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**Rural Industries Research and
Development Corporation**

Muntries

The domestication and improvement of *Kunzea pomifera* (F.Muell.)

**A report for the Rural Industries Research
and Development Corporation**

by Tony Page

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Foreword

Many Australian native plant foods have the potential to broaden the culinary and nutritional composition of the human diet, both in Australia and worldwide. *Kunzea pomifera* (muntries) is a species that occurs naturally in south-eastern South Australia and western Victoria and produces an edible berry which was highly valued by Aboriginal peoples and is now being grown in commercial plantations on a modest scale.

Current cultivation of muntries is based primarily on selections taken from its natural populations. The present study was undertaken to evaluate the potential for its domestication by examining its plant morphological and molecular variation, its breeding system including its intra- and interspecific cross-compatibility, and the potential for successful vegetative propagation.

An idealised commercial plant form, or ideotype, of *Kunzea pomifera* has been proposed as a result of the present study that would be suited to commercial production. It could be achieved through breeding and selection to exploit the plant to utilize the morphological variation found in its natural populations.

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Abbreviations

AMOVA	analysis of molecular variance
ANOVA	analysis of variance
DNA	deoxyribose nucleic acid
IBA	indole-3-buturic acid
Kk	Ki Ki
Ki	Kangaroo Island
Lh	Lake Hawdon
M	molar
mL	millilitre
mm	millimetre
μ L	microlitre
mM	minimolar
μ M	micromolar
NMDS	non-metric multidimensional scaling
p	p-value
pers. comm.	personal communication
psi	pounds per square inch
RAPD	random amplified polymorphic DNA
S.A.	South Australia
UPGMA	unweighted pair group method with arithmetic means
w/v	weight by volume

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Executive Summary

The Australian flora contains a number of species related to current crop species (Brown and Brubaker 2000) and a large number of species with edible fruit, seed, leaf and roots, which provided sustenance to, and were highly valued by, Aboriginal peoples (Low 1991). The little work undertaken to domesticate Australian edible plant species in recent history, may reflect our failure to appreciate the value of such species, rather than a deficiency in the species amenability for domestication. Thus present day challenges appear to be offered to the agriculturalist and horticulturalist to evaluate the potential for, and possibly effect, domestication of certain species of the Australian flora. Of particular interest in this undertaking, is the possibility for domestication of species that have the potential to broaden the culinary and nutritional composition of the human diet, both in Australia and worldwide. It is in this context that the present study was undertaken, to examine the potential for the domestication of *Kunzea pomifera* (muntries) by providing a detailed knowledge of aspects of the species such as its breeding system, plant morphological and molecular variation and the potential for successful vegetative propagation, which were considered relevant to evaluating the feasibility for its domestication.

Kunzea pomifera is a prostrate Australian native shrub species that produces edible berries that were prized by Aboriginal peoples (Clarke 1998) and is now considered to have commercial potential (Graham and Hart 1997). The plant is currently grown commercially in Victoria and South Australia on a somewhat limited scale for both the fresh, and processed, fruit markets (Page 1999). Further development of muntries as a commercial horticultural crop requires improvement in both the level and consistency of fruit quality and yield. The development of improved cultivars, through plant breeding, would be a prerequisite for this improvement, and complementary with the development of appropriate cultural practices.

The objectivity of plant breeding activities would be heightened by a sound knowledge of both the genetic basis and extent of variation for commercial characters in natural populations of the species and its breeding biology. Therefore fundamental research on morphological and genetic variation and the breeding system in muntries is necessary to develop the berry as an Australian crop plant. The principal aim of this investigation was to elucidate the potential for developing cultivars that would be suitable for the commercial production of muntries as an Australian crop plant. The species has various common names based on the south-eastern South Australian Aborigines reference to it as mantari (Clarke 1998), such as muntari, monterry, munter berry, muntries, munthari. In the context of this study the crop plant and the berry will be referred to as muntries.

To accomplish this aim, field collections comprising 345 accessions from 30 populations across the natural distribution of *K.pomifera* as cuttings and grown in a single environment in the Burnley nursery and used as the basis for this research (Section 1). Methods for vegetative propagation of the species were investigated, including the possibility of modifying its habit from a prostrate to a more upright form. Studies were made of the effects of exogenous application of IBA at different concentrations, and artificial heating of the rooting media, on the initiation of adventitious roots of its cuttings (Section 2). The feasibility for grafting *K.pomifera* as a scion on to the upright species *K.ambigua* and *K.ericoides* to confer upright plant habit to the commercial muntries crop was ascertained (Section 3). An examination was made of the possible presence of topophysis in *K.pomifera* by taking cuttings from upright and prostrate positions in the canopy of the mother plant and assessing the effect on the habit of the resulting plants (Section 4).

Levels of both morphological and molecular variation within and between three natural populations of *K.pomifera* were determined. Morphological variation was evaluated (Section 5) by measuring leaf shape and size, mean stomatal, oil-gland and trichome density and the measurement of molecular variation was undertaken using the randomly amplified polymorphic DNA (RAPD) technique (Section 6). Several aspects of the reproductive biology of *K.pomifera* were investigated to determine the feasibility of routine breeding. The viability of both fresh and stored pollen (under a cool

desiccated state) was determined by *in vitro* germination in a liquid culture medium (Section 7). The capacity for the storage of pollen under such conditions was further evaluated by the development of viable seed following its use in artificial pollination in the routine breeding of *K.pomifera*. The onset and duration of stigma receptivity was assessed to establish the optimal stage of floral development for undertaking artificial pollination (Section 8). This was ascertained through studies of peroxidase activity on the stigma, pollen germination and pollen-tube growth in the pistil and seed set following artificial pollination of flowers at different ages. Studies of the breeding behaviour of *Kunzea pomifera* were undertaken to elucidate its self-incompatibility and the intra- and interspecific cross-compatibility with *K.ambigua* and *K.ericoides* (Section 9). These studies entailed the evaluation of pollen germination and pollen-tube growth in the pistil and seed set following artificial pollination of flowers when the stigma was receptive.

An ideal commercial plant form, or ideotype, of *K.pomifera*, was proposed (Section 10), which included an appropriate combination of plant characters expected to contribute, both directly and indirectly, to the efficient production of muntries of consistent and high yield and quality for the marketplace.

1. Sampling field populations of *Kunzea pomifera*

The systematic collection of 345 accessions from thirty populations across the geographical range of *K.pomifera* was undertaken in the autumn of 1999 to obtain representative sampling of its genetic variation within and between populations. The variation between populations was sampled by collecting five to ten accessions from twenty-five populations. The variation within populations was sampled by collecting at least fifty accessions in three core populations located in Ki Ki, Lake Hawdon and Kangaroo Island in South Australia. The collection strategy for the three core populations was based on a predetermined minimum number of individual plants that would most likely encompass all alleles in the population with a frequency of greater than 0.05 with a confidence level of 95% for an outbreeding species. Given that the microclimatic conditions around a plant growing in its natural habitat can be a better indicator of the likely nature of genetic adaptation to an environment than plant phenotype (Marshall and Brown 1975; Falk and Holsinger 1991), each of the three populations of *K.pomifera* sampled intensively (Ki, Kk and Lh) were 'stratified' by microclimate and a systematic sample was taken from each microclimate. The Kangaroo Island (Ki) population was divided into two microclimates according to the exposure to direct coastal exposure *i.e.* those occurring on the primary coastal dune and those on the secondary dune. In the Ki Ki (Kk) population the population was divided into three microclimates, those occurring on a south facing slope or the flat in which the soil profile had been highly modified by highway construction or those individuals occurring on flat but in what appeared to be largely undisturbed soil. The Hawdon (Lh) population was separated into three different microclimates according to topography, for those that occurred at the top of a hill, on its gradient or at the bottom. To collect the stratified random sample the distance across each microclimate/population was estimated using a measuring wheel (Trumeter TR5000) and one sample was taken at every $1/n$ (where n = the total number of samples, see Table 1) of the total distance. Populations were sampled by taking vegetative material for the production of cuttings, of which three replicates per accession were planted into 200mm- diameter pots and arranged in a randomised block design at Burnley Horticultural College. These plants were used for evaluating vegetative propagation, morphological and genetic variation, and reproductive biology of the species.

In its natural habitat *K.pomifera* has the ability to layer from its main branches (Toelken 1986; Elliot and Jones 1993; King 1998) and therefore, genetically identical individuals can typically occur in close proximity to each other. This dictates that each sample needed to be at least three metres from others. Since *K.pomifera* can layer naturally, collection of vegetative propagation material was a feasible option for introducing wild plants into cultivation. From previous wild collections, 71% of cutting material initiated roots and 98% survival rate of accessions was achieved (Page 1998). This is encouraging considering that the timing of field collection of cuttings for this collection was made during a relatively dormant period (June). An advantage of vegetative propagation is that the accession will be genetically identical to the wild mother plant (ortet) and will typically take less time to develop flowers and fruit than seed-propagated plants. This is of particular importance in the present study, because the growing of field collections to flowering is needed to undertake analyses of the breeding system of the species.

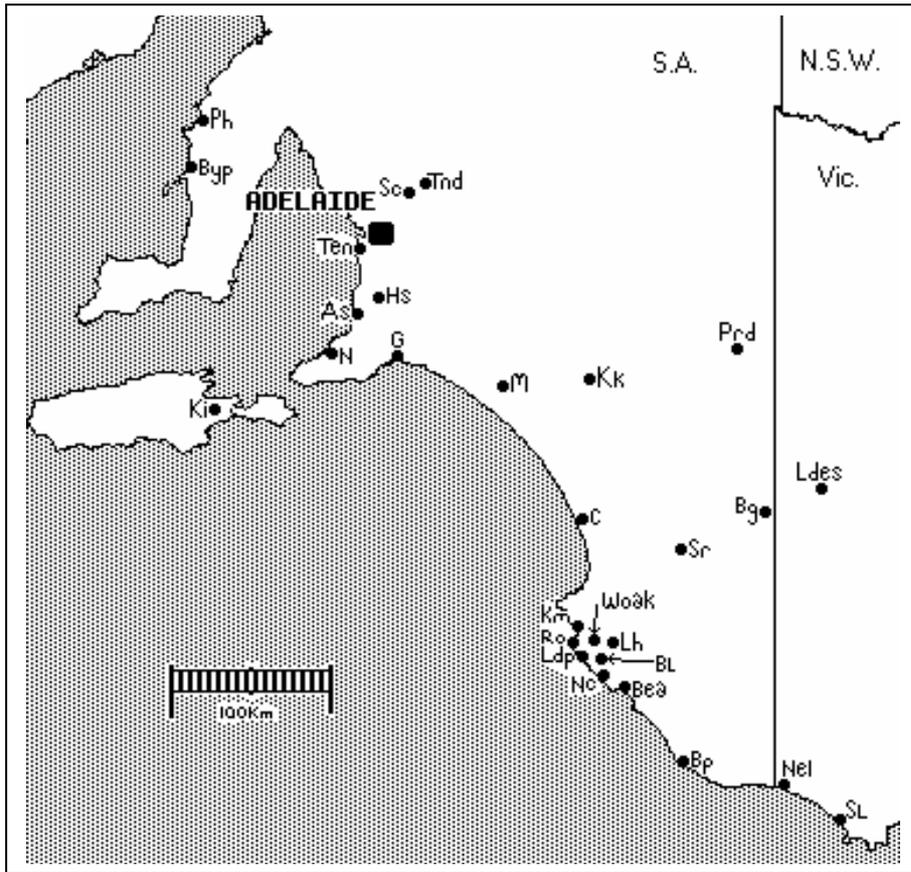
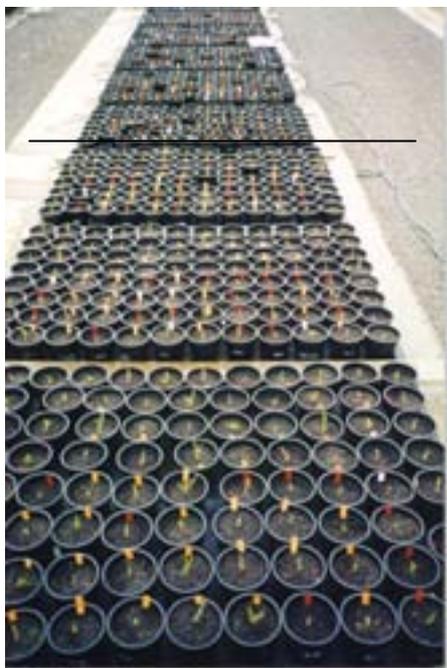
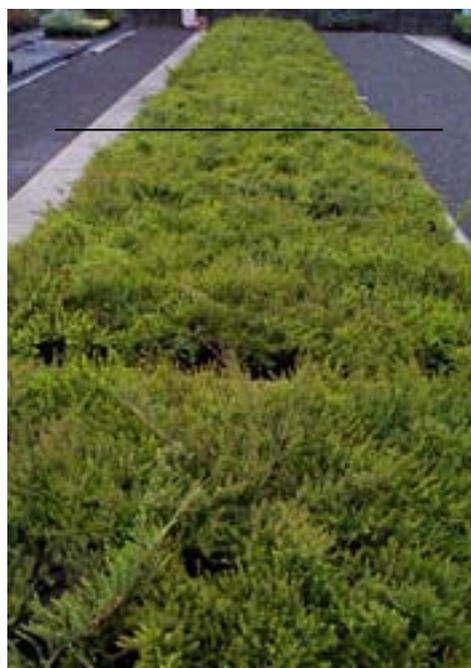


Figure 1: Collection sites across the geographic range of *Kunzea pomifera*. Each symbol represents a population that was sampled. (Population details in Table 1.)



(a)



(b)

Plate 1: Rooted cuttings of field collections watered by capillary irrigation at the Burnley nursery in (a) September 1999 and (b) January 2000. Area from foreground to horizontal state represents one replication of randomised accessions.

Area of collection in South Australia and Victoria	Population designation	Numbers of accessions collected	Latitude	Longitude
Coastal South East	Swan Lake (SL)	8	38° 35' 52"	141° 23' 36"
	Nelson (Nel)	8	38° 04' 52"	141° 15' 01"
	Woakwine (Woak)	8	37° 10' 59"	139° 52' 43"
	Robe (Ro)	16	37° 09' 51"	139° 46' 01"
	Little Dip (Ldp)	5	37° 10' 59"	139° 45' 58"
	Nora Creina (Nc)	10	37° 19' 07"	139° 51' 03"
	Bogg Lane (BL)	5	37° 17' 27"	139° 51' 01"
	Hawdon (Lh) *	52	37° 15' 38"	139° 55' 15"
	Guichen Bay (Km)	6	37° 14' 46"	139° 50' 98"
	Beachport (Bea)	8	37° 28' 19"	140° 02' 21"
	Bangalow Point (Bp)	6	37° 55' 14"	140° 24' 55"
Inland South East	Little Desert (Ldes)	9	36° 26' 57"	141° 47' 55"
	Stewart Range (Sr)	8	36° 50' 24"	140° 24' 39"
	Bangham (Bg)	10	36° 36' 28"	140° 54' 34"
	Pinaroo (Prd)	12	36° 12' 02"	140° 40' 38"
	Ki Ki (Kk) *	50	35° 38' 11"	139° 49' 22"
Fleurieu Peninsula	Goolwa (G)	11	35° 31' 01"	138° 46' 22"
	Hardy Scrub (Hs)	12	35° 09' 53"	138° 35' 33"
	Normanville (N)	5	35° 27' 01"	138° 18' 18"
	Aldinga (As)	10	35° 17' 48"	138° 26' 51"
	Tennyson (Ten)	3	34° 52' 57"	138° 29' 00"
Coorong	Meningie (M)	10	35° 31' 33"	139° 21' 32"
	Coorong (C.)	5	36° 39' 28"	139° 51' 15"
Northern Lofty	Tanunda (Tnd)	9	34° 30' 55"	138° 58' 49"
	Sandy Creek (Sc)	5	34° 36' 09"	138° 51' 15"
Yorke Peninsula	Balgowan (Byp)	5	34° 17' 36"	137° 30' 06"
	Port Hughes (Ph)	3	34° 04' 22"	137° 33' 08"
Kangaroo Island	Kangaroo Island (Ki) *	52	35° 47' 02"	137° 51' 50"
Total		345		
Cultivated Selections	Roger's selection (SES)	1		
	Bonney's aborescent (Ba)	1		
	ANPI	4		
	King	5		

Table 1: Details of collections made from natural populations of *K.pomifera* for the present study, plus four cultivated selections of the species. *Major collections for studies of genetic polymorphism in *K.pomifera*.

2. Vegetative propagation of *Kunzea pomifera*

2.1 Abstract

The influence of IBA treatment of cuttings, heat treatment of the rooting media, and different genotypes, on the percentage of rooted cuttings, the number of roots per cutting and mean root length were evaluated in *K.pomifera*. The percentage of rooted cuttings increased significantly with exogenous application of IBA at concentrations of 1000, 3000 and 5000ppm compared with no auxin. However, exogenous IBA application had no effect on the number of roots or mean root length per rooted cutting. The optimum concentration of IBA for percentage of rooted cuttings and number of roots per cutting varied between accessions of *K.pomifera*, but it appeared that 3000ppm IBA (in a 50% ethanolic solution), would be generally suitable for successfully propagating different genotypes of *K.pomifera*. Heating the rooting media to a temperature of $24 \pm 2^\circ\text{C}$ appeared to have little, to no, effect on any of the root traits measured, compared with un-heated media (within a temperature range of $16\text{-}25^\circ\text{C}$ and therefore the species can be propagated successfully in rooting media with a temperature ranging from $16\text{-}26^\circ\text{C}$. Cuttings could be produced for all accessions where the percentage of struck cuttings, under optimal IBA concentrations for each accession, ranged from 68-96%. Significant variation for all measured root traits was found between accessions, indicating that selection for these characters may be used in breeding programmes to maximise successful vegetative propagation in commercial cultivars.

2.2 Introduction

The success of muntries (*Kunzea pomifera*) as a commercial fruit crop will depend partly on the availability of high-yielding, well-adapted cultivars that produce high quality fruit. Considering the allogamous nature of its breeding system, and consequently, the largely heterozygous characteristics of its individual plants in the natural state, it must be ensured that in commercial populations of *K.pomifera* high levels of heterozygosity are maintained for maximum commercial performance. Thus, while vegetative propagation of the species will be an important activity in multiplying desirable genotypes, commercial plantations must always comprise a range of different genotypes, which, through out-crossing will most likely confer high vigour in terms of high fruit yield and quality. *K.pomifera* is a prostrate species that layers naturally along its primary stems (Elliot and Jones 1993; Jeanes 1996), and therefore, vegetative propagation by cuttings may be a cost-effective method for reproducing desirable genotypes for commercial production (Spanos *et al.* 1999). The aim of the present study was to determine the effect of different concentrations of exogenous auxin applied to cuttings (in the form of indole butyric acid (IBA)), and the application of heat ($24 \pm 2^\circ\text{C}$) to the cutting medium, on the initiation and growth of roots in semi-hardwood cuttings in *Kunzea pomifera*.

2.3 Materials and methods

Semi-hardwood cuttings 8cm in length were taken from terminal stems of the stock-plants (for accessions used See below). The propagating medium, comprised a 1:2:9 parts (by volume) of sieved peat moss, Perlite grade P500 and medium-grade pine bark (6mm) with 'Saturaid' wetting agent at 750g / 500L. Cuttings were taken from the apices of each stem arising from a lateral stem, and leaves on the lower 30mm of each cutting were removed, and the 'stripped' lower sections were dipped in a solution of 50% EtOH containing various concentrations of IBA (See below) for 10 seconds before being placed in the rooting medium. Trays containing 100-30mL cells were filled with propagation mix and each cutting was placed in a single cell. They were grown under natural photoperiod in a greenhouse under 70% shade cloth, with a temperature range of $16\text{-}25^\circ\text{C}$, and a relative humidity of 90%, maintained through a fogging system (Envirocare-MF200). The application of heat at the base propagation tray was delivered through a system of polypropylene pipes containing heated water, to maintain the temperature of the rooting medium at $24 \pm 2^\circ\text{C}$. Cuttings were checked daily for moisture requirement and manually irrigated as required.

Two separate cutting experiments were undertaken. In the first, semi-hardwood cuttings 100 cuttings from each of four accessions of *K.pomifera* (Ki2f, Ki7c, Lh 1g, Lh1i) were dipped for 10 seconds in a solution of 50% EtOH (control) or 50% EtOH with indole-3-butyric acid (IBA) at concentrations of either 1000, 3000 and 5000ppm with 24 cuttings per from each accession per treatment. In the second experiment involving 50 cuttings from each of eight accessions of *K.pomifera* (Ba, Ki2f, Ki7b, Ki7c, Lh1i Lh1g, Lh2g and Lh3k) heat ($24 \pm 2^{\circ}\text{C}$) treatment was applied to the bottom of the cutting with a control wherein the trays were not heated for 25 cuttings from each accession per treatment. Cuttings were harvested after 6 weeks (IBA experiment) or 10 weeks (bottom-heat experiment) from being placed in the rooting-medium and records were taken of the number of primary roots per cutting ($\geq 0.5\text{mm}$ in length) and the length of each root. Root length was measured manually using digital display vernier callipers with 0.01mm increments (Toledo Digical 150).

2.4 Results

In the first experiment a total of 39% of cuttings from the control (0ppm IBA) exhibited root initiation, which was significantly ($P=0.001$) lower than 1000- (70%), 3000- (84%) and 5000-ppm (75%) IBA over all accessions. The positive effect of IBA application, on the percentage of cuttings that initiated roots was consistent across all four accessions of *K.pomifera*. In Ki2f, Ki7c and Lh1i no significant differences were found in the percentage of roots initiated between the IBA concentrations, but the Lh1g cuttings treated with 3000ppm produced a significantly ($P<0.05$) greater percentage of rooted cuttings than 1000ppm, but not significantly different from that with 5000ppm. No significant ($P=0.263$) interaction was found between accessions and IBA treatments.

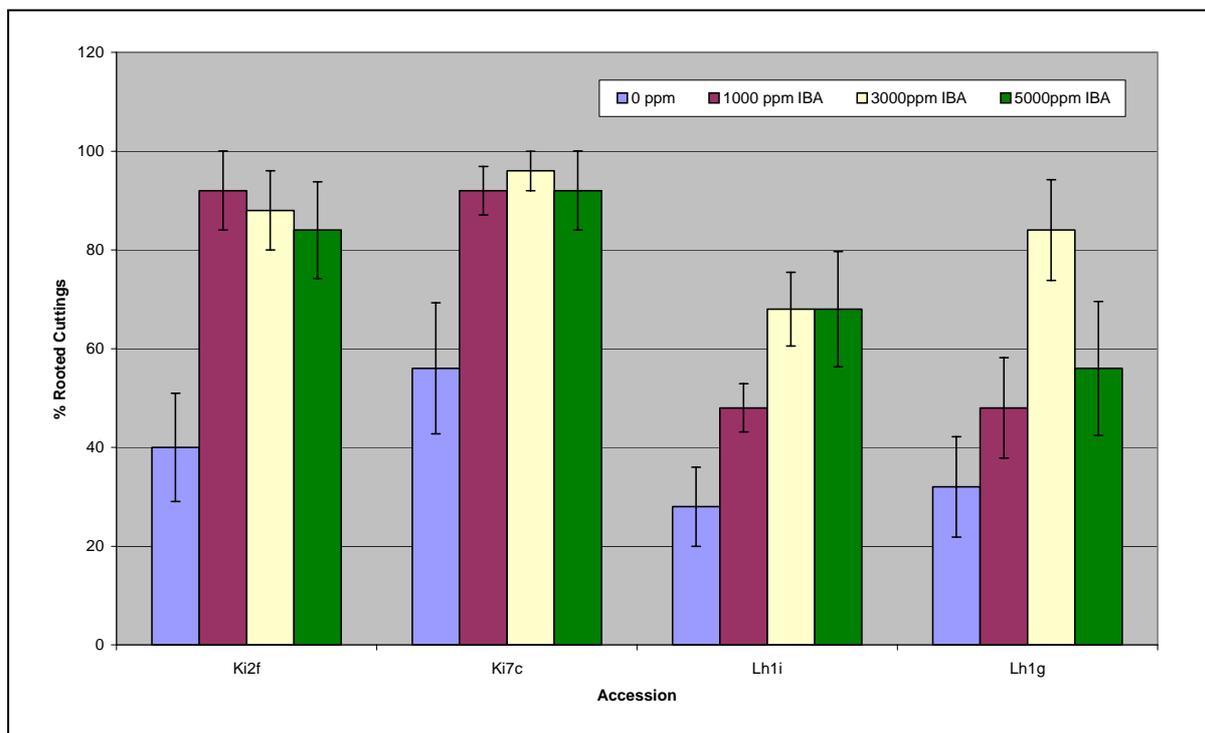


Figure 2: Percentage of cuttings initiating roots in four accessions of *K.pomifera* in response to different IBA treatment. Standard errors are indicated by vertical bars

In the bottom-heat experiment, across all accessions there were no significant ($p=0.740$) differences in the percentage of rooted cuttings, mean number of roots initiated ($p=0.604$) or mean root length ($p=0.096$) per cutting between the bottom-heat treatment and the control. Throughout the ten weeks of the experiment 8.5% of the control and 7.5% of the bottom-heat-treated cuttings, had not initiated roots.

2.5 Discussion

This study demonstrated that the application of exogenous IBA, at concentrations of 1000, 3000 and 3000ppm, to cuttings of *K.pomifera* has a beneficial effect on the induction of roots. While the favourable effect of IBA application in such concentrations, for the induction of roots, was consistent in all four accessions, the optimum concentration of IBA, *i.e.* that giving the greatest percentage of rooted cuttings, differed between accessions. For Ki2f, Ki7c and Lh 1i there was no significant difference in the percentage of rooted cuttings between any of the three IBA concentrations. For Lh1g cuttings no significant difference was found in its level between 3000 and 5000ppm. A significant observation of the present experiment was that the percentage of rooted cuttings was increased by the application of endogenous IBA, but an optimal concentration could be identified between 1000 – 5000ppm.

In three of the four accessions of *K.pomifera* the application of IBA (in one or more concentrations) had a significant positive effect on the number of roots initiated per cutting relative to the control, with no indication of an optimal IBA concentration for maximum root number. The application of IBA to the cuttings of *K.pomifera*, at concentrations between 1000-5000ppm is likely to increase the number of roots initiated per cutting but the optimal concentration appears to be genotype-specific. It appears that the application of exogenous IBA to cuttings of *K.pomifera* has little effect on the growth extension of roots once they have been initiated.

There was an indication, in the present study, of a differential genotype response in *K.pomifera* to heating of the rooting medium in terms of root number initiated on a cutting. Accessions Ki7b and Ki7c produced a significantly greater root number per cutting in response to the application of heat to the rooting medium, than the control. Conversely the accessions Ba and Ki2f were significantly lower in root number per cutting with this temperature treatment than the control. In the Lh accessions of *K.pomifera*, heating the rooting medium for cutting propagation had little, to no, effect on the percentage of rooted cuttings, the mean number of roots per cutting and mean root length. Considering that an adequate number of roots for growth and development of the ramet were initiated in both treatments for each accession, it is proposed that heating the rooting medium is not required to ensure the production of viable clones in this species, when cuttings are struck in a greenhouse with environmental conditions similar to those of this study *i.e.* 90% relative humidity of 90% and a temperature range of 16-25°C. However for the routine propagation of particular cultivars it would be necessary to optimise the temperature of the rooting medium to maximise the percentage of rooted cuttings.

Because of very high levels of self-incompatibility in *K.pomifera* the commercial production of the crop will require plantings consisting of a number of genetically different genotypes to ensure cross-pollination and subsequent fruit development. In *Melaleuca alternifolia* clonal production poses as viable option to preserve and multiply gene combinations in genotypes selected in a recurrent selection programme, which are advantageous for commercial production (Doran and Baker 2000). Considering that the economic product of this crop, the essential oils, is from vegetative parts of the plant, the establishment of clonal plantations for commercial production of the oil is a feasible proposition. These authors proposed, however, that such plantations should desirably be based on a 'number' of genetically distinct clones, to maintain genetic diversity in commercial plantings to minimise the risk of pest and disease outbreaks. Likewise hybrid eucalypt clones, produced in Aracruz (Brazil), are planted in clonal blocks throughout plantations to offset potential disadvantages of monoculture production such as unchecked pest and disease infestations (Zobel 1992). Therefore while populations comprising different genotypes would be essential to maximise levels of cross-pollination and fruit production in *K.pomifera*, they could also serve to reduce the potential for pest and disease infestation, compared with a genetically uniform population.

2.6 Conclusion

The application of exogenous IBA to cuttings of *K.pomifera* at concentrations of 1000, 3000 and 5000ppm gave increased frequency of rooted cuttings compared with no auxin. Cuttings treated with 3000ppm IBA also increased the number of roots per cutting than compared with no auxin. Within each accession little variation was found between different concentrations of IBA for achieving a maximal level of cutting production. It appears that IBA, at a concentration of 3000ppm (in a 50% ethanol solution), could be used for the routine propagation of *K.pomifera*, in a pinebark-based rooting media (approx. 24-25% air-filled porosity), under shaded (70%), greenhouse conditions (16°-25°C) with a relative humidity of 90%. Under optimal IBA concentration, the percentage of cuttings that produced roots in a particular *K.pomifera* accession ranged from 68% to 96%, indicating that in the commercial propagation of the species a consistently high level of successful propagation could be achieved. However, there was significant variation between accessions for the level of successful rooting, root number and length of cuttings, indicating that the level of successful vegetative propagation could be increased by selection

3. Grafting of *Kunzea pomifera*

3.1 Abstract

Kunzea pomifera was grafted as the scion on to *K.ambigua* and *K.ericoides* rootstocks using the side-veneer technique, giving a mean success rate of 50-100% for *K.ambigua* and 25% for *K.ericoides*. Successful grafting between these species can provide potentially useful modification of plant form, especially tall erect stems and the possible extension of the edaphic range of adaptability of *K.pomifera* its commercial cultivation.

3.2 Introduction

Selection, breeding, and the use of appropriate cultural practices of crop management are important components of the domestication process of a 'wild' plant species. The domestication of muntries would involve improvement of potentially important characters such as yield, disease resistance and fruit quality through the application of these techniques (Pickersgill 1986). The potential exists to rapidly transform the species from a wild to a domesticated form, thus providing a new horticultural industry and a new taste choice to the range of berry-type fruits currently available commercially. Grafting is an important activity in the commercial production of a whole range of horticultural plants. Its function is to combine the desirable features of a horticultural species (as a scion) with those of a stock plant, either a member of the same species, or a related species, which confers a greater level of vigour or measure of tolerance/resistance to a particular edaphic and/or biotic factor(s) than possessed by the root system of the scion plant. The aim of the present study was to determine the success rate of grafting *K.pomifera*, as scions, on to the rootstocks of *K.ambigua* and *K.ericoides*, with the view of conferring *K.pomifera* with erect plant habit for its commercial production.

3.3 Materials and methods

An initial grafting experiment was undertaken in 1999 using the side-veneer grafting technique (Hartmann *et al.* 2002) of *K.pomifera* on to *K.ambigua*. The scion material for the grafting experiment was collected from the Guichen Bay (Km) population and stored at 4°C for 10 days before grafting, and the rootstock plants of *K.ambigua* were grown in small pots, which were somewhat restricted in vigour. The *K.ambigua* specimens were grown from seed obtained from Nindethana Seeds (W.A) and the plants were approximately 1m in height at the time of grafting. A total of 50 grafts were carried out using semi-hardwood cuttings of *K.pomifera* as scions, which were approximately 5mm in diameter and were positioned on the stock of *K.ambigua* at heights of 60-80cm. The main leader of the rootstock of *K.ambigua* was cut and 20% of the foliage below the graft was removed. The cuts were made using a No. 11 disposable scalpel blade, and unions were bound using flexible self-adhesive Parafilm®. The plants were then placed in a greenhouse possessing a fogging system, which maintained a relative humidity of approximately 90%. The removal of 50% of the remaining leaves on the rootstock was undertaken at bud burst on the scion, and the remaining leaf material on the stock was removed when the scion exhibited vigorous growth. The successful grafts were then potted into 125mm-diameter pots and placed in an unheated greenhouse to acclimatise for 4 weeks, before being placed outdoors at the Burnley Nursery. Six months after removal from the greenhouse, twelve of the grafted plants were planted in the field, for ongoing assessment.

The grafting trial in 2000 employed the use of two rootstock species, *Kunzea ambigua* and *Kunzea ericoides*, and two environmental treatments, consisting of either a greenhouse environment with relative humidity at approximately 90%, or outside with the scion enclosed in a 'zip lock' plastic bag for protection from desiccation. Using a single accession of *K.pomifera* (Km t2) as the scion material, a total of 10 grafts were made on to rootstocks of *K.ambigua* and 16 on to *K.ericoides* on the 10th April 2000. Half the number of the grafts for each rootstock treatment were allocated to each environment for a total of 16 weeks, after which they were assessed for successful graft union. Successful grafts were grown for a further 16 weeks in a heated greenhouse. Plants were potted from 200mm to 300mm-diameter PVC pots and grown outside and irrigated twice daily for 5 minutes using drip-

irrigation (Antelco Shrubber[®] 360°). Four grafted individuals (two with *K.ambigua* and two with *K.ericoides* rootstocks) were potted into 400mm-diameter pots (an example of each is seen in Plate 2).

3.4 Results

Those grafts that formed callus at the union of the rootstock and scion and exhibited vigorous growth of scions were recorded as successful. In 1999, 25 of the 50 grafts of *K.pomifera* on to *K.ambigua* were successful, giving a success rate of 50. Of the successful grafts of *K.pomifera* on to *K.ambigua* 3 died, 5 months later, but there was no evidence of incompatibility at the graft union for these individuals. The remaining 44% (22 plants) survived in pots for a further six months and 12 of these were planted in the field and have exhibited vigorous growth for four years after grafting, with no visible signs of incompatibility between the *K.pomifera* scion and the *K.ambigua* rootstock. During this time, however, flowering has not occurred on any of these field-grown plants.

In 2000, all of the five grafts of *K.pomifera* on to *K.ambigua* rootstocks, which were located in the greenhouse, revealed a successful union between scion and rootstock, and of five plants located outside, four were found to have a successful union. For *K.ericoides* used as rootstocks, only two out of eight grafts resulted in a successful union in both the greenhouse and outside environments. Grafts survived in pots for three years (Plate 2) and those grown in 300mm-diameter pots flowered prolifically on both rootstock species during the spring of 2002, resulting in the development of fruit (Plate 3b). Those that were potted in 400mm-diameter pots also flowered during this season, but flower yield in them was substantially lower than those grown in 300mm-diameter pots and, consequently, with a high fruit yield on the latter (Plate 3).



Plate 2: Plants of *Kunzea pomifera* grafted on the rootstock of *Kunzea ericoides* (left) and *K.ambigua* (right) two years after grafting.

3.5 Discussion

Given that the scion material for the grafting experiment in 1999 was collected from a wild population at Guichen Bay, South Australia and stored at 4°C for 10 days before grafting and that the rootstock plants were restricted in vigour, the grafting conditions were not considered to be optimal. The successful graft union rate of 50% found between *K.pomifera* scions and *K.ambigua* rootstocks under such conditions is an indication that a graft union between these two species can be readily achieved. Greater vigour in the scion and stock plants may be likely to improve the success rate of grafted plants, which would be necessary for the efficient commercial production of grafted plants.

For the grafts made between *K.pomifera* and *K.ambigua* in 2000, 90% resulted in a successful union between them. All grafted individuals placed in the humidity-controlled greenhouse were found to have successful graft unions. Grafts enclosed in plastic bags and placed outside exhibited a union rate of 80%. While a greater number of grafts would be required to determine if there was any significant difference in union rate between the outdoor and greenhouse environments, it indicates that grafting *K.pomifera* in the field appears to be feasible.

Grafts between *K.pomifera* (scion) and *K.ericoides* (rootstocks) made in 2000 exhibited a success rate of 25% in both the outside, and greenhouse environments, indicating that while it is possible to develop grafted plants between these species, the success rate is much lower than those between *K.pomifera* (scion) and *K.ambigua* (rootstocks). Considering that no difference in success rate of grafts was found between the outdoor and greenhouse environments in grafts between *K.pomifera* and *K.ericoides*, it appears that factors other than the climatic environment may operate to limit the frequency of successful graft unions between them.



Plate 3: Grafted plants using *K.pomifera* accession Kmt1 as the scion and *K.ambigua* as stock with (a) no fruit production when grown in 400mm-diameter PVC-pots and (b) substantial fruit production when grown in a 300mm-diameter PVC-pots.

The majority of grafted individuals between *K.pomifera* (scion) with each of *K.ambigua* and *K.ericoides* as rootstocks were found to grow vigorously for 3–4 years after grafting with no evidence of incompatibility between them over that period, as reported by McKenzie (1984), Lewis (1985) and Hartmann *et al.* (2002). Levels of flower production fruit development were substantially greater in grafted individuals in which rootstock vigour was reduced, as in 300-mm diameter PVC pots relative to that in 400-mm diameter pots, or that in field-grown grafts. In *Eucalyptus globulus* Hasan and Reid (1995) found floral induction was promoted in seedlings by a factor(s) associated with reduced pot

size. In the cultivation of *K.pomifera* anecdotal evidence indicates that large amounts of vegetative growth, without flower-bud initiation of flowers and associated fruit development, was often observed under 'luxuriant' growing conditions (Glowinski 1991; Elliot and Jones 1993; Page 1999). While the positive association between apparent root restriction and fruit yield was observed consistently in the present study, further evaluation of this relationship is required, to determine the factors involved in fruitfulness and cultural practices in field plantings of *K.pomifera*.

Knowledge of the ranges of climatic and edaphic adaptation in *K.ambigua* and *K.ericoides* can assist in identifying the potential environments in which *K.pomifera* could be produced commercially when grown as grafted plants with these related species as rootstocks. Elliot and Jones (1993) described *Kunzea ambigua* as being capable of growing in a wide range of soils and climatic conditions. Judd (1990) found that *K.ambigua* colonised coastal heath environments at Wilson's Promontory even in the absence of disturbance, and described it as an invasive shrub in this part of its distribution. At Wilson's Promontory *K.ambigua* occurs in both siliceous and granitic soils (never on calcareous soils) with a wide range of moisture availability, from the dry soils of granite outcrops to those subject to seasonal waterlogging (by ground-water) in coastal heaths (Judd 1990). Ashton and Webb (1977) proposed *K.ambigua* to be particularly tolerant of drought stress, since they found a large relative water content (60%) in its cell walls of its leaves and stems and a capacity for rapidly reducing the water potential, after a lowering of the relative water content, of its shoots. Judd (1990) demonstrated that seedlings of *K.ambigua* and *K.ericoides* had the capacity for enhanced survival during drought stress by substantially reducing their osmotic potential at wilting point. He also proposed that drought tolerance is enhanced in both species by the capacity to accumulate osmotically active solutes, and in *K.ambigua*, by the capacity of its leaves to tolerate desiccation.

K.ericoides has a more widespread geographical distribution than *K.ambigua*, occurring from western Victoria to south-eastern Queensland (Stanley and Ross 1986; Sellar 1996; Harden 2002) and also occurs in New Zealand as extensive, uniform stands with individuals growing up to 20m high (Esler and Astridge 1974). A revision of the genus *Kunzea* is being currently undertaken by Toelken (pers. comm. 2003) who proposed that the name *K.ericoides* will refer only to the New Zealand occurrences and that those in Australia will be further classified into six separate species. Until this revision is completed it is proposed that this species should be discussed in terms of its current classification as *K.ericoides*.

Since both *K.ambigua* and *K.ericoides* can grow to heights of 2-5m and 2-8m respectively (Elliot and Jones 1993; Sellar 1996; Harden 2002), another potential benefit of grafting *K.pomifera* on to these species as rootstocks is the production of upright forms of *K.pomifera* from grafting on to single-stemmed rootstocks at a height of approximately two metres. Such upright forms could potentially improve the efficiency of its commercial production by providing greater ease of crop maintenance and harvesting of fruit. Standard forms of plants, achieved through top-grafting on to tall rootstocks, have been reported primarily for the development of plants with a habit having ornamental merit (Mezitt 1982; Wilson 1984; Bakker 1998). Crossen (1990) produced weeping standard forms of *Grevillea* for ornamental horticulture, by splice-grafting the prostrate species *G. 'Poorinda Royal Mantle'* on to the upright rootstocks of *G.robusta*, which were grown to approximately 1.8m before grafting.

It is likely that the production of grafted plants would be more labour-intensive and therefore more expensive than the production of plants from either seed or cuttings. Further investigation into the economics of producing grafted plants at a height of 1.8-2.0m is consequently necessary to determine the feasibility of producing such grafts commercially. However propagation of grafted plants could be used as an interim measure for producing upright plants for cultivation until cultivars of *K.pomifera* with an erect, upright habit have been bred.

3.6 Conclusion

In the present study a high level of success was achieved (50-100%), from grafts of *K.pomifera* on to rootstocks of *K.ambigua*. A lower level of successful grafting (25%) was achieved between *K.pomifera* (as scion) on to *K.ericoides*. By using such methods, it would be possible to produce upright forms of *K.pomifera* that are suited to the commercial production of its fruit (muntries). These grafted plants could feasibly extend production of *K.pomifera* beyond the calcareous sandy soils of its natural distribution, to the wide range of soils types, to which both *K.ambigua* (Judd 1990) and *K.ericoides* (Cook *et al.* 1980; Judd 1990; Allen *et al.* 1992) appear to be adapted. While the high grafting success between *K.pomifera* and *K.ambigua*, found in the present study, indicates the potential for producing upright forms of *K.pomifera* for commercial production, further investigation would be required to establish its feasibility in terms of providing the potential for high yield and fruit quality of plants from this graft combination.

4. The influence of topophysis on vegetative propagation in *Kunzea pomifera*

4.1 Abstract

The position on the mother plant of *K.pomifera*, from which a cutting was taken, was examined in terms of its effect on the habit of the resulting ramets in two semi-upright and two prostrate forms of the species. Cuttings were taken from primary stems that grew in either an upright orientation (60-90° to the horizontal), or prostrate (0-30° to the horizontal). In three of four accessions of *Kunzea pomifera* the effect of the position of the cutting, on the height, and height-to-breadth ratio, of the resulting ramets was not significant after 17 months growth. With one accession (Ba), however, ramets were significantly greater in height-to-breadth ratio, when cuttings were taken from upright, compared with prostrate, stems.

4.2 Introduction

The prostrate plant habit of *K.pomifera* poses a possible limitation to the successful domestication of the species for efficient commercial production of its fruit. A more upright plant habit would be more desirable in terms of weed, pest and disease control and manual and, possible, mechanical harvest of the fruit. For these reasons the present study was concerned with examining the topophysis phenomenon and its use, in vegetative propagation, for the possible circumvention of the prostrate habit in the commercial production of *K.pomifera*.

Topophysis and its definition have been reviewed by Olesen (1978) and Dodd and Power (1988), both giving a slightly different interpretation of the phenomenon. Olesen (1978) described topophysis as a phenomenon in which vegetative propagules (scions, buds and cuttings) maintained the branch-like growth habit of the parental shoots from which they were removed. Dodd and Power (1988) considered a broader explanation in which the phenomenon was highlighted by its persistence in cuttings, but not confined to shoots removed from the ortet, for vegetative propagation. They defined topophysis as constituting any differences in morphology, anatomy and physiology, developed from 'positional' effects within a plant, that are retained in plants propagated from detached shoots. This account acknowledges Molisch's original description since many characteristics can be passed from ortet to ramet and encompassed by topophysis, whereas other descriptions focused primarily on the retention of lateral (plagiotropic) growth in the ramet. The description of the phenomenon by Dodd and Powers (*loc. cit.*) differed from earlier definitions, in that it was associated with plant development and does not require intervention of propagators to be recognised as a phenomenon. In the present study of topophysis in *K.pomifera* the description of Dodd and Power (*loc. cit.*) will be used and where it demonstrated to occur as morphological differences persisting between ramets taken from cuttings from different parts of the ortet, it will be regarded as topophysis.

Topophysis is a phenomenon that has had particular relevance to the propagation of coniferous trees (Olesen 1978; Stemmer *et al.* 1982; Wühlisch 1984; Ritchie *et al.* 1997), wherein these studies, have sought to understand the phenomenon in terms of maintaining, in commercial cultivation, the naturally occurring habit of a particular species. The present study examined the possible occurrence of topophysis in *K.pomifera* and its possible significance in attempting to achieve a more erect plant habit for its commercial production. It was considered that selection of cutting material exclusively from upright stems, could possibly alter the prostrate nature of the shrub, to a more upright habit. Although topophysis is rarely reported in native Australian species with prostrate habit, Campbell (1999) indicated that the selection of cutting material from prostrate stems on the ortet is important in maintaining a prostrate habit in the ramet in such species as *Acacia maxwellii*, *Dampiera rosmarinifolia*, *Myoporum parvifolium* and *Grevillea* cultivars. However, the occurrence and degree of topophysis is species-dependent (Molisch 1938), and its occurrence in ramets of *K. pomifera* is yet

to be evaluated. This study examined the possible occurrence of topophysis in the vegetative propagation of the prostrate species *Kunzea pomifera* to ascertain whether it could be exploited to derive plants of more upright habit in such propagation. The possible impact of topophysis on the breeding and domestication of the species is also discussed.

4.3 Materials and methods

Two accessions of *K.pomifera* (Ki7b and Ba) were selected to represent the upright plants (height to breadth ratio of greater than 60%, with an overall height of greater than 40cm) and two accessions (Lh3k and Lh2g) were selected to represent prostrate plants (having a height to breadth ratio less than 30%, with an overall height less than 20cm). The canopy of each ortet was divided into prostrate and upright primary stems based on their angles to the horizontal, with upright stems between 60 and 90°, and prostrate stems between 0 and 30° to the horizontal. These two cutting treatments (prostrate and upright) were taken from each of four accessions of *K.pomifera* (Ba, Ki7b, Lh3k and Lh2g). A total of 50 cuttings were taken for each of the eight ortet/ramet treatment combinations on 1st May 2001 and propagated using 1000ppm IBA under the conditions described in (Section 2.3). After ten weeks the cuttings were transferred into 100mm-diameter PVC pots and grown outside at the Burnley nursery with overhead irrigation. Plants were sufficiently spaced so that there was no contact between any of the ramets during growth. All ramets were measured for height and breadth, on 28th January 2002, and twenty ramets from each of the eight treatment combinations were re-potted into 140mm-diameter pots and grown under the conditions previously described. These plants were measured for height and breadth on 28th November 2002.

4.4 Results

After seven months growth no significant differences in height were found between the ramets produced from cuttings taken from upright and prostrate stems for the accessions Ki7b Lh2g and Lh3k. However, the cuttings taken from upright stems of Ba, produced ramets with a significantly greater height than those from prostrate stems after seven months growth (Figure 3). After 17 months growth no significant differences in height were found between the ramets produced from the upright and respective prostrate sources for any of the accessions (Figure 3).

