. Armstrong & S. H. James, 1993. Cytoevolutionary patterns in Rutaceae. Plant Systematics & Evolution, 187:1-28

Vogts, M., 1982. South Africa Proteaceae, know them and grow them. Struik, Cape Town, South Africa, pp156-159

Yan Guijun, Fucheng Shan, N George, D Growns, J. A. Considine. (2001) Chromosome number and intraspecific ploidy variation in *Chamelaucium uncinatum* Schauer. *Euphytica* (in preparation)

7. Concluding Summary

The aim of the project was to study the potential for creating new commercial varieties from hybrids between different species of the genus *Leucadendron*, which do not normally interbreed in nature, and to overcome the barriers that may prevent hybridisation, germination of seed, and the subsequent development of a new range of *Leucadendron* varieties for Australian growers.

To this end we have completed development of a protocol involving the following sequence:

1. selection of suitable parents for crossing to produce hybrids with the desired characteristics.
2. collection and long term preservation of pollen to enable wide crosses to be made.
3. harvest and germination of hybrid seed resulting from successful crosses, according to the appropriate temperature regime.
4. identification of barriers that prevent the successful fertilisation of pollinated flowers.
5. confirmation of the absence of variation in chromosome numbers and ploidy levels that might render the objectives of the project unattainable within the given time frame.
6. establishment of hybrid seedlings on the properties of six growers who have financed the industry contribution to the project and who are collaborating in evaluating the hybrids.

Completion of this project has now established a basis for confidently moving to the next phase. This will involve the extension of the above protocol to include the efficient selection of superior hybrids for trialling and their subsequent market evaluation and eventual release to Australian growers.