Speeding-up the Release of New Hybrid Leucadendrons

A report for the Rural Industries Research and Development Corporation

By Guijun Yan & Ralph Sedgley

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Foreword

The first phase of the Australian Leucadendron breeding program UWA46A started in mid-1998 with financial support from RIRDC and a group of protea growers. Outstanding progress has been made (Yan et al. 2001). Time is of the essence, for growers who have invested in this project and are looking for an early return on their considerable investment, and for the industry at large, which urgently needs new varieties to invigorate its offerings to the world market. Hence the need for methods for making early selections and for techniques to rapidly multiply them and so hasten their evaluation and distribution for planting out.

The second phase of the project was a tremendous success. A total of more than 23,000 hybrid seeds was produced by the project. Altogether, 5,319 plants representing most successful cross combinations have been trialled on the farms of contributing growers and so far 251 selections have been made, 10 of which have been fast-tracked among the contributing growers and three of them have been registered for Plant Breeders Rights (PBR). Our research into the development of molecular and morphological markers and the establishment of a rapid propagation system were highly successful. So far 8 scientific papers have been published or accepted for publication in refereed journals. Armed with the scientific outcome and the bank of about 17,000 hybrid seeds and more than 200 elite selections, the project has the potential to release new Leucadendron varieties for many years to come.

This project was funded from RIRDC core funds which are provided by the Australian Government.

This report is an addition to RIRDC’s diverse range of over 1500 research publications. It forms part of our Wildflowers and Native Plants R&D sub-program which aims to identify and develop new fresh products.

Most of our publications are available for viewing, downloading or purchasing online through our website:

- purchases at www.rirdc.gov.au/eshop

Peter O’Brien
Managing Director
Rural Industries Research and Development Corporation
Acknowledgments

We would like to express my sincere gratitude to the Protea Growers Pty. Ltd. for both their financial support and their continuing enthusiasm in gathering genetic resources and trialling of hybrids produced from this project. We are also indebted to Mr Ben Croxford, who has worked on the entire project as a Research Officer and has made an enormous contribution to the project.

The contributing Protea growers/exporters in alphabetical order are:
Abbey Farm - Cheryl Foster, Paul Cook
Amarillo Proteas - Ralph and Grace Sedgley
Annie Brook Flower Farm - Wally and Dawn Lewis
Busselton Proteas - John Daykin
Cox Farm - William Morris Cox
Collina Export - Brian Harris
Golden West Flowers - Bob Ward
Muchea Gold – Paul and Lynn Hoffman
Sattler + Co – Vern and Chris Sattler
Tanridge – Tom, Joan and Nick Anthoine
Total Flower Exports - Tony Dick
WAFEX - Craig Musson

We also thank Fucheng Shan, Made Pharmawati, Helen Liu and Patrick Finnegan for scientific and technical support to this project.
# Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>°C</td>
<td>degrees Celsius</td>
</tr>
<tr>
<td>μM</td>
<td>micromolar</td>
</tr>
<tr>
<td>2n</td>
<td>somatic ploidy number</td>
</tr>
<tr>
<td>2x</td>
<td>diploid</td>
</tr>
<tr>
<td>BAP</td>
<td>6-benzylaminopurine</td>
</tr>
<tr>
<td>cpDNA</td>
<td>chloroplast DNA</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribose nucleic acid</td>
</tr>
<tr>
<td>dNTP(s)</td>
<td>2’-deoxy nucleotide triphosphate(s)</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylene diamine tetra-acetic acid</td>
</tr>
<tr>
<td>F₁</td>
<td>first generation hybrid</td>
</tr>
<tr>
<td>F₂</td>
<td>second generation hybrid</td>
</tr>
<tr>
<td>FDA</td>
<td>Fluorescein diacetate</td>
</tr>
<tr>
<td>g/L</td>
<td>grams per litre</td>
</tr>
<tr>
<td>IBA</td>
<td>indole-3-butyric acid</td>
</tr>
<tr>
<td>M</td>
<td>molar</td>
</tr>
<tr>
<td>mL</td>
<td>millilitre</td>
</tr>
<tr>
<td>mm</td>
<td>millimetre</td>
</tr>
<tr>
<td>mM</td>
<td>millimolar</td>
</tr>
<tr>
<td>MS</td>
<td>mixture of salts as per Murashige and Skoog (1962)</td>
</tr>
<tr>
<td>n.a.</td>
<td>not applicable</td>
</tr>
<tr>
<td>NAA</td>
<td>naphthalene acetic acid</td>
</tr>
<tr>
<td>NaOH</td>
<td>sodium hydroxide</td>
</tr>
<tr>
<td>p</td>
<td>p-value</td>
</tr>
<tr>
<td>PAUP</td>
<td>phylogenetic analysis using parsimony</td>
</tr>
<tr>
<td>PCB</td>
<td>para-dichlorobenzene</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>pers. comm.</td>
<td>personal communication</td>
</tr>
<tr>
<td>std dev.</td>
<td>standard deviation</td>
</tr>
<tr>
<td>w/v</td>
<td>weight by volume</td>
</tr>
<tr>
<td>x</td>
<td>basic ploidy number</td>
</tr>
</tbody>
</table>
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Executive Summary

Introduction
This report summarises the second phase of the Australian Leucadendron breeding project. The first phase of the project resulted in a reliable protocol for the wide hybridisation of leucadendrons through interspecific hybridisation. The protocol included details of parent selection, pollen viability assessment and pollen storage, pollination, seed collection and seed germination and planting out of hybrids for evaluation. Considerable numbers of hybrid seeds were also produced.

Research objectives and general approach
Time is of the essence, for growers who have invested in this project and are looking for an early return on their considerable investment, and for the industry at large, which urgently needs new varieties to invigorate its offerings to the world market. This second phase of the project researches into the methods for making early selections and for techniques to rapidly multiply them and so hasten their evaluation and distribution for planting out. To quickly reduce the large number of hybrids into a core elite selection, both molecular and morphological markers are needed. Also in order for the access of new materials by the growers, a quick multiplication method either by small cutting or tissue culture is essential. The major objective of the project is to quicken the selection process and to deliver the new materials to the growers in a speedy way.

Outcomes and implications
The second phase of the project was highly successful. More than 23,000 hybrid seeds were produced by the project. Altogether, 5,319 plants representing most successful cross combinations have been trialled on the farms of contributing growers from which 251 selections have been made; 10 of these have been fast-tracked among the contributing growers and three of them have been PBR'ed (See Appendices). Our research into the development of molecular and morphological markers and the establishment of a rapid propagation system were also successful. So far 8 scientific papers have been published or accepted for publication in refereed journals (See Appendices). Armed with the scientific outcome and the bank of about 17,000 hybrid seeds and more than 200 elite selections, the project now has the potential to deliver new Leucadendron varieties to the industry for many years to come.
Recommendations

On the basis of the findings of this research, the following recommendations are made:

1. That the report be published and made available to wildflower producers and advisory agencies.
2. That the successful breeding and genetic studies in Leucadendron be extended to the breeding of other wildflowers.
3. That the best practice of marketing, promotion, adoption, and commercialisation of new wildflower varieties be studied to support the current wildflower breeding projects.
1. Search for sex linked DNA markers in Leucadendron

Abstract. *Leucadendron* is successfully cultivated in Australia. All *Leucadendron* species described so far are strictly dioecious. There is a market preference for male or female flowers depending on the species. Earlier studies suggested a single gene sex controlling system as most families segregated into males and females in a 1:1 ratio. The availability of sex-linked markers would be useful for marker assisted breeding in *Leucadendron*. We used bulk segregant DNA analysis to search for DNA markers linked to the sex determining gene(s). Sixty-six genotypes from 11 species were examined using 42 RAPD and 34 ISSR primers. The universal DNA markers across all species were not identified. However, a number of sex-linked RAPD and ISSR markers were identified in individual species. These species-specific markers are being investigated further to confirm their reliability before being used in the breeding program. Suppressive subtraction hybridisation (SSH) of genomic DNA was also conducted to identify genome differences between male and female genotypes in *L. discolor*. Almost identical signal intensities were observed between inserts probed with *RsaI*-digested male genomic and female genomic DNA. This indicated the high degree of genome homology between male and female *L. discolor*. Further approaches, such as RAMP (Randomly Amplified Microsatellite Polymorphism) and MFLP (Microsatellite Fragment Length Polymorphism) are being attempted to find universal markers.

1.1 Introduction

*Leucadendron* is one of the main wild flower groups grown in Australia. The flowers often have colourful bracts surrounding a central flower head. The bracts, head and upper leaves are highly desired by the cut flower and foliage industry. All of the 80 *Leucadendron* species described so far are strictly dioecious, i.e. there are male and female individuals. There is a market preference for male or female flowers depending on the species. It normally takes 2-3 years for seedlings to flower in breeding programs. Therefore early determination of gender in hybrids is highly desired to the *Leucadendron* breeders.