For the prostrate accessions Lh2g and Lh3k, no significant differences were found in the ramets for either height or height-to-breadth ratio, between the cutting treatments during the first seven months of growth. After this time the cuttings taken from upright stems in the genotypes Ba and Ki7b, produced ramets with a significantly greater height-to-breadth ratio than those from prostrate stems (Figure 4).

After 17 months growth, no significant differences were found between cutting treatments for plant height or height-to-breadth ratio in the ramets of each of Lh 2g, Lh3k and Ki7b (Figure 4). However in Ba, a significantly ($p=0.001$) greater plant height-to-breadth ratio was found in the ramets that developed from cuttings taken from upright, compared with, prostrate stems (Figure 4).

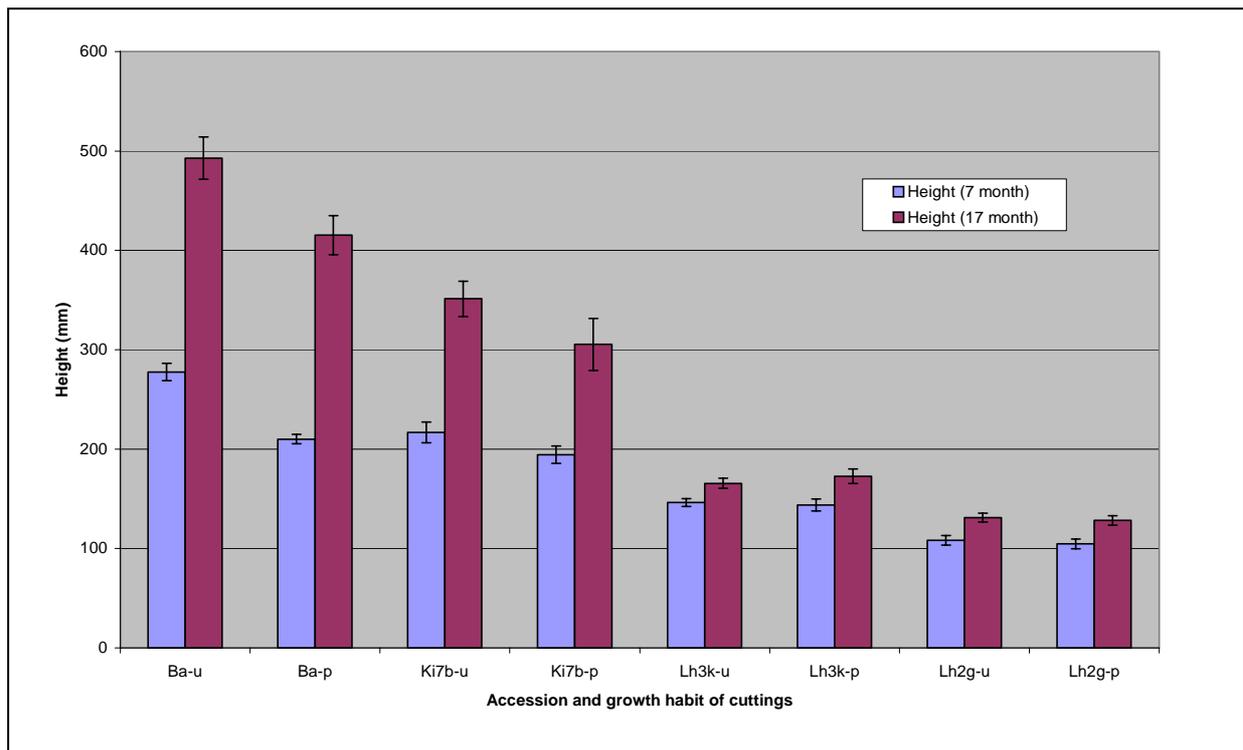


Figure 3: Mean plant height for each ortet/ramet of four accessions of *K.pomifera*, seven and 17 months after propagation. The first letters are the accession code for the growth habit of the cuttings on the ‘mother’ plant, namely u= upright and p=prostrate. Vertical bars represent standard errors.

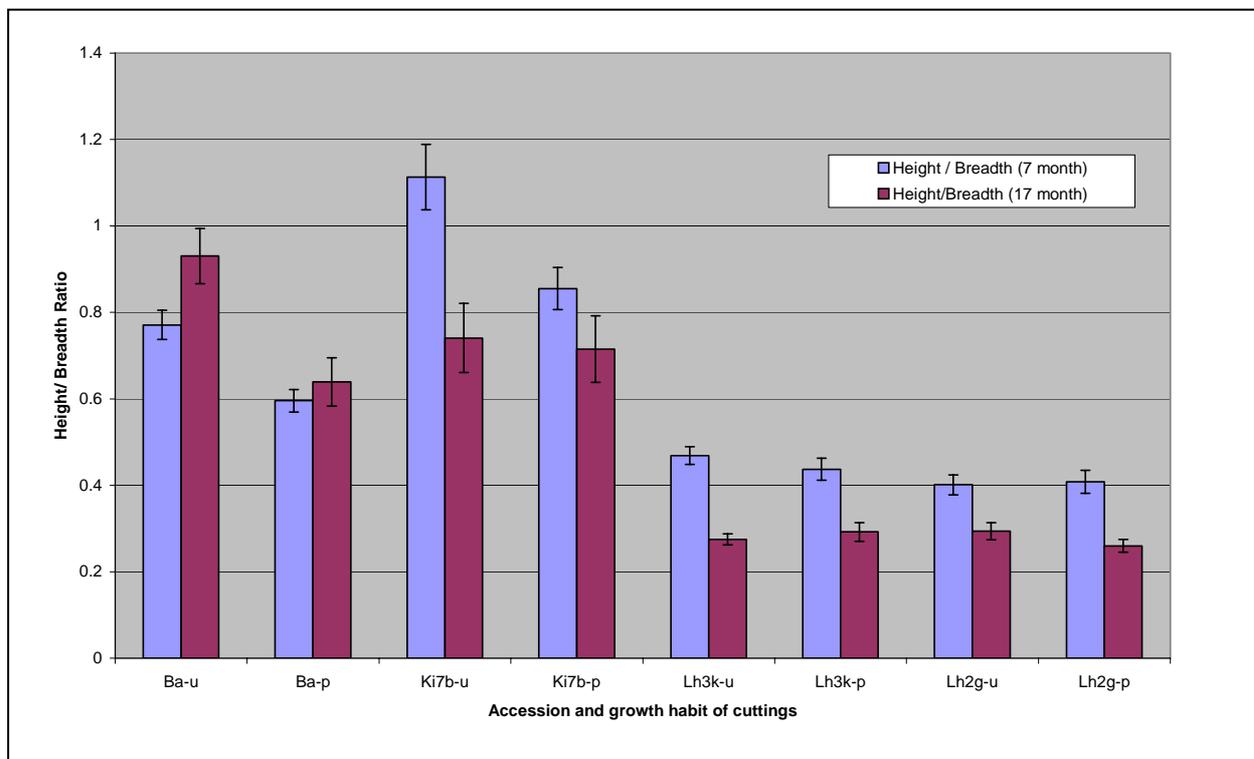


Figure 4: Mean height-to-breadth ratio for ortet/ramets derived from four accessions of *K.pomifera*, seven and 17 months after propagation (u= upright and p= prostrate). Vertical bars represent standard errors.

After 17 months growth, there were significant ($p < 0.001$) differences in the mean height of ramets between all accessions including both upright and prostrate sources of cuttings and a significantly

($p < 0.001$) greater height-to-breadth ratio for the upright (Ba and Ki7b) compared with the prostrate (Lh2g and Lh3k) accessions but no significant differences were found between accessions within each group for height-to-breadth ratio. All ramets from the prostrate ortets (Lh3k and Lh2g) were classed as prostrate (height-to-breadth ratio less than 30%, and height less than 20cm) at the conclusion of the study, indicating that these ramets maintained the habit of the ortet. The ramets of Ba from both upright and prostrate sources were classed as upright (height-to-breadth ratio greater than 60%, with on overall height greater than 40cm). For Ki7b the ortets of each treatment had a height-to-breadth ratio greater than 60% but the mean height for each treatment in this accession was 328mm, and thus not classed as upright. The Ki7b ortet had a height of 450mm after 24-months growth and it was assumed that the ramets would eventually attain the height of the ortet with an additional seven-months growth.

4.5 Discussion

After seventeen months growth three of four accessions of *K.pomifera* (Lh2g, Lh3k and Ki7b) showed no significant difference for height or height-to-breadth ratio between ramets developed from prostrate or upright classified cuttings. Therefore it can be concluded that topophysis was not manifest in the three wild accessions (Ki7b Lh2g and Lh3k)

What appeared to be transitory topophysis was observed in the accession Ki7b because after seven months growth the height-to-breadth ratio of the resultant plant was significantly greater in ramets taken from upright, than prostrate, stems, but after 17 months no distinction in this ratio could be made between ramets taken from the two different sources on the ortet. Power *et al.* (1988) found that the growth habit of ramets of *Sequoia sempervirens* was initially influenced by the position in the tree from which cuttings were taken, but reversion to upright growth occurred over time as the expression of the genotype appeared to assume increasing influence over plant habit. Ritchie *et al.* (1997) also found transitory topophysis in Douglas-fir (*Pseudotsuga menziesii*) ramets, in which plagiotropic growth rapidly reverted to orthotropic growth when they were transplanted from pots to open ground.

In the *K.pomifera* accession Ba, ramets with a significantly greater plant height and ratio were produced from cuttings taken from upright, than prostrate, stems on the ortet after seven months, but not after 17 months of growth. Despite this, the height-to-breadth ratio was significantly greater its ramets from upright than prostrate stem cuttings throughout the period of evaluation. It appears that physiological differences between upright and prostrate stems had an effect on the habit but not on the overall height of the resulting ramets. Considering that topophysis was found to be transitory in the accession Ki7b and that no differences in height were found between cutting types (upright vs. prostrate) for the selection Ba it is likely that topophysis is only transitory in the Ba ramets. Further investigation is required, however, to determine if topophysis, manifested as differences in the height-to-breadth ratio of the plant between ramets of erect versus prostrate origins of the accession Ba, is transitory, as observed for the accession Ki7b.

Cultivars of *K.pomifera* growing to approximately 2m high without artificial training would be desirable for commercial production in minimising establishment costs of a planting and would facilitate more efficient crop maintenance and harvest. Significant variation was found between accessions of *K.pomifera* in both plant height and the height-to-breadth ratio of the plant, but no accession in the present collection was found to grow over 500mm in height. The Ba accession is of particular interest as a possible source of more erect habit in the breeding of upright forms of *K.pomifera*. Ramets derived from it and grown in the present study grew consistently taller than any other accession from the populations of Kangaroo Island (Ki), Ki Ki (Kk) and Lake Hawdon. However to attain a height of 2.5m in the Ba accession formative pruning and training was required. The development of cultivars that could grow naturally to a height of 2m is, therefore, likely to require further collection and evaluation within the species for upright phenotypes. Another possible approach for the incorporation of erect plant habit in *K.pomifera* is hybridisation and selection using species having an upright habit such as *K.ambigua*.

4.6 Conclusion

In the present study topophysis was found to be absent from prostrate accessions of *K.pomifera* but was manifest as a transitory effect in those accessions classed as upright. Selection of cuttings from upright stems in *K.pomifera* did not produce ramets with a more upright habit. While most accessions of *K.pomifera* exhibit prostrate to semi-erect plant habit, one genotype, namely Ba, possesses an upright habit, but it is likely to require formative pruning and training to produce an appropriate plant habit for commercial production. Further collection within the species for upright phenotypes, and/or interspecific hybridisation with species having an upright habit, such as *K.ambigua*, is therefore recommended to exploit different sources of upright plant habit for its incorporation in commercial forms of *K.pomifera*.

5. Leaf morphology variation in *Kunzea pomifera*

5.1 Abstract

Leaf morphology variation in *Kunzea pomifera* was studied in three populations (located at Kangaroo Island, Lake Hawdon and Ki Ki, South Aust.) with the aim of using these traits as an index of genetic variation within and between populations for other plant characters. The traits measured were leaf area, length, width, elongation, and stomata, oil-gland and trichome densities and the ratio of adaxial to abaxial leaf-surface densities of stomata, oil-glands and trichomes. Significant variation was found within these populations for all seven leaf characters, with evidence for significant differences between populations for many of the traits. For instance, the inland Ki Ki population was of smaller leaf size than the two coastal populations of Kangaroo Island and Lake Hawdon. The presence, or absence, of trichomes was another differentiating trait wherein, the Ki Ki population typically possessed an abundance on both leaf surfaces, but in the other two populations trichomes were almost completely absent. Both the evolutionary and practical breeding implications of this variation are discussed

5.2 Introduction

Most leaf morphology and anatomy traits in plants appear to be reasonably heritable, as indicated by the reliance on these characters for plant classification of both inter and intra-specific levels (Kottek *et al.* 1990; Cook and Ladiges 1991; Fontenelle *et al.* 1994; Haron and Moore 1996; Webster *et al.* 1996; St-Laurent *et al.* 2000). Therefore variation in leaf morphology could serve as a useful index of general genetic variation in population studies. The development of this species as a significant horticultural crop will necessitate a breeding programme to produce forms that have the genetic potential for producing high yield and high fruit quality. The domestication of this species demands access to the full range of its natural variation, whose evaluation can assist the development of selection and breeding strategies to achieve this goal. The aim of the present study was to examine the level of variation in leaf characters within and between three natural populations of *K.pomifera* that can serve as an indicator of broad variation for quantitative characters important to developing the species as a crop

5.3 Materials and methods

Populations of *K.pomifera* were sampled in April and May 1999 at three main collection sites located at Lake Hawdon (Lh; 37°15'35"S 139°55'08"E), Kangaroo Island (Ki; 35°51'06"S 137°45'02"E) and Ki Ki (Kk; 35°38'11"S 139°49'22"E) in South Australia. A total of 123 individual plants were randomly sampled (Lh-41, Ki-42, Kk-40 individuals) and approximately 20 stem cuttings from each accession were collected for propagation in the Burnley Nursery (See Section 1). Once the cuttings had formed a root system, 3 replicates of each accession were planted in 200mm-diameter PVC pots on a capillary bed in a randomised block design (Plate 1). The plants were grown for approximately 10 months outdoors before the leaf morphological measurements for the present study were taken.

Ten fully-expanded mature leaves, each from actively-growing axillary shoots, were removed by hand from each plant. These were scanned, for morphological characters, (Hewlett Packard® ScanJet 5p scanner) into a computer, using Corel Photopaint. The file was converted to an 8 bit greyscale format and saved as a Windows bitmap. The Uthscsa Image Tool 2.00 program was used to measure leaf area, perimeter, length, width, elongation for the ten leaves of each accession from each of the three replicates.

Six of the ten scanned leaves, for each plant, were randomly selected and fixed to a microscope slide with UHU® superglue, three with abaxial surfaces upwards and three with them downwards. These slides were placed under a flat weight (5kg), to ensure an even impression in the adhesive. After an hour the slides were removed and the leaves were peeled away from the slide with forceps. The

superglue remained on the slide and bore an imprint of the leaf surface. These slides were viewed under a light microscope at x10 magnification. Two images per leaf imprint of 0.37 mm² were scanned into a file using Media Grabber 1.2 and saved as an 8-bit greyscale TIFF format. The images were printed through Microsoft Photo Editor 3.0 with a Hewlett Packard Laser Jet 4000N. Oil glands, stomata and trichomes were counted by eye from each printed image (0.37mm²) by marking and registering the tally on a Colby hand-tally counter.

5.4 Results

Leaf area (\log_{10}) and elongation ($-1/\text{elongation}$) were transformed, to remove deviation from normal distribution and unequal variance in the data for these characters. Leaf size (\log_{10} area, length and width) and shape ($-1/\text{elongation}$) exhibited significant variation ($p < 0.001$) both within and between the three populations of *K. pomifera*, using a one-way analysis of variance. There were significant differences ($p < 0.001$) between populations for mean stomata, oil-gland and trichome densities (Table 2) but the ratio of adaxial to abaxial densities for both stomata and oil-glands did not significantly discriminate the populations (data not presented).

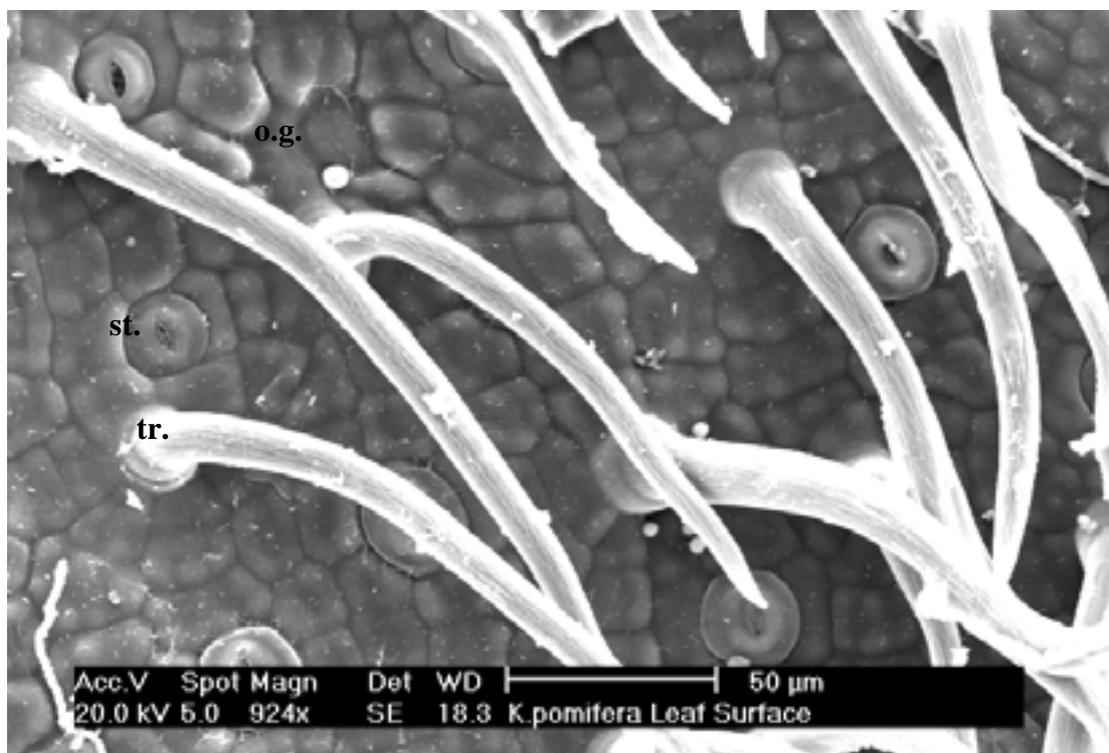


Plate 4: Scanning electron micrograph of the leaf surface of *Kunzea pomifera* showing: (o.g.) oil-glands, (st.) stomata and (tr.) trichomes.

Mean leaf area was significantly ($p < 0.001$) lower in the Kk, than both Lh and Ki populations. Mean leaf length in the Lh population was significantly ($p < 0.001$) greater than that of Ki and Kk, but mean leaf width was significantly ($p < 0.001$) lower in the Kk population than either Lh or Ki, suggesting that the shape of the former is markedly different from that of the latter two. In one-way analysis of variance, all populations could be separated on the basis of leaf elongation. Individuals of the Ki population had the least elongated leaves with a mean length 1.2 times the width, with Lh 1.4 times, and Kk, the most elongated, with leaf length 1.5 times that of width.

Leaf character	Mean character value of populations		
	Kangaroo Is. (Ki)	Lake Hawdon (Lh)	Ki Ki (Kk)
Area (mm ²)	31.4, ±0.91 , a	32.13, ±0.81 , a	20.99, ±0.63 , b
Length (mm)	7.11, ±0.12 , a	7.83, ±0.14 , b	6.68, ±0.13 , a
Width (mm)	5.95, ±0.09 , a	5.68, ±0.08 , a	4.45, ±0.08 , b
Elongation (ratio)	1.21, ±0.02 , a	1.40, ±0.03 , b	1.53, ±0.37 , c
Mean stomata density	170.51, ±2.92 , a	158.32, ±2.72 , b	182.37, ±2.71 , c
Mean oil-gland density	20.44, ±0.58 , a	16.85, ±0.45 , b	17.45, ±0.4 , b
Mean trichome density	0.11, ±0.04 , a	0.04, ±0.02 , a	52.3, ±5.31 , b
Principal component 1	-0.09, ±0.18 , a	-1.13, ±0.16 , a	2.12, ±0.18 , b
Principal component 2	1.05, ±0.13 , a	-0.62, ±0.19 , b	-0.47, ±0.16 , b

Values in rows followed by a common letter are not significantly different ($P < 0.05$)

Table 2: Mean values ± standard errors for seven morphological characters and the first two principal components generated from them for three populations (Ki, Lh and Kk) of *K.pomifera*

Stomata and oil glands were present on both leaf surfaces in all populations. The mean density of stomata of the two leaf surfaces was significantly ($p < 0.001$) different between each of the three populations and varied from 158 in the Lh, to 182 stomata per mm² in the Kk, population. The mean oil-gland density of the two leaf surfaces was significantly ($p < 0.001$) greater for the Ki, than the Lh and Kk populations, which were not significantly different. Leaf trichomes, on both surfaces, is a character that can be described in terms of (a) the frequency of individual plants with or without trichomes and (b) the mean density of trichomes in individuals on which they occurred. Trichomes were present on at least one of the leaf surfaces in 90.0% (36/40) of Kk, 26.2% (11/42) of Ki and 14.6% (6/41) of Lh individuals. The trichomes on the Lake Hawdon accessions were present on the adaxial surface only. On the Kangaroo Island accessions, trichomes were present, primarily on the adaxial surface (9/11), but with one accession they were present on the abaxial surface only, and with another they were present on both leaf surfaces.

Accessions in the Lh and Ki populations possessed leaves with trichomes of mean densities less than 1/mm² for both surfaces combined. Three accessions in the Kk population also exhibited trichome densities less than 1/mm², two of which possessed them on the adaxial surface only. The mean trichome density (on both leaf surfaces) for the Kk population was 52.3 per mm² and the value for those individuals with abundant (greater than 1/mm²) trichomes was 63.35 per mm². Among accessions with abundant trichomes the adaxial surface had an average of 2.7 times that on the abaxial surface.

Stomata and oil-glands were present in higher densities on the adaxial, than the abaxial, leaf surface for individuals in the three populations. The ratios for stomata and oil-glands (adaxial to abaxial), although significantly ($p < 0.05$) different, were of similar levels, with means of 1.67 and 1.75 respectively. The trichome density ratio (adaxial to abaxial) was substantially higher (2.66) than stomata and oil-gland ratios for those individuals with abundant (>1/mm²) trichomes on both leaf surfaces. For accessions with sparse trichomes (<1/mm²), 90% (18/20) had a greater density on the adaxial than the abaxial surface. It was not possible to test the difference between populations for trichome density ratio, because all but one accession in the Lh and Ki populations had values of zero. No significant differences between populations were found for stomata or oil-gland density ratios (adaxial to abaxial).

The hierarchical partitioning of variation for each trait, which was analysed by a nested ANOVA, revealed that percentage variation was lowest between individual replicates (4.4-14%) for all characters, indicating that little environmental variation existed between replicate blocks in the experiment. For leaf area, leaf width and mean trichome density, a greater proportion of phenotypic variation occurred between, rather than within, populations (Table 3). A similar level of variation for

leaf elongation occurred within, as between, populations, but for all other characters, most of the variation was that within populations. For principal component one (PC1) a high proportion (73.1%) of variation was found between populations but for principal component two (PC2) a reasonably high proportion (55.1%) was found within populations (Table 3).