In *Leucadendron*, distinguishable sex chromosomes have not been identified. Our earlier studies from artificial hybridizations suggested a single gene controlling system in sex determination in *Leucodendron* as most families segregate in a 1:1 ratio (Croxford *et al.*, 2003). DNA molecular markers, if available, could provide a quick, economic and highly specific means of determining gender at the seedling stage.

In this study, random amplified DNA polymorphisms (RAPD) and inter-simple sequence repeats (ISSR) were applied to detect sex-linked markers. Suppressive subtraction
hybridization (SSH) of genomic DNA was also used to identify genome differences between male and female genotypes in *L. discolor* in order to search for sex-specific sequences.

## 1.2 Materials and Methods

### 1.2.1 DNA extraction

For RAPD and ISSR analyses, young leaves were collected from three male and three female genotypes from each of the following 11 species, *L. conicum, L. discolor, L. floridum, L. galpinii, L. gandogeri, L. laureolum, L. procerum, L. rubrum, L. salignum, L. spissifolium,* and *L. uliginosum*. Genomic DNA was extracted using CTAB method (Rogers and Bendich 1994) and equal proportions of three male or female DNA samples from the same species were bulked together to be used as PCR DNA templates. For SSH analysis, DNA was extracted by DNeasy Plant Mini Kit (Qiagen, Clifton Hill, Victoria, Australia).

**RAPD analysis**

Forty two random decamer primers (Table 1.1) were used in the PCR amplification. The PCR was performed in a Hybaid OmniGene Thermal Cycler, following the method by Yan *et al.* (2002). The RAPD products were separated by 1.6% agarose gel electrophoresis stained with ethidium bromide.

### 1.2.2 ISSR analysis

Thirty four ISSR primers (Table 1.2) were tested. PCR amplifications were performed in 25 μl reactions. Amplification and visualization of PCR products were carried out as described by Pharmawati *et al.* (2005).
Table 2. ISSR primers used in this study

<table>
<thead>
<tr>
<th>ISSR primer</th>
<th>Sequence (5’-3’)</th>
<th>ISSR primer</th>
<th>Sequence (5’-3’)</th>
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<tr>
<td>UBC813</td>
<td>CTCTCCTCCTCTTT</td>
<td>UBC870</td>
<td>GACAGACAGACAGACA</td>
</tr>
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<td>UBC814</td>
<td>CTCTCCTCCTCTTA</td>
<td>UBC881</td>
<td>GGGTGGGTGGGTGTTG</td>
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<td>UBC815</td>
<td>CTCTCCTCCTCTTG</td>
<td>UBC888</td>
<td>EDBCACACACACACAC</td>
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<td>UBC817</td>
<td>CACACACACACACAA</td>
<td>UBC889</td>
<td>DBDACACACACACAC</td>
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<td>UBC820</td>
<td>GDTTGTTC</td>
<td>UBC890</td>
<td>HVHVTGTTGGTGGTG</td>
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<td>UBC824</td>
<td>TCTCTCCTCCTCTG3</td>
<td>UBC891</td>
<td>HVTGTTGGTGGTGTTG</td>
</tr>
<tr>
<td>UBC826</td>
<td>ACACACACACACACAG</td>
<td>UBC894</td>
<td>TCTCTCCTCCTCTCA</td>
</tr>
<tr>
<td>UBC836</td>
<td>AGAGAGAGAGAGAGY</td>
<td>UBC895</td>
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</tr>
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<td>UBC843</td>
<td>GCTCTCCTCTCCTC</td>
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<td>UBC898</td>
<td>TCTCTCCTCCTCTRA</td>
</tr>
</tbody>
</table>

Single letter abbreviations for mixed base positions: Y=(C, T), R=(A, G), B=(non A), D=(non C), V=(non T), H=(non G)

1.2.3 SSH analysis

Hybridisation of driver and tester
DNA of female *L. discolor* was used as driver and male as tester. The driver and tester DNA were prepared according to Diatchenko *et al.* (1996). The tester DNA was divided into two groups and each was annealed with a different adaptor (adaptor 1 and adaptor 2 respectively). Hybridization of tester with driver to obtain hybrid DNA with different suppression adaptors was performed. The amplification of subtracted DNA was done in two steps; the primary and secondary PCR amplifications according to Diatchenko *et al.* (1996).

Cloning of the subtracted DNA and genomic library construction
The purified PCR products were inserted into pGEM-T vector and the ligated products were transformed into *E. coli* JM109 competent cells. Clones containing insert were detected by color selection of colonies according to Sambrook *et al.* (1987). The inserts were amplified in 25 μl reaction mixture containing 1 x PCR buffer, 2.5 μM each of primer NP1 and NP2, 2 mM MgCl2, 0.2 mM of each dNTP, 1 U of Taq polymerase (Eppendorf). The amplification program was: one cycle of 95°C for 3 min, 25 cycles of 94°C for 30 sec, 68°C for 30 sec, 72°C for 1.5 min followed by one cycle at 72°C for 7 min.

Differential screening of subtracted *L. discolor* DNA library
Amplified inserts were arrayed on positively charged nylon membranes to perform dot-blotting analysis. The membranes were screened by hybridization with Rsal digested
genomic DNA from male and female *L. discolor* labeled by DIG (Roche, Castle Hill, NSW, Australia) according to manufacturer’s instruction.

**1.3 Results and Discussion**

**1.3.1 RAPD markers**

Considerable DNA polymorphisms were generated but most primers produced identical amplification fragments for bulked male and female samples. No sex-specific band common to all species was found by RAPD analysis. Potential species-specific sex markers were identified using some primers in some species. Of the 42 primers screened, seven primers produced 11 markers linked with male genotypes and five primers produced six markers linked with female genotypes. Some RAPD primers, such as SK19, generated potential sex-linked markers for several species. In verification, these primers were used to screen three male and three female individuals in the species (Figure 1.1).

![Figure 1.1.](image)


**1.3.2 ISSR markers**

ISSR has the advantages over RAPD due to the longer primers used that gives more stringent annealing temperatures and provides higher reproducibility (Nagaoka and Ogihara, 1997). Since 42 RAPD primers did not identify universal sex markers in *Leucadendron*, ISSR markers were generated to detect sex-specific fragments. Five primers out of 34 primers tested gave putative sex-specific products (UBC826785, UBC836540, UBC852500, for *L. laureolum*, and UBC858900, UBC8681080 for *L. discolor*). However, further testing involving more plant individuals showed that the fragments were not sex-specific (Figure 1.2).
Figure 1.2. ISSR patterns of male and female *L. laureolum* using primer UBC836. Left: ISSR markers in three male and two female plants. Right: ISSR banding patterns from 13 male and 11 female *L. laureolum* individuals. The UBC836\textsubscript{540} band (arrowed) is not present in the 13 male individuals suggesting that it is not sex-dependant DNA fragment.

It was reported that the frequency of sex-linked markers found in dioecious plants correlated to the total size of the genome, and the relative size of the chromosomal segments that determine sex (Kafkas, *et al*., 2001). The bigger the genome and the smaller the segments that determine sex, the more difficult it will be to find sex-specific markers. The size and the complexity of *Leucadendron* genome are still unknown. However, the high number of common ISSR bands between male and female *Leucadendron* plants suggested that the two genomes were quite homologous. Therefore, more primers need to be screened in order to find universal sex-specific fragments.

1.3.3 SSH signals

Bulked segregant analysis using RAPD and ISSR markers did not identify universal sex markers in *Leucadendron*. SSH was reported as the most powerful method for isolating differentially expressed transcripts and identifying differentially presented DNA (Diatchenko, *et al*., 1996, Akopyants, *et al*., 1998). SSH of genomic DNA was then conducted to identify genome differences between male and female *L. discolor* plants.

Figure 1.3. Hybridisation analysis of randomly selected clones from the subtracted library of male *Leucadendron discolor* genomic DNA. (A). PCR amplicons were dot-blotted on nylon membrane and hybridized with probes made from *RsaI* digested male genomic DNA of *L. discolor* using DIG labeling. (B). Hybridisation of amplicons using prepared probes from
RsaI digested female genomic DNA of *L. discolor*. One hundred clones from a total of 248 clones are shown. Dots in column 11 of each blot are positive controls of an ISSR fragment (rows A to D) and genomic DNA (rows E to H) in serial dilutions.

From a total of 248 inserts of SSH product where male genomic DNA was used as a tester, no insert demonstrates differentially presented DNA between male and female plants (Figure 1.3). Similar signal intensities were observed between inserts of male SSH probed with RsaI-digested male genomic and female genomic DNA. This implies that the male and female genome of *L. discolor* are rather homologous which is consistent with the results indicated by RAPD and ISSR analysis. The results also suggest that the region of sex determining gene(s) in *Leucadendron* is very small, making the identification of sex-linked gene more difficult. Further approaches, such as RAMP (Randomly Amplified Microsatellite Polymorphism) and MFLP (Microsatellite Fragment Length Polymorphism) are being attempted to find universal markers.