Leaf morphological character	Percentage of variation for characters . . .	
	Within populations	Between populations
Leaf area	35.9%	64.1%
Leaf length	68.5%	31.5%
Leaf width	32.9%	67.2%
Leaf elongation	51.9%	48.1%
Mean stomata density	70.3%	29.7%
Mean oil-gland density	73.4%	26.6%
Mean trichome density	29.2%	70.8%
PC1	22.1%	77.9%
PC2	65.9%	34.1%

Table 3: Partitioning of variation within and between three populations of *K.pomifera* (Ki, Kk and Lh) for seven leaf morphological characters accounted for by principal components (PC1 and PC2) generated from these characters.

The graphical patterns of variation across the seven morphological characters were revealed by plotting the first two components of PCA, wherein the first principal component, accounting for 48.1% of the total variation substantially comprising variation in leaf size (area, width and length) contrasting with mean stomata and trichome density. The Kk population differed significantly ($p < 0.05$) from both the other two populations in each of the previously mentioned characters except leaf length, where it differed significantly ($p < 0.05$) from Lh only (Table 2). The first principal component clearly discriminated Kk from both the Ki and Lh populations, which were not significantly different from each other, as indicated in a plot of PC1 and PC2 (Figure 5) and in its one-way ANOVA. The apparent divergence between the Kk population from each of Ki and Lh in PC1 reflects the findings from the nested ANOVA for PC1, wherein 73.1% of the total variation in this component was that between populations.

The second principal component, accounted for of 23.5% of the total variation in the seven leaf morphological characters, accounted for the contrast in leaf length and elongation with mean leaf oil-gland density. In leaf elongation and mean oil-gland density the Ki population was significantly ($P < 0.001$) different from the other two populations and in leaf length it was significantly ($P < 0.001$) different only from Lh. While the discrimination was not as clear as that of the Kk population from both Ki and Lh with PC1, that for PC2 significantly ($p < 0.001$) distinguished the Ki population from Lh and Kk (Table 2).

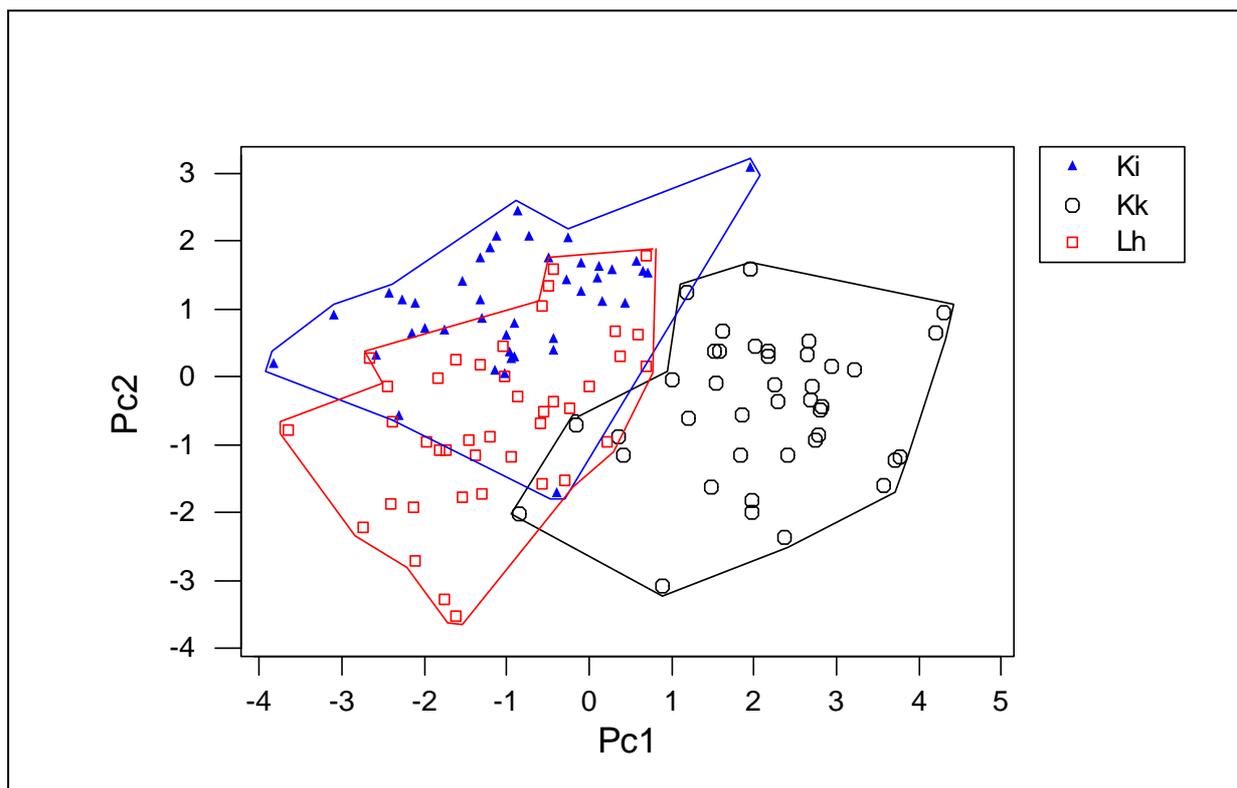


Figure 5: Principal component analysis using seven leaf morphological characters in three populations of *K.pomifera*. PC1 and PC2 are indices of variation in all characters and account for 71.6% of the total variation.

5.5 Discussion

5.5.1 Character variation within and between populations

5.5.1.1 Variation within populations

While significant differences were found between at least two populations for all leaf characters measured in the present study, most of the variation was found within populations for mean leaf length, stomata and oil-gland densities. It is likely that similar magnitudes of variation could also exist within natural populations of *K.pomifera* for phenotypic characters important for the breeding and domestication of the species. Similarly, as in the present study, in *Eucalyptus camaldulensis* considerable variation was found within populations for leaf characters such as leaf length, width, thickness and mean stomata and oil gland densities (James and Bell 1995).

5.5.1.2 Variation between populations

While the Kk population is closer geographically to both Ki and Lh (approximately 185km from each), than the latter populations are to each other (approximately 250km apart), it was found to be distinct from both populations in the principal component analysis. The clear divergence of the Kk population from both the Lh and Ki populations may be an indication that it has adapted to different selective forces, associated with climatic and edaphic factors, than those of the Lh and Ki populations. These findings are also an indication that divergence between populations may exist for other characters that are of interest to the breeding and domestication of *K.pomifera*. The level of divergence occurring between populations was, however, found to vary according to a particular leaf character. For instance, mean leaf area, width and trichome density in Kk were significantly different from both Ki and Lh, but in mean oil gland density Ki was significantly greater than both Kk and Lh. These results were supported also by the partitioning of variation, as indicated by the nested ANOVA, wherein it was found that the level of variation was greater between, than within, populations for some characters, such as mean leaf area and width and trichome density. However the reverse was found for mean leaf length and stomatal and oil-gland density. Whiffin and Bouchier (1992) found that three

clusters of like individuals within a population of *Eucalyptus radiata*, based on leaf and fruit morphology were not as distinct from one another, or correlated with groups formed based on volatile oil variation. Based on morphological characters such as flower and fruit dimensions (Marginson and Ladiges (1988) demonstrated that two eucalypt populations (Barossa Range and Fleurieu Peninsula, South Australia) were likely to be *Eucalyptus baxteri*, but for leaf volatile oil data these same populations were classified as *E.arenacea* (Whiffin and Ladiges 1992). These results indicate that the level of divergence between species and/or populations is dependant upon the particular character being measured and hence the value of techniques such as principal component analysis, which use variation in a number of characters, to evaluate population differentiation.

5.5.1.3 Leaf area and shape

In the present study the mean leaf area was significantly greater in the cooler coastal populations Ki and Lh than the warmer inland population Kk. Similar phenotypic patterns, of leaf size being larger in cooler, compared to warmer, conditions was reported for *E.leucoxylon* (Boland 1978), but in *Eucalyptus camaldulensis*, James and Bell (1995) reported that leaf size tended to increase from cool coastal, to warm inland, populations. Population differentiation has been found in *Eucalyptus globulus* ssp. *globulus*, wherein mean leaf area of individuals in a Tasmanian population of was almost twice that of individuals occurring in a Wilson's Promontory population (James and Bell 2001). Shorter leaves in *E.globulus* were found on trees in regions with shallow soils or low precipitation (Kirkpatrick 1975) cited in (James and Bell 2001).

Based on water use efficiency models for plant leaves, a reduction in leaf size leads to concomitant reduction in the boundary-layer resistance to the diffusion of water out of, and CO₂ into, the leaf, and leads to increased convective heat transfer leading to lower leaf temperatures than larger leaves under identical environmental conditions (Parkhurst and Loucks 1972; Gates 1980). Parkhurst and Loucks (1972) proposed that water use efficiency (CO₂ assimilated / H₂O transpired) would be maximised in smaller leaves in climates with high air temperatures and light intensity, and would thus confer an adaptive advantage in such environments. Leaf width among eleven species of *Chuquiraga*, which was positively correlated with leaf area, was negatively associated with both mean summer and winter temperatures, with temperature variation accounting for approximately 70% of the variation in leaf width (Ezcurra *et al.* 1997). In *Quercus petraea* Bruschi *et al.* (2003) found smaller leaves with higher stomata density in those positioned in the outer part of the crown compared with those from the inner crown, and proposed that environmental heterogeneity between these two microenvironments such as greater cold, heat, sun radiation and water stress, were contributory factors to the observed within-tree variation in these leaf characters. Considering that in *K.pomifera*, the Kk population occurred in an environment with a more extreme temperature range with a greater number of clear days than the Ki and Lh populations, it appears that a reduced leaf surface area found in individuals in the Kk population may confer adaptation to such an environment.

In *K.pomifera* mean leaf length was significantly greater in the Lh, than the Ki and Kk populations, with the latter populations being somewhat similar in mean leaf length. For mean leaf width, however, no significant difference was found between the Lh and Ki populations, but both these populations had significantly wider leaves than the Kk population. While all three populations differ in leaf shape, as seen in the differences exhibited between them for elongation (Table 2), the possible adaptive significance of such variation is unclear.

5.5.1.4 Stomatal density

The greater density of stomata found on the adaxial, compared with the abaxial, leaf surface of *K.pomifera* was consistent for the three natural populations, with a mean across populations of 1.6 times more stomata on the on the upper, than the lower, surface. For stomatal density ratio these findings are contrary to that found in *E.alba*, *E.urophylla* (Pryor *et al.* 1995), *Populus trichocarpa*, *P.deltoides* and their hybrid (Drew and Chapman 1992) wherein greater numbers of stomata were found on the abaxial, compared with the adaxial, surface of the leaf. Ridge *et al.* (1984) found variation in the ratio of stomata density between leaf surfaces amongst *Eucalyptus* spp. wherein hypostomatous leaves occurred in *E.calophylla*, *E.resinifera*, *E.saligna* and *E.marginata* whereas

amphistomatous leaves were found in *E.globulus*, *E.maculata* and *E.wandoo*. In *E.camaldulensis* Pereira and Kozlowski (1976) found a similar density of stomata on both leaf surfaces (amphistomatous leaves), but James and Bell (1995) found that a greater density of stomata on the abaxial than the adaxial leaf surface for the same species. Hypostomatous leaves were found in *E.globulus* wherein no stomata occurred on the adaxial leaf surface (Pereira and Kozlowski 1976), but (Ridge *et al.* 1984) found they were amphistomatous in this species and likewise in *E.globulus* ssp. *globulus* (James and Bell 2001).

The occurrence of stomata on both leaf surfaces (amphistomatous leaves) increases the boundary layer conductance and consequently total leaf conductance compared with those with stomata on the abaxial surface only (hypostomatous leaves) (Mott *et al.* 1982; Mott and Michaelson 1991). Parkhurst (1978) found that the amphistomatous leaves were positively associated with leaf thickness, which caused a reduction in the intercellular diffusion distance for CO₂ relative to hypostomatous leaves. The level of amphistomy has been positively associated with irradiance in *Ambrosia cordifolia* (Mott and Michaelson 1991) and *Eucalyptus globulus* ssp. *globulus* (James and Knox 1993). Furthermore Smith *et al.* (1998) found that among five Western Australian plant communities, there was a positive relationship between the period total daily sunlight and mean annual rainfall at a particular location and the proportion of species having amphistomatous leaves. In *K.pomifera*, however, there was no significant difference in stomatal density ratio between three natural populations despite differences in the duration of sunlight exposure and mean monthly rainfall between the environments of these populations. The adaptive significance of a significantly greater number of stomata on the adaxial than the abaxial surface, as found in *K.pomifera*, is yet to be determined.

In the present study it was found that in *K.pomifera* leaf area was negatively correlated with stomatal density, wherein larger leaves had a lower density of stomata. Mean stomatal density over both leaf surfaces was highest in the Kk population, which experiences higher mean maximum temperature and greater number of days over 30°C than the two coastal *K.pomifera* populations. In *E.camaldulensis* (James and Bell 1995) leaf stomatal density tended to be associated positively with aridity. While further evaluation of the adaptive significance of the greater mean stomatal density in the Kk population, compared with Ki and Lh populations is required, the higher density found in the Kk population could possibly be involved with an adaptation involving greater regulation of water loss and/or CO₂ uptake in its individuals.

5.5.1.4 Oil-gland density

Oil-glands were found, like that of stomata, to have a greater density on the adaxial surface than on the abaxial surface in the three populations of *K.pomifera*. No significant differences were found between the three populations for oil-gland ratios (*i.e.* adaxial: abaxial leaf surfaces), and across the three populations there was a mean of 1.8 times more oil-glands on the adaxial than the abaxial, surface. *K.pomifera* is similar to *E.camaldulensis* in that, on the adaxial leaf surface, oil-glands are of greater size and higher density than those on the abaxial surface and compared with stomata, these oil-glands are substantially larger than those on the abaxial surface (James and Bell 1995).

The mean density of oil-glands averaged over both leaf surfaces was significantly greater on individual accessions in the Ki population than the Kk or Lh populations. A significantly greater oil-gland density was found on the leaves of individuals occurring in a Tasmanian population of *Eucalyptus globulus* ssp. *globulus* than in a population located on Wilson's Promontory in Victoria (James and Bell 2001). It was proposed that the presence of oil glands in *Eucalyptus camaldulensis* may be a general adaptive character for herbivore defence (James and Bell 1995). It is unlikely that variation in oil-gland density and/or oil-gland size would be associated with resistance to the primary leaf herbivore of *K.pomifera*, the looping caterpillar larvae of some *Macalla* spp. (Lepidoptera) that form characteristic webs within the shrub canopy (Nielsen and Common 1991; Wrigley and Fagg 1993). Vourc'h *et al.* (2001) found, however, that increased monoterpene concentration in the leaf oils of *Thuja plicata*, provided a greater defence against grazing by the black-tailed deer, which is a significant leaf herbivore. Further investigation of the relationship between chemical composition of

leaf oils and herbivore defence could be undertaken to determine if such positive associations exist with *K.pomifera*.

5.5.1.6 Trichome density

In the accessions with abundant trichomes (a minimum 16.2 in Kk3q to 117.4 Kk2f per mm²) the density was 2.7 times greater on the adaxial, than the abaxial, leaf surface. Given the complete divergence of the Kk population from each of the Ki and Lh populations in mean leaf trichome density over all individuals, it is possible that there are different selection pressures operating on individuals of the Kk population, than in the Ki and Lh populations insofar as trichomes may be of significant adaptive value. The mean maximum daily temperature and the number of days exceeding 30°C (per calendar month) was greater, and mean daily minimum and the number of days below 2°C was lower, in the area of with the Kk population than that for the Ki and Lh populations. It is possible that these climatic factors have contributed to the differentiation between these populations. Johnson (1980) found unicellular trichomes were consistently present on young leaves of a wide range of *Leptospermum* spp. but they persisted in the mature leaf for only some species, such as *L.lanigerum*, *L.longifolium* and *L.recurvum*. Variation in the persistence of leaf trichomes in mature leaves was found between individuals of *L.lanigerum*. This author proposed that the persistence of leaf trichomes might have contributed to protection of the leaf from solar radiation, leading to a reduction in transpiration compared to glabrous leaves, but conceded that further investigation was required to substantiate this hypothesis.

In a review of the adaptive significance of plant trichomes Levin (1973) proposed that intraspecific variation in trichome density was often associated with ecogeographical variation. Variation in the mean density of leaf trichomes in *Origanum vulgare*, for instance, appeared to be positively associated with temperature, mean hours of sunshine per annum and negatively associated with the level of summer precipitation (Kokkini *et al.* 1994; Bosabalidis and Kokkini 1997). In *Glycine max* Woolley (1964) demonstrated that the presence of leaf trichomes significantly reduced water loss from detached leaves by 21% compared with leaves where the trichomes were artificially removed with a razor. Lenssen *et al.* (2001) also found that glandular and simple leaf trichomes retarded water loss from cut leaves from the forage crop *Medicago sativa* (alfalfa or lucerne) and the mean density of such trichomes was negatively correlated with the drying rate of leaves in the species. These authors also indicated that, in alfalfa, simple leaf trichomes had a greater effect over water loss from the leaves than glandular leaf trichomes. In the desert plant species *Encelia farinosa*, Ehleringer *et al.* (1976) found that leaf pubescence reduced leaf absorptance to 29% of the available photosynthetically-active solar radiation (400-700nm). Leaf temperature and the rate of photosynthesis were reduced in *E.farinosa*, due to reflection of solar radiation, caused by leaf trichomes, than in the glabrous-leaved species *E.californica*, wherein 84% of photosynthetically-active radiation was absorbed by its leaves (Ehleringer *et al.* 1976; Ehleringer and Mooney 1978; Ehleringer and Björkman 1978a). Ehleringer and Björkman (1978b) proposed that in spring and summer leaf pubescence in *E.farinosa* sufficiently reduced leaf temperature, relative to the ambient temperature, to allow photosynthesis to occur at near optimal temperatures for the species. These authors also found that leaf trichomes acted as a selective radiation reflector, wherein reflection of near-infrared radiation (which does not contribute to photosynthesis) increases more rapidly than the visible radiation spectrum, (in which photosynthetically-active radiation occurs) as leaf trichome density increased. These findings of these authors highlighted the adaptive importance of leaf trichomes to high temperatures in natural populations of *E.farinosa*. In *Olea europaea*, however Grammatikopoulos *et al.* (1994) found that the artificial removal of leaf trichomes had no significant effect on leaf temperature or CO₂ uptake under exposure to white-light radiation (400-700nm). However when the light spectrum was widened to include UV-B radiation (270-320nm) CO₂ uptake was reduced and temperature was slightly increased in leaves without, than those with, trichomes. The slight increase in temperature in leaves without trichomes in this treatment was attributed to increased stomatal resistance to diffusion of water vapour. These authors proposed that leaf trichomes in this species were predominantly a protective adaptation against exposure to UV-B radiation and not for reducing the heat load on its leaves. Given that leaf trichomes, in *K.pomifera*, are abundant only on individuals in the inland population (Kk), their presence in this species may have adaptive significance for its survival in a climate with a more

extreme temperature range and a significantly higher number of clear days per month, than the two coastal populations.

In many plant species the presence and density of leaf trichomes have been positively associated with resistance to insect feeding and/or ovipositioning (Levin 1973) such as the leaf herbivore *Pieris rapae* in *Brassica rapa* (Ågren and Schemske 1993) and whitefly oviposition in *Medicago lupulina* (Goertzen and Small 1993). In *Verbascum thapsus*, grasshoppers (Acrididae) preferentially consumed mature glabrous leaves, over young pubescent leaves (Woodman and Fernandes 1991), but when these authors manually removed leaf trichomes from immature leaves with a razor, no preference, by these herbivores was observed, towards any leaf type. Manually-shaven immature leaves were also found to have significantly higher evapotranspirative water-loss rates than the control leaves in which trichomes were present on the leaf surface. These findings indicate the likelihood that leaf trichomes, present on the surface of immature leaves, had a dual function in *Verbascum thapsus*, by limiting leaf herbivory and evapotranspiration. While the presence of leaf trichomes has been found in a number of plant species, to reduce damage from herbivores and ovipositioning by certain insect species, further study of their possible role in plant protection in *K. pomifera* appears to be necessary, particularly for the deterrence of the larvae from *Macalla* spp. that have been found to consume its leaves under cultivation (Page 1999).

The mean density of leaf trichomes was found to exhibit Mendelian inheritance in *Trifolium incarnatum* (Knight 1969), *Glycine max* (Bernard and Singh 1969), *Triticum turgidum* (Leisle 1974), *Helianthus* (Harada and Miller 1982), *Gossypium* (Lee 1985) and *Brassica carinata* (Velasco *et al.* 2001). Simple inheritance indicates that selection for trichome density may be successful, in these species, for breeding pubescent and or glabrous cultivars. Quantitative inheritance for leaf trichome density was found in *Brassica rapa*, (Ågren and Schemske 1992) and *Phaseolus vulgaris* (Park *et al.* 1994). The inheritance of the expression of leaf glandular trichomes in *Medicago sativa* was found to be complex and polygenic but incorporation of this character from undated germless in commercial cultivars, for perceived pest-resistance benefits, was considered feasible, given that it exhibited a narrow sense heritability of 0.55 ± 0.17 (Kitch *et al.* 1985). Ågren and Schemske (1992) demonstrated that selection for either high or low trichome densities on the leaf margin of the self-incompatible species *Brassica rapa*, resulted in significant divergence between lines, with a realized heritability of 0.38 for this character. Given that phenotypes possessing leaf trichomes exhibited greater resistance, than glabrous-leaved phenotypes, to the Lepidopteran herbivore *Pieris rapae*, Ågren and Schemske (1993) proposed that the development of resistant cultivars was possible through recurrent selection for high trichome density in agricultural varieties.

5.6 Conclusion

All seven leaf morphological characters measured in the present study, exhibited significant variation between three natural populations of *Kunzea pomifera*, indicating the likelihood that, for these characters, natural selection has produced substantial genetic differentiation between these populations. The Kk population exhibited clear differentiation from the two coastal populations, Ki and Lh, as revealed from principal component, discriminant function and cluster analysis of variation in these leaf characters. The Kk population, comprised phenotypes that typically had smaller, more elongate leaves with higher leaf stomatal density and a much greater abundance of leaf trichomes on both leaf surfaces than either Ki or Lh populations. The Kk population was found to grow in a climate with a greater number of clear days, lower humidity and more extreme temperatures than the latter two populations. Of the three populations the two coastal populations are the most geographically distant from each other, but they are the most similar in terms of leaf area, width and trichome density and climatic variables of number of days over 30°C, and under 2°C per month and number of clear days per month. This indicates that the similarities in morphological characters are possible adaptations to similar prevailing environmental conditions. The variation observed in leaf morphological characters, revealed in the present study, indicates the likelihood for variation also within and between these populations for other plant characters, and of particular interest are those that are significant for the successful domestication and further breeding of the species.

6. Genetic polymorphism in *K.pomifera*

6.1 Abstract

The genetic relationship between 29 individuals from three natural populations of *Kunzea pomifera*, and 9 cultivated accessions of the species was investigated using the random amplified polymorphic DNA technique (RAPD). Twenty-one RAPD primers were screened, two of which generated a total of 36 reproducible polymorphic amplification products. Analysis of molecular variance revealed