**1.4 Literature Cited**


Pharmawati M, Yan G, Finnegan P, 2005. Molecular variation and fingerprinting of
Leucadendron cultivars (Proteaceae) by ISSR markers. Annals of Botany 95: 1163-
1170.

Plant Molecular Biology Manual D1, 1-8.

Spring Harbor, USA: Laboratory Press.

Yan G, Shan F, Plummer JA, 2002. Genomic relationships within Boronia (Rutaceae) as
revealed by karyotype analysis and RAPD molecular markers. Plant Systematics and
Evolution 233: 147-161
2. The Search for Morphological Markers in Leucadendron

Abstract Early assessment and selection of hybrids are important in breeding programs in order to increase breeding efficiency. A study was conducted to correlate traits measured at seedling stage and economically important traits at adult stage in Leucadendron wide hybrids. Seedling traits such as plant height, stem diameter and internode length of 33 hybrids between L. laureolum and L. procerum, 31 hybrids between L. salignum and L. procerum and 33 hybrids between L. gandogeri and L. laureolum were measured when the seedlings were about 7 months old. Adult traits such as sex, flower colour and productivity were recorded when the plants were 3 years old and were correlated to the seedling traits. Results from the family of L. laureolum and L. procerum indicated that sex can be predicted from the seedling growth habit. At seedling stage, male plants were significantly taller than female plants (p=0.015), had significantly longer internodes (p=0.009) and thicker stems (p=0.037). More vigorous seedlings, taller (p<0.01), longer internodes (p=0.04) and higher internode number (p<0.01) tended to develop more colourful bracts with red tinges. Seedling trait – number of nodes has a moderate correlation but significant regression with adult trait – total stem production (r = 0.479). However, the same trends were not clear in the other two families L. salignum with L. procerum and L. gandogeri with L. laureolum. No other seedling traits were found to be strongly correlated to adult traits but internode length at seedling stage was found to be moderately correlated to the percentage of saleable stems (>45cm) harvested (r=0.334-0.429).

2.1 Introduction

The breeding of woody perennial hybrids is a long term and expensive process and any methods allowing for the early selection of hybrids at the seedling stage could increase the efficiency of the program. Morphological markers have been used in the early selection of hybrids in the breeding of a number of woody plants which have a long juvenile phase (Janick & Moore, 1996). For example, soluble solid content (SSC) in the leaf petiole of seedlings has been found to be correlated to the fruit sugar content in apples and therefore SSC in the leaf petiole of seedlings has long been used to predict the sweetness of apples (Nybom, 1959). Anthocyanin colouring of stems of seedlings was also used for the early selection of flower colour in numerous plants (Janick & Moore, 1996). Breeding efficiency can be greatly improved if useful morphological markers can be identified and employed in the breeding program.

An experiment was set up to search for correlations between seedling traits and mature traits in Leucadendron.
2.2 Materials and Methods

Controlled crosses were performed between different species of *Leucadendron* using the method described by Yan et al (2001). Six families with more than 30 or close to 30 seedlings were selected for the development of morphological markers (Table 2.1). These plants were grown in 50mm tree tubes containing a 3 parts nutrient free potting mix : 1 part perlite (v:v) plus slow release low Phosphorus fertilizer (Macrocote grey) under shade at the University of Western Australia for 6-8 months.

The following traits were recorded; height (mm), number of nodes, mean internode length (mm), basal stem diameter (mm), number of branches, hypocotyl colour and stem colour when the seedlings were about seven months old. These plants were then planted in the field on a contributing grower's property in Coorow approximately 350 km NNE of Perth. These plants were grown along with many other interspecific hybrids as part of the ongoing industry trialling of hybrids originating from the breeding program. These plants received the same fertigation and maintenance as other *Leucadendrons* being grown commercially on the property.

**Table 2.1.** Summary of Morphological marker experiment planted in year 2002

<table>
<thead>
<tr>
<th>Code</th>
<th>Female parent</th>
<th>Male parent</th>
<th>Total No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>GW</td>
<td>O13-045 L. gandogerii (LGB06)</td>
<td>L. laeoleum (LLB01)</td>
<td>33</td>
</tr>
<tr>
<td>GW</td>
<td>167-199 L. laeoleum (LLB02)</td>
<td>L. salignum (LSL02)</td>
<td>33</td>
</tr>
<tr>
<td>GW</td>
<td>207-238 L. laeoleum (LLB04)</td>
<td>L. procerum (LPB04)</td>
<td>32</td>
</tr>
<tr>
<td>GW</td>
<td>304-334 L. salignum (LSB11)</td>
<td>L. procerum (LPB04)</td>
<td>31</td>
</tr>
<tr>
<td>GW</td>
<td>375-402 L. uliginosum (LUA03)</td>
<td>L. conicum (LCF01)</td>
<td>28</td>
</tr>
<tr>
<td>GW</td>
<td>403-432 L. uliginosum LUA05</td>
<td>L. conicum (LCF01)</td>
<td>30</td>
</tr>
</tbody>
</table>

Flower size, flower colour, stem length, sex and productivity of each plant grown from the seedling were recorded at flowering season when the plants were three years old. Correlation and regression analyses were performed to study the relationships between traits at seedling stage and traits of mature plants. Early expressed traits correlating well with traits of mature plants can be used as morphological markers for early selection.
2.3 Results and discussion

2.3.1 The family of L. laureolum X L. procerum

A total of 32 hybrids were planted from this family in 2002 of which 31 survived. Among them, 11 males and 10 females flowered in year 2005. Correlation analysis (Table 2.2) indicated that seedling trait – number of nodes at seedling stage has a moderate correlation but significant regression with adult trait – stem production (r = 0.479, Figure 2.1a); both internode length and internode number at seedling stage had strong correlation with seedling height (r = 0.795, r = 0.651, Figure 2.1b, c) and adult trait - total stem production and percentage of more than 45cm stems in total stems were highly negatively correlated (r = -0.705, Figure 2.1d). Adult trait - bract length also had a moderate positive correlation but significant regression with another adult trait - percentage of more than 45cm stems in total stems (r = 0.468). Other traits measured do not seem to be significantly correlated (Table 2.2).
Table 2. Correlation matrix between traits measured at seedling and adult stages of interspecific hybrids from *Leucadendron laureolum* with *L. procerum*

<table>
<thead>
<tr>
<th>Adult trait: No. of &gt;45cm stems</th>
<th>1.000</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adult trait: &gt;45cm stems/Total stems</td>
<td>0.346</td>
</tr>
<tr>
<td>Seedling trait: basal stem diameter</td>
<td>0.092</td>
</tr>
<tr>
<td>Seedling trait: height</td>
<td>0.254</td>
</tr>
<tr>
<td>Seedling trait: internode length</td>
<td>0.334</td>
</tr>
<tr>
<td>Adult trait: bract length</td>
<td>0.011</td>
</tr>
<tr>
<td>Seedling trait: No of nodes</td>
<td>0.045</td>
</tr>
<tr>
<td>Adult trait: No. of total stems</td>
<td>0.240</td>
</tr>
<tr>
<td>&gt;45 stem</td>
<td>&gt;45/Total</td>
</tr>
<tr>
<td>diameter</td>
<td>length</td>
</tr>
</tbody>
</table>
Figure 2.1. Regressions between traits measured in hybrids between *Leucadendron laureolum* and *L. procerum*. Seedling trait – number of nodes at seedling stage has a moderate regression with adult trait – stem production (a); both internode length and internode number at seedling stage have strong regression with seedling height (b, c) and adult trait - total stem production and percentage of more than 45cm stems in total stems are highly regressed (d).
There is a clear relationship between sex and seedling height at seven month old (Table 2.3). Male plants grew significantly faster than female plants ($p = 0.015$). Males also have longer internode length ($p = 0.009$) and thicker stem diameter ($p = 0.037$) at seedling stage. However, the relationship between sex and node number is not significant ($p = 0.191$).

**Table 2.3.** The relationship between sex and seedling traits

<table>
<thead>
<tr>
<th>Sex</th>
<th>Seedling height (cm)</th>
<th>Node number</th>
<th>Internode length (cm)</th>
<th>Diameter (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>235.27</td>
<td>45.18</td>
<td>5.12</td>
<td>4.43</td>
</tr>
<tr>
<td>Female</td>
<td>197.20</td>
<td>43.1</td>
<td>4.42</td>
<td>4.06</td>
</tr>
<tr>
<td>Significance</td>
<td>S</td>
<td>N</td>
<td>S</td>
<td>S</td>
</tr>
</tbody>
</table>

There is no correlation between stem colour and seedling height, internode number, internode length and stem diameter at seven month (Table 2.4). However, adult trait - bract colour appears to be correlated with seedling height. Plants with low seedling height tended to produce flowers with pure yellow bracts, whereas taller seedling tended to grow yellow flowers with red tinges ($p=0.004$). As node number and internode length are correlated to seedling height, they tend to show similar relationship with bract colour, i.e. seedlings with more nodes and longer internode length tended to produce flowers with red-tinged bracts ($p=0.041$, $p=0.008$). Bract colour was not correlated with stem diameter at seedling stage ($p=0.296$).

**Table 2.4.** The relationship between bract colour and seedling traits

<table>
<thead>
<tr>
<th>Bract colour</th>
<th>Seedling height (cm)</th>
<th>Node number</th>
<th>Internode length (cm)</th>
<th>Seedling Diameter (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Red tinges</td>
<td>243.30</td>
<td>46.30</td>
<td>5.19</td>
<td>4.39</td>
</tr>
<tr>
<td>Pure yellow</td>
<td>193.36</td>
<td>42.27</td>
<td>4.42</td>
<td>4.13</td>
</tr>
<tr>
<td>Significance</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>N</td>
</tr>
</tbody>
</table>

2.3.2 The family of L. gandogeri (LGB06) X L. laureolum (LLB01)

All the 33 hybrids planted in 2002 survived. Among them 18 males and 14 females flowered in year 2005. Correlation analysis (Table 2.5) indicated that seedling trait – internode length at seedling stage had a moderate correlation but significant regression with adult trait – percentage of saleable stems more than 45 cm long ($r = 0.429$, $p = 0.016$, Figure 2.2a); both internode length and internode number at seedling stage have strong regression with seedling height ($r = 0.626$, $r = 0.750$, Figure 2.2b, 2.2c) and adult trait - total stem production and number of more than 45cm stems are moderately negatively correlated ($r = -0.469$, Figure 2.2d). There is
Table 2.5. Correlation matrix between traits measured at seedling and adult stages of interspecific hybrids from *Leucadendron gandogeri* with *L. laureolum*

|                        | Adult trait: No of >45cm stems 1.000 | Adult trait: >45cm stems/Total stems 0.638 | 1.000 | Seedling trait: basal stem diameter -0.042 | 0.053 | 1.000 | Seedling trait: height 0.225 | 0.172 | 0.014 | 1.000 | Seedling trait: internode length 0.429 | 0.089 | -0.290 | 0.626 | 1.000 | Adult trait: bract length 0.087 | -0.271 | -0.031 | 0.246 | 0.316 | 1.000 | 1.000 | Seedling trait: No of nodes -0.068 | 0.150 | 0.241 | 0.750 | -0.038 | 0.047 | 1.000 | Adult trait: No of total stems -0.469 | 0.151 | 0.101 | 0.002 | -0.160 | -0.229 | 0.135 | 1.000 |
|------------------------|-------------------------------------|---------------------------------------------|-------|---------------------------------------------|-------|-------|----------------------------------|-------|-------|-------|----------------------------------|-------|-------|-------|-------|----------------------------------|-------|-------|-------|-------|----------------------------------|-------|-------|-------|----------------------------------|-------|-------|-------|----------------------------------|-------|-------|-------|
| >45 stem               |                                      | >45/Total                                   | Stem  | Height                                      | internode | bract | internode | Total                     | diameter | length | length | number | Stem |
Figure 2.2. Regressions between traits measured in hybrids between *Leucadendron gandogeri* with *L. laureolum*. Seedling trait – internode length at seedling stage has a moderate regression with adult trait – percentage of salable stems more than 45 cm long (a); both internode length and internode number at seedling stage have strong regression with seedling height (b, c) and adult trait - total stem production and percentage of more than 45cm stems in total stems are moderately regressed (d) also a regression between number of more than 45 cm stems and the percentage of more than 45cm stems in total stems (p=0.001).
The obvious positive relationship between saleable stems > 45cm and the percentage of saleable stems in total stems, and the negative relationship between total stems and the percentage of saleable stems in total stems, were observed where the percentage of saleable stems in total stems was calculated as 

\[ \% = \frac{45\text{cm}}{\text{Total}}. \]

No obvious relationship was found between adult traits such as stem colour, bract colour, sex and seedling characters including stem diameter, height, internode length and number.

2.3.3 The family of L. salignum (LSB11) X L. procerum (LPB04)

Plants were too small to assess most traits other than sex and stem colour. Neither adult traits were found to be correlated to seedling traits (P >0.3). However, there is a slight chance that adult stem colour might be correlated to seedling height (p = 0.09) with more vigorous plants (higher seedling) tend to grow pink adult stems and shorter seedlings tend to grow more creamy adult stems.

2.4 Literature Cited


3. Efficient cutting propagation of potential new varieties

Abstract. Both stem diameter and length of the cuttings were found to influence rootstrike of female Leucadendron varieties ‘436’ and ‘1424’. Thin cuttings of 100mm long produced the highest and most consistent root strike rate. However, similar trends were not found in a male variety ‘Pixy Red’ indicating that male plants might be easier to propagate than female plants in Leucadendron.

3.1 Introduction

Clonal propagation of Leucadendron varieties is required to reproduce selected plants. The fastest and most reliable method uses large semi-hardwood cuttings struck in autumn following the vegetative summer growth flush (Malan, 1992). However large Leucadendron bushes often contain potential cuttings that can vary significantly in both length and diameter. Many shorter and/or thinner cuttings are often overlooked as it is believed that this material is less successful and may take a longer period of time to propagate. Material available in summer may be too soft while material in winter and spring when the plants are flowering is often considered too mature and not ideal for propagating.

However, when propagating material is limited the potential benefits from using smaller and/or thinner cuttings or of taking cuttings throughout the year are numerous. An experiment was designed to determine if a more efficient use of available plant material is possible. The idea of reducing the size of cuttings to increase their number has been suggested or implemented by many authors working with similar plants including Leucadendron (Rodriquez Perez, 1992), Telopea (Ellyard and Butler, 1985), Alloxylon (Donovan et al, 1999) and Proteaceae plants in general (Malan, 1992).

The experiments reported here looked at the effect of stem length and diameter on the vegetative propagation of 3 hybrid Leucadendrons from cuttings. For one of the varieties cuttings were attempted during 2 different seasons, in May prior to flowering and again in November after flowering. These results and their implications are discussed below.

3.2 Materials and methods

3.2.1 Plant material

Material for the first cv. Pixy Red trial was collected from Amarillo Proteas (Karnup, Western Australia) on the 14 May 2002 approximately 4 months prior to flowering and was divided into stems with a diameter greater than 3mm (thick stems) or less than 3mm (thin stems).

Material for the second Pixy Red trial was collected from Amarillo Proteas in (Karnup, Western Australia) on the 3 November 2002 approximately 1 month after flowering and was divided into stems with a diameter greater than 3mm that had flowered (thick stems) and stems with a diameter less than 3mm that had not flowered (thin stems).

Material for the ‘1424’ trial was collected at Tanridge (Busselton, Western Australia) on the 28 March 2003 approximately 6 months prior to flowering and consisted of thick primary stems with a diameter greater than 2.5mm from which grew thinner secondary stems with a diameter less than 2.5mm.

Material for the ‘436’ trial was collected from Anniebrook Flower Farm (Busselton, Western Australia) on the 22 August, 2003 just prior to flowering and consisted of stems with a diameter greater than 3mm which had initiated flowers (thick stems) and stems with a diameter less than 3mm with no flowers (thin stems).
In all trials, 42 thick and 42 thin stems were prepared at each of 4 different lengths; 50mm, 75mm, 125mm and 225mm. The diameter of each cutting was recorded and the leaves from the lower half were removed except for the smallest cuttings where only the top two leaves were left. Immediately prior to insertion into Clonex Purple (3 g/L IBA) and then the propagation media, the bottom 25mm or so was removed such that the final cut was just below a node and final lengths of cuttings were 25mm, 50mm, 100mm and 200mm. The cuttings were placed into the prepared hole and soil firmed. Once each tray was full, the cuttings were watered and placed into a controlled environment misting room. Throughout the preparation process all stems were kept shaded, moist and cool.

3.2.2 Propagation media

A standard propagation mix containing 3 parts perlite, 2 parts crushed quartz and 1 part peat was used for all experiments. The mix was placed into square pots each 48mm across the top and 60mm deep with 42 pots filling a standard nursery tray. Prepared pots were steam pasteurized at 60°C for 1 hour before a hole was made in the propagation mix to a depth approximately one third of the cutting length.

3.2.3 Rooting environment

Cuttings were set in a misting chamber inside a glasshouse under 50% shadecloth and received irrigation each morning and humidity controlled misting throughout the day. Temperature was thermostatically controlled and averaged 21°C.

3.2.4 Scoring the trials

The progress of cuttings was followed over the next few months and they were scored according to their survival, the condition of the foliage, the presence of roots coming through the bottom of the pot and the growth of new shoots. The condition of foliage was given a score according to the percentage of the foliage that had died, if less than 33% of foliage was dead, the cutting was given a foliage score of 1, between 33% and 66% of the foliage dead the score equals 2 and greater than 66% of the foliage dead the score equals 3. Dead cuttings (i.e. dead stem) were removed from the analysis of foliage scores.

After 12 weeks the cuttings were removed from the misting house (14 weeks for 1424) and placed into a shadehouse where they received irrigation in the morning only. Surviving cuttings were fertilized with slow release fertilizer (Macrocote Grey).

3.3 Results

Figure 3.1 shows the rooting percentage for ‘436’ at 12 weeks and ‘1424’ at 14 weeks for thick and thin stems for the 4 length categories (25mm, 50mm, 100mm or 200mm). In all cases thin stems have struck at a much higher rate than thick stems except with 25mm stems of ‘1424’ where no cuttings survived until week 12. The large difference in rootstrike between thin and thick stems is most prominent in the longer stems. For example with genotype ‘1424’, 81% of the 100mm long thin stems had struck roots while less than 10% of the thick cuttings of the same length had struck roots.

The other trend that can be identified in Figure 3.1 is that longer thin stems of these genotypes strike roots at a higher percentage than shorter stems. For both genotypes, long, thin stems (100mm or 200mm) have struck at more than double the rate of thin short stems (25mm or 50mm). The difference in rootstrike between longer and shorter stem lengths is much less significant for thick material. For example in genotype ‘1424’ less than 10% of thick stems had struck roots regardless of stem length.
A two way analysis of variance (ANOVA) of these results indicate that both the length and diameter of the cuttings significantly influenced the rootstrike by 12 weeks (P value <0.05) for these genotypes. When the results from both genotypes are combined 50.6% of thin cuttings struck roots compared with only 15.2% of thick cuttings. Therefore in this experiment by 12 weeks, thin cuttings struck roots at more than 3 times the rate for thicker cuttings. The effect of cutting thickness was further analyzed using a two-sample T test to compare the actual thickness of cuttings with rootstrike. This was found to be very significant (P value <0.01) for varieties 1424 (mean thickness of 1.79 mm for rooted cuttings compared to 2.70mm for unrooted) and 436 (mean thickness of 2.79mm for rooted cuttings compared to 3.49mm for unrooted).

ANOVA also identified cutting length to significantly influence rootstrike for these genotypes (P value <0.05). The combined mean strike rates for genotypes ‘1424’ and ‘436’ for cuttings of length 25, 50, 100 and 200mm were 16.1%, 24.4%, 51.8% and 39.3% respectively indicating that on average 100mm long cuttings were most likely to have struck by 12 weeks.

![Figure 3.1](image-url)  
*Figure 3.1 The effect of cutting length and thickness on the rootstrike of Leucadendron varieties ‘436’ and ‘1424’. Thin cuttings of ‘436’ have a diameter less than 3mm, thin cuttings of 1424 have a diameter less than 2.5mm.*

Figure 3.2 shows the effect of stem diameter and length on the rooting of ‘Pixy Red’ material collected prior to flowering in May and after flowering in November. These results differ from those observed with ‘1424’ and ‘436’ in that there are no clear trends linking stem length or diameter to rootstrike by 12 weeks. When the combined results from the 2 ‘Pixy Red’ trials were analysed, ANOVA confirmed that there is no significant effect of stem thickness on rooting (40.5% of thin cuttings struck compared with 39.3% of thick cuttings) or stem length on rooting (31.5%, 30.9%, 45.8% and 57.1% respectively for cuttings 25mm, 50mm, 100mm and 200mm long).
Figure 3.2 The effect of cutting length and diameter on the rootstrike of *Leucadendron* variety ‘Pixy Red’ for cuttings taken at 2 different seasons. Thin cutting have a diameter less than 3mm, thick cuttings have a diameter greater than 3mm.

Likewise, a two sample T-test could not identify a significant influence of actual cutting diameter on rootstrike.

Part of the reason for being unable to demonstrate significance is probable due to some unexpected results. Some poor results were observed with the shortest cuttings e.g. 2.4% rootstrike for 25mm long thick cuttings taken in May but this contrasts with the 55% rootstrike observed when 25mm long thick cuttings were taken in November. Also worth noting is the high rooting percentage observed for both thick and thin stems 200mm long taken in November. Despite these anomalies, it would appear that choice of material is less important in predicting rootstrike of ‘Pixy Red’ than it is for varieties ‘1424’ and ‘436’.

Figure 3.2 also shows the effect of propagation during two different seasons, before flowering in May and after flowering in November. Once again no clear difference can be observed between the 2 seasons. Although there is a slightly higher overall rooting rate in November, for some length and width categories rooting results were better in May. The results from the two different dates for ‘Pixy Red’ are similar in that there is no clear trend linking stem length or diameter to rootstrike as observed with genotypes ‘436’ and ‘1424’.

Although trends could be identified across all genotypes, ‘Pixy Red’ was the least influenced by cutting material with some reasonable rooting rates even with short, thick cuttings while 1424 was the genotype most significantly affected by cutting material with only long and thin cuttings striking at a high percentage. This trend is closely related to survival rates (Fig. 3.3) which clearly shows ‘Pixy Red’ to be relatively unaffected by the sort of cutting material whereas survival of 1424 cuttings is highly dependant on both the length and thickness of cutting material.
Figure 3.3 The survival of cuttings from 4 length categories and 2 thickness categories for 3 Leucadendron varieties. Thin cuttings of ‘Pixy Red’ and ‘436’ have a diameter less than 3mm, thin cuttings of ‘1424’ have a diameter less than 2.5mm.

The condition of foliage and the presence of new shoots were also recorded for each of the treatments each week. Generally thicker cuttings received higher foliage scores (the higher the score the higher the percentage leaf death) with high early foliage scores indicating the likely failure of that cutting to strike roots and survive. Very few new shoots were observed during the first 12 weeks after taking the cuttings and these were almost all on the long cuttings. Even up to week 20 only a small percentage of the cuttings from these trials had grown new shoots.

3.4 Discussion

These results illustrate that the stem diameter and length of the cuttings influence rootstrike by 12 weeks for Leucadendron varieties ‘436’ and ‘1424’. Thin cuttings (diameter <3mm for ‘436’, <2.5mm for ‘1424’) 100mm or 200mm long produced the highest and most consistent root strike rate. Cutting diameter is likely to be related to stem woodiness; thicker stems are more woody and may therefore be slower or less able to strike roots. There is also likely to be an affect of stem maturity. Leucadendrons put on a fresh flush of growth each season following flowering in spring. As these shoots are rapidly growing over late spring and summer they are referred to as softwood. As this growth extension stops and the stems thicken late in summer and early autumn the material is referred to as semihard wood and finally hardwood as it begins to flower. However the progression of maturity from soft to semihard to hard is an ongoing process. There is also likely to be more mature and less mature stems on the one bush at the same time and in fact for a long Leucadendron stem the basal part may be semihard or even hard while the tip is still soft. With this in mind it is likely that thinner stems chosen at any given time are likely to be less mature than the thicker stems. There are many reports of softwood providing better propagating material for many species including several Proteaceae genera.
Conospermum mitchellii is one such species where softwood cuttings were nearly twice as successful as either semihard or hardwood cuttings (Perry and Trueman, 1999) and this may help to explain why in general thinner cuttings were more successful than thicker cuttings.

For variety ‘Pixy Red’ neither cutting length nor diameter had any significant effect on rootstrike. Also cuttings taken in May prior to flowering should have been less mature and may in fact be expected to strike at a higher percentage than the more mature post flowering cuttings collected in November but this difference was not observed. This suggests that either 1) no further maturation of the wood occurred between May and November, or 2) that any increase in maturity did not effect rootstrike or 3) that any effect of decreased rooting ability due to increase in stem maturity was offset by other factors which increased rooting ability e.g. warmer temperatures and increased light in November relative to May.

It is likely that a combination of these 3 factors is responsible. ‘Pixy Red’ is a male Leucadendron variety. For many Leucadendron species and varieties male plants have stems that are thinner and less woody than those formed on female plants. The reason for this is likely to be that stems supporting female flowerheads have to be able to support the weight of the developing seed head and seeds contained there in, where as male flowerheads wither and die soon after anthesis and thus do not have to support the same weight. This may suggest why ‘Pixy Red’ cuttings are not as significantly influenced by stem maturity as predicted by thickness or season than the female varieties ‘1424’ and ‘436’.

These results have produced some evidence to support the idea that using much smaller cuttings could increase the total number of cuttings produced by increasing the potential number of cuttings available, especially with variety ‘Pixy Red’. This is because large cuttings e.g. 200mm long could be cut into several smaller cuttings or shorter pieces that would normally be overlooked could be included. Caution should be observed however as success using smaller cuttings was often much lower after 12 weeks and resulted in very high fatality rates for ‘1424’ cuttings. Never-the-less, thick cuttings 25mm long of ‘Pixy Red’ struck at more than 50% by 12 weeks in the November trial and a high percentage of 50mm and 25mm cuttings (except ‘1424’) were still alive (Fig 3.3) many of which did later strike roots. This suggests that small cutting method could be a valuable tool if many plants are required from a small amount of material. Small cuttings have been reported successful for other Proteaceae species including Leucadendron ‘Safari Sunset’ (Rodriquez Perez, 1992) which took 36 weeks to obtain 20% success from single node cuttings. The length of time to strike, the low success rate and slow subsequent growth make this treatment less than ideal and it is not recommended unless there is a shortage of material. It is completely unsuitable for the propagation of ‘1424’ under the conditions tested.

‘1424’ has been one of the most consistently difficult varieties to propagate to date and the result of 81% rooted using thin 100mm cuttings is a significant improvement on many other attempts to propagate this variety. Genotypic influences such as these have been reported in other Proteaceae e.g. Personia virgata, (Ketelhohn et al 1998) and other unrelated genera (Rodrick and Zsuffa, 1992) and indicate the importance for ongoing trials to further refine the conditions for each genotype being propagated.

The propagation of these Leucadendron varieties at these times using the material described is possible and the results were influenced by cutting length and thickness for genotypes ‘1424’ and ‘436’. If material is not limited we recommend using long thinner cuttings although other material may be used with varying degrees of success for some varieties such as ‘Pixy Red’. Rootstrike by 12 weeks was only a percentage of those cuttings still alive for most varieties and many cuttings continued to strike until week 20 at least. This is a long time from a nursery's point of view and corresponds to an increase in the price of production. By taking cuttings in summer when material is less mature (softwood) and temperatures are warmer it is hoped that the process can be speeded up. Small unpublished trials during our program have shown very high strike rates within 6 weeks over summer for many Leucadendrons.
3.5 Literature cited


4. Micropropagation of Leucadendron

Abstract A collaborative Leucadendron breeding program based at The University of Western Australia has produced thousands of Leucadendron hybrids which are currently being trialled. Tissue culture techniques were investigated as a possible method of quickly producing commercial quantities of plants from limited material available for any new selection. Multiplication of Leucadendron hybrids has been achieved on MS medium containing 20 g L\(^{-1}\) sucrose and 3 g L\(^{-1}\) Phytogel. We found that the multiplication rate varied between genotypes but in general increasing the concentration of 6-benzylaminopurine (BAP) in the media from 0.025 mg L\(^{-1}\) to 1 mg L\(^{-1}\) significantly increased the number of shoots produced but decreased their length. BAP at 0.1 mg L\(^{-1}\) gave the highest number of useful shoots for the three hybrids tested. Rooting percentages depended on genotype and other treatment. The four genotypes tested were successfully rooted and deflashed by dipping microcuttings in Clonex Purple® (3g L\(^{-1}\) indole-butyric acid (IBA)) and striking in propagation mix or Growool®. Culturing shoots on media containing IBA at 2 mg L\(^{-1}\) resulted in 0 to 100% of shoots forming roots in vitro depending on genotype while transferring plantlets from this media to propagation mix after 5 weeks resulted in 16.7 to 99.4% survival 6 weeks later. Root systems formed in propagation mix were superior in terms of root number and length than those formed in Growool® or agar. It was also shown that rootstrike and deflasking could occur simultaneously in a glasshouse.

Key words: micropropagation, Leucadendron, Proteaceae, multiplication, rootstrike, deflasking, plant growth regulators

4.1 Introduction

Flowers and foliage of Leucadendron species and cultivars are an important cutflower export of Australia. The majority of stems sold are from the cultivar 'Safari Sunset' which was bred in New Zealand 40 years ago (Matthews, 1983). However several other species and cultivars are being grown which contain traits useful in the breeding of new varieties. A breeding program based on controlled pollination between selected Leucadendron germplasm began in Western Australia in 1998 as a collaboration between commercial growers, The University of Western Australia and the Rural Industries Research and Development Corporation. The project produced thousands of hybrid plants and are now being trialled. However, due to the cost of trialling the amount of propagation material available for any selected genotype will initially be very limited. Both tissue culture and cutting propagation were investigated to maximize propagation efficiency following the decision to release a new variety.

Cutting propagation is the usual method of multiplying Leucadendron varieties but for some genotypes the type of material and/or the season affect the success rate. The development of tissue culture techniques may allow for rapid year round production from a very limited amount of material. Tissue culture involves firstly sterilizing and initiating the desired genotype into in vitro culture. Successfully initiated cultures are then multiplied by manipulating their morphology and growth using plant growth regulators. Of particular importance at this stage are the cytokinins. Our preliminary investigations have shown that 6-benzylaminopurine (BAP) an artificial cytokinin incorporated into the multiplication media at concentrations between 0.025 mgL\(^{-1}\) and 1 mgL\(^{-1}\) can support many Leucadendron cultures and influence their branching and shoot production. This research, will quantify the effect of BAP concentration on the number and the quality of shoots produced from 3 different Leucadendron culture lines. Successful initiation and multiplication of Leucadendron 'Safari Sunset' (Perez-Frances et al, 1995, Dias Ferreira et al 2002), Leucadendron discolor (Perez-Frances et al, 2000) and a large variety of related genera have been reported. However multiplication is often variable and rootstrike and deflasking are reported as being problematic.

Initial attempts to induce rootstrike in vitro by culturing shoots on agar solidified media containing 3-indolebutyric acid (IBA) or naphthalene acetic acid (NAA) were largely unsuccessful. While some shoots did form roots this was inconsistent between varieties and resulting roots were stunted and
fragile leading to problems with deflasking. Other methods were investigated including striking of in vitro grown cuttings in a standard cutting propagation mix, perlite or Growool®. The results from these trials were very positive and two experiments were designed to determine the relative advantages of these different methods for rootstrike and deflasking of in vitro grown Leucadendron material. Methods such as these have been reported by many researchers as being more successful than in vitro agar based rootstrike for a variety of related genera including Conospermum (Newell et al 2002), Stirlingia (Bunn and Dixon 1992) and Telopea (Offord and Campbell, 1992).

4.2 Materials and methods

4.2.1 Plant material

All culture lines used in the following experiments were initiated by surface sterilizing seeds for 20 minutes in a domestic bleach solution (Whiteking) diluted to 1% available Chlorine and germinated under aseptic conditions in vitro. Each line was assigned a 3-digit code. The code and parentage of the lines are: 005 L. floridum x L. procerum, 007 L. linifolium x L. procerum, 008 L. spissifolium x L. strobilinum and 009 L. linifolium x L. galpinii. These cultures were chosen to represent the main sub sections used in our crossing program; alata, trigona and villosa.

4.2.2 Media

All media used contains full strength MS salts and vitamins (Murashige and Skoog, 1962), 20 gL⁻¹ sucrose and 3 gL⁻¹ phytogel. Multiplication media also contained 6-benzylaminopurine (BAP) at various concentrations (see 2.3). Rooting media contained no BAP but had 3-indolebutyric acid (IBA) incorporated at 2 mg L⁻¹. The pH of all media was set at 6.0 and 50ml of media was dispensed into each 250ml polycarbonate culture vessel prior to autoclaving at 121°C for 15 minutes. Each culture vessel is fitted with a Teflon vent spot (Flora Laboratories). Prior to these experiments, all genotypes had been growing in media containing 0.025 mgL⁻¹ (0.1µM) BAP and subcultured every 8 to 12 weeks.

4.2.3 Multiplication

20mm long shoot segments without axillary bud break and without an apical meristem from the culture lines 005, 007 and 008 were used. 7 shoots were placed per culture vessel with 5 culture vessels (35 shoots) per treatment per genotype. The treatments varied only in the concentration of BAP, either 0 mgL⁻¹, 0.025 mgL⁻¹, 0.1 mgL⁻¹, or 1.0 mgL⁻¹ (0, 0.11, 0.44, and 4.4µM). The cultures were incubated at 25°C with a photoperiod of 16 hours and a photon flux of 40µmol m⁻²s⁻¹. After 49 days, all new shoots were counted and their lengths measured. For each genotype / treatment combination the mean number of shoots and the mean length of shoots and their standard errors were calculated.

4.2.4 Rooting Experiment 1

Shoots from 3 culture lines 005, 007, 008 were exposed to 4 rootstrike treatments. Different media were used in the rooting experiment (Table 4.1).
Table 4.1. Growth regulator and substrates trialed for the rootstrike of *in vitro* grown *Leucadendron* shoots.

<table>
<thead>
<tr>
<th>Rootstrike treatment</th>
<th>Growth regulator</th>
<th>Substrate for first 5 weeks</th>
<th>Substrate from 6 – 11 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>2mg/L IBA</td>
<td>Agar solidified MS</td>
<td>Agar solidified MS</td>
</tr>
<tr>
<td>B</td>
<td>2mg/L IBA</td>
<td>Agar solidified MS</td>
<td>Perlite : Sand : Peat in cellpack</td>
</tr>
<tr>
<td>C</td>
<td>Clonex Purple 3 g/L IBA</td>
<td>Growool® in vitro</td>
<td>Growool® in vitro</td>
</tr>
<tr>
<td>D</td>
<td>Clonex Purple 3 g/L IBA</td>
<td>Perlite : Sand : Peat in vitro</td>
<td>Perlite : Sand : Peat in cellpack</td>
</tr>
<tr>
<td>E</td>
<td>Clonex Purple 3 g/L IBA</td>
<td>Perlite : Sand : Peat in cellpack</td>
<td>Perlite : Sand : Peat in cellpack</td>
</tr>
</tbody>
</table>

4.2.3.1 Agar solidified media. 30mm shoots had the lower third of their leaves removed prior to shoots being inserted approximately 10mm into rooting media. 6 shoots were placed in each jar and 6 jars were used per genotype.

4.2.3.2 In vitro Growool®. 3 cubes of Growool® (25mm x 25mm x 40mm) were placed in each 250ml polycarbonate culture vessel fitted with a Teflon vent spot (Flora Laboratories). The cubes were soaked in deionised (DI) water and then allowed to drain prior to autoclaving at 121°C for 15 minutes. 30mm shoots had the lower third of their leaves removed prior to being dipped in Clonex Purple® containing 3gL⁻¹ IBA and inserted 10mm into the Growool®. 1 shoot was inserted into each Growool® cube, 4 jars each containing 3 shoots were prepared per genotype.

4.2.3.3 In vitro propagation mix. 250ml polycarbonate culture vessels fitted with a Teflon vent spot (Flora Laboratories) had the bottom 25mm filled with a propagation mix containing 3 parts perlite, 2 parts crushed quartz (sand) and 1 part peat. Vessels and soil were autoclaved at 121°C for 15 minutes. 30mm shoots had the lower third of their leaves removed prior to being dipped in Clonex Purple® and inserted 10mm into the propagation mix. Shoots were gently watered with sterile DI water. 6 jars each containing 6 shoots were prepared per genotype.

4.2.3.4 Cell Pack and plastic cover. A nursery propagation tray containing 64 individual cells each 34mm x 34mm x 50mm (cellpack) was filled with propagation mix as above prior to being autoclaved at 121°C for 15 minutes. 30mm shoots had the lower third of their leaves removed prior to being dipped in Clonex Purple® and inserted 10mm into the propagation mix. Shoots were watered with sterile DI water and allowed to drain. 1 shoot was inserted per cell, 16 shoots per genotype. A rigid plastic cover was placed over the tray and the completed tray was then sealed inside a plastic bag. All treatments were incubated at 25°C with a photoperiod of 16 hours and a photon flux of 40µmol m⁻² s⁻¹. 5 weeks after the start of the trial the survival and rootstrike for each treatment was assessed. Half of the cuttings struck in agar were aseptically transferred to cellpacks containing sterile propagation mix, sealed and then placed in the grow room. Living shoots from the in vitro soil treatment were also transferred as above.

9 weeks after the start of the trial the cellpacks containing shoots from the agar, in vitro soil and cellpack treatments and the Growool® treatments were transferred to a misting chamber inside a glasshouse under 50% shadecloth. The plantlets in cellpacks were watered and fertilized once a week. After 11 weeks the covers were gradually opened. The Growool® cubes were removed from their jars and placed in an open mesh tray. Once covers were removed irrigation was via humidity controlled misters. 20 weeks after the start of the trial all surviving plantlets were potted up.
4.2.4 Rooting experiment 2

60 Shoots from culture line 007 were subjected to 3 rooting treatments. 30 of the shoots from each treatment were incubated in a grow room at 25°C with a photoperiod of 16 hours and a photon flux of 40µmol m⁻²s⁻¹ and the other half placed into a misting chamber inside a glasshouse under 50% shadecloth.

4.2.4.1 Agar solidified media. Media and technique was as described in treatment 1 for rooting experiment 1.

4.2.4.2 Growool® Materials and techniques are similar to those described in treatment 2 for rooting experiment 1 except that Growool® was placed directly onto open mesh nursery trays and then treated the same as for the cell pack trials.

4.2.4.3 Cellpack Materials and techniques were as described in treatment 4 for rooting experiment 1.

After 4 weeks the survival for Growool® and cellpacks and rootstrike for agar treatment were assessed. Cuttings struck in agar were aseptically transferred to cellpacks containing sterile soil as described above, sealed and then placed in the misting house. Cuttings in Growool® and cell packs that were in the grow room were transferred to the misting house. 2 weeks later the bags were removed and plants were sprayed with fungicide. The plastic covers were removed 2 weeks later.

4.3 Results and discussion

4.3.1 Multiplication

Increasing the concentration of BAP from 0.025 mgL⁻¹ to 1.0 mgL⁻¹ increased the number of shoots but decreased the average length of shoots for all genotypes trialled (Figures 4.1 and 4.2). Leonardi et al (2001) and others have observed similar responses with other woody species where the number of shoots increases to maximal value after which higher concentrations of BAP either produce no further effect or have a negative effect on shoot number. Genotype 005 appears to reach the maximal number of shoots at a concentration of BAP of 0.1 mgL⁻¹ while the other 2 genotypes showed further increased shoot number at 1.0 mgL⁻¹ s indicating that a higher level may produce even more shoots.

The short length of shoots produced at high BAP concentrations limit their usefulness for rootstrike. Deformities such as vitrification and excessive callus growth have been observed. For these genotypes a BAP concentration of 0.1 mgL⁻¹ gave the maximum number of useful shoots. Although the trend was the same for all genotypes the actual number of shoots produced at this concentration varied significantly with OO5 producing more than 8 shoots while the other 2 genotypes produced about 5 shoots. Shoot lengths at this optimal level were also different between genotypes with 007 having an average shoot length more than double the other 2 genotypes.

The tradeoff between number and length of shoots may be solved by the addition of gibberellic acid (GA) into the media to promote shoot elongation. The addition of 1 mgL⁻¹ or 2 mg L⁻¹ GA allowed Dias Ferreira et al (2003) to use BAP at concentration of 2 mgL⁻¹ and still achieve reasonable shoot length when multiplying Leucadendron 'Safari Sunset'. However, Perez-Frances et al, (1995) found no significant effect of using a combination of BAP and GA on the multiplication of Safari Sunset in vitro. Further experiments are needed to see if improved multiplication will result from using combinations of BAP and GA for these genotypes.
4.3.2 Rooting Experiment 1

Genotype 009 responded well to all treatments with regards to rooting, but for the other 2 genotypes rootstrike in agar solidified media was poor. For genotype 005 transfer from agar to propagation mix resulted in 94.4% rootstrike after 11 weeks while only 11.1% of cuttings left in agar media struck roots. This suggests that it is not the initial concentration of IBA that is important but either the continual exposure to IBA, or that agar solidified media was not suitable for the growth of these roots. Dias Ferreira et al (2003) found that rooting of *in vitro* grown *Leucadendron* 'SafariSunset' cuttings...
only occurred after exposing shoots to 1gL⁻¹ IBA for a brief period followed by transfer to medium free of IBA. Gorst et al (1983) found root growth to be stunted with continual contact with IBA and used riboflavin to photo-oxidize the IBA and successfully improve root elongation. Newell et al (2002) showed that even in the absence of IBA, root initiation and growth was stunted in agar based media relative to propagation mix for the woody perennials they studied. Further evidence to suggest that continual exposure to agar media containing IBA is detrimental to root growth was that many roots growing from above the surface of the media grew more frequently and longer than roots below the surface of the media. All roots formed in agar were fragile with many breaking off at the deflasking stage.

The use of a quick dip in a much higher concentration of IBA (3g/L) followed by transfer to hormone free substrate supports this treatment, as can be seen with treatments C, D and E (Table 4.2). Although the percentage rootstrike in Growool® is similar to the percentage rootstrike in propagation media the roots were more like those formed in agar being less in number, shorter and in some cases forming above the surface of the Growool®. It is possible that transfer to hormone free substrate allowed root initiation to occur but that low aeration at the rooting zone in Growool® had a detrimental effect on subsequent root growth. By comparison roots formed in propagation mix were quite different (see Figure 4.3). These roots were greater in number, longer and initiated from all subterranean parts of the stem.

Striking in vitro grown cuttings directly into propagation mix has significant benefits over agar based rootstrike followed by transfer to soil, both in the quality of resulting roots and in reduced time and costs. It is worth noting that all materials used in these experiments had been autoclaved and all manipulations for the first 5 weeks were carried out under lamia flow thus reducing contamination by pathogenic fungi and bacteria at this fragile stage. Some shoots were lost in the period between 5 and 11 weeks due to Botrytis and other unidentified pathogens which may be controlled by fungicide application suggesting that the actual success rates after 11 weeks could have been much higher.

Table 4.2. The effect of different rooting treatments on the rootstrike of 3 Leucadendron Hybrids. Values for rooted shoots followed by the same letter are not significantly different at the 5% level.

<table>
<thead>
<tr>
<th>Rooting treatment</th>
<th>Genotype</th>
<th>Number shoots treated</th>
<th>Rooted shoots @ 5 weeks (%)</th>
<th>Rooted shoots @ 11 weeks (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>009</td>
<td>18</td>
<td>44.4bcd</td>
<td>100.0g</td>
</tr>
<tr>
<td>B</td>
<td>009</td>
<td>18</td>
<td>38.9bc</td>
<td>88.9fg</td>
</tr>
<tr>
<td>C</td>
<td>009</td>
<td>12</td>
<td>58.3cde</td>
<td>75.0defg</td>
</tr>
<tr>
<td>D</td>
<td>009</td>
<td>36</td>
<td>97.2f</td>
<td>69.4cdef</td>
</tr>
<tr>
<td>E</td>
<td>009</td>
<td>16</td>
<td>93.8ef</td>
<td>93.8fg</td>
</tr>
<tr>
<td>A</td>
<td>008</td>
<td>18</td>
<td>0.0a</td>
<td>0.0a</td>
</tr>
<tr>
<td>B</td>
<td>008</td>
<td>18</td>
<td>0.0a</td>
<td>16.7ab</td>
</tr>
<tr>
<td>C</td>
<td>008</td>
<td>12</td>
<td>41.7bcd</td>
<td>100.0g</td>
</tr>
<tr>
<td>D</td>
<td>008</td>
<td>36</td>
<td>58.3cde</td>
<td>83.3efg</td>
</tr>
<tr>
<td>E</td>
<td>008</td>
<td>16</td>
<td>81.3def</td>
<td>81.3defg</td>
</tr>
<tr>
<td>A</td>
<td>005</td>
<td>18</td>
<td>11.1ab</td>
<td>11.1a</td>
</tr>
<tr>
<td>B</td>
<td>005</td>
<td>18</td>
<td>0.0a</td>
<td>94.4g</td>
</tr>
<tr>
<td>C</td>
<td>005</td>
<td>12</td>
<td>41.7bcd</td>
<td>33.3abc</td>
</tr>
<tr>
<td>D</td>
<td>005</td>
<td>36</td>
<td>63.9cde</td>
<td>41.7bcd</td>
</tr>
<tr>
<td>E</td>
<td>005</td>
<td>16</td>
<td>43.8bcd</td>
<td>50.0bcd</td>
</tr>
</tbody>
</table>
Figure 4.3. Rootstrike and growth of tissue cultured cuttings under different conditions – genotype 008.
A. Excessive growth of callus and abnormal root formation in agar solidified medium;
B. Healthy root formation and growth in sand : peat : perlite medium.

4.3.3 Rooting experiment 2

This experiment further confirmed that rootstrike and deflasking could occur simultaneously and suggests that further benefits could be achieved if this occurs in a glasshouse when compared to grow room (Table 4.3). The main differences are the light intensity in glasshouse (between 100 to 350 µmol m⁻²s⁻¹) and in grow room (40µmol m⁻²s⁻¹) and temperature which fluctuates between 10.5°C and 28.6°C with a mean of 21.1°C in the glasshouse and is constant 25°C in the grow room. Root strike in agar was higher in the misting house than the grow room. This may have been as a result of faster breakdown of IBA in increased light thus reducing the inhibitory effect on root growth or as a result of greater photosynthesis and hence growth at the higher light level. Rootstrike could not be measured for Growool® and propagation mix without disturbing the substrate and possibly damaging the young roots. However there was a significant difference in the number of shoots showing root growth through the bottom of the tray after 8 weeks in propagation mix for those shoots cultured in the misting house when compared to those that were in the grow room initially. Although some roots could be seen growing from shoots in Growool® near the stem no roots had reached the bottom or sides of the Growool® after 8 weeks. This further confirms our earlier observations that root growth in Growool® was stunted relative to propagation mix. Previous experience has shown root growth in Growool® is best when the Growool® is allowed to dry out more in-between watering. If a protocol could be developed to ensure Growool® is not over watered subsequent root growth is likely to be improved.

About a quarter of the shoots transferred directly to propagation media collapsed and died within 1 week regardless of the location. This is not thought to be due to a toxic effect of using a relatively high concentration of IBA (3g/L) or reduced humidity on the aerial parts of the shoots as those struck in Growool® were treated identically in these regards and suffered no losses. It is likely that the bass of these cuttings were in large air filled pockets that occur in the propagation mix and thus the shoots died from not being able to maintain a transpiration stream. This may also have been responsible for the death of some of the shoots in the previous trial and may be overcome by modifying the propagation media or ensuring good contact between the media and stem. No losses occurred when
the agar grown shoots were transferred to propagation media, possibly as a result of better contact between the shoot and the media.

These results suggest that there are advantages in simultaneous rootstrike and deflasking of in vitro grown shoots in the glasshouse.

**Table 4.3.** The effect of rooting substrate and environment on the rootstrike and survival of in vitro grown shoots of *Leucadendron* genotype 007. Values for rooted shoots followed by the same letter are not significantly different at the 5% level.

<table>
<thead>
<tr>
<th>Rooting substrate</th>
<th>Environment</th>
<th>Rooted shoots @ 4 weeks (%)</th>
<th>Surviving shoots @ 4 weeks (%)</th>
<th>surviving shoots @ 8 weeks (%)</th>
<th>% with roots visible through bottom of tray</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agar</td>
<td>Tissue culture room</td>
<td>13.3a</td>
<td>100a</td>
<td>100a</td>
<td>0</td>
</tr>
<tr>
<td>Growool®</td>
<td>Tissue culture room</td>
<td>NA</td>
<td>100a</td>
<td>90.0ab</td>
<td>0</td>
</tr>
<tr>
<td>Perlite Sand Peat</td>
<td>Tissue culture room</td>
<td>NA</td>
<td>70.0b</td>
<td>43.3c</td>
<td>10.0b</td>
</tr>
<tr>
<td>Agar</td>
<td>Misting glasshouse</td>
<td>30.0a</td>
<td>100a</td>
<td>100a</td>
<td>0</td>
</tr>
<tr>
<td>Growool®</td>
<td>Misting glasshouse</td>
<td>NA</td>
<td>100a</td>
<td>86.7ab</td>
<td>0</td>
</tr>
<tr>
<td>Perlite Sand Peat</td>
<td>Misting glasshouse</td>
<td>NA</td>
<td>76.7b</td>
<td>73.3b</td>
<td>63.3a</td>
</tr>
</tbody>
</table>

4.4. Literature Cited


Appendices

Released varieties

1. **00 AP 084** - a hybrid between *L. laureolum* and *L. floridum*. The plant produces long stems, yellow bracts and nice looking conical cones. The saleable stage is about late August to September, later than *L. laureolum* and earlier than *L. floridum*.

2. **1325** – a hybrid between *L. salignum* and *L. procerum*. It is a male plant with long single stems, red-head and yellow brackets. It is very productive.

3. **1386** – a hybrid between *L. uliginosum* and *L. discolor*. It is a male plant, very vigorous and productive. The flowers are multi-headed with striking color and long availability.

4. **436** – a hybrid between *L. coniferum* and *L. discolor*. It is a female plant with long and single stems. The plant is vigorous, productive and can be picked for a long time.

5. **772** – a hybrid between *L. floridum* and *L. discolor*. It is a female with long stems. The plant is vigorous and productive. The flowers are multi-headed and can be picked for a long time.

6. **802** – a hybrid between *L. floridum* and *L. gandogeri*. It is a female plant. The flowers are multi-head with bright yellow brackets and it is pickable for a long time.

7. **868** – a hybrid between *L. floridum* and *L. procerum*. It is a male plant with yellow multi-head. It is very vigorous and productive.

8. **Claire’s Beauty** – a hybrid between *L. procerum* and *L. salignum*. It is a female plant with very long stems and very nice colour. It has been PBR protected.

9. **Pixy Red** – a hybrid from uncontrolled pollination between neighbouring female (*L. salicifolium*) and clonal population of male plants (*L. procerum*) in absence of other pollinating plants. It has been selected because of its vigour, erect form, with numerous stems after pruning terminal shoot, distinctive large bright red male flower. It has been PBR protected.

10. **Ruby Red** - a hybrid between *L. uliginosum* and *L. salicifolium*. The plant produces multi-headed long stems. The striking feature of this plant is that all the small cones will turn purple red in November to December, when there is strong market. It has been PBR protected.
Publications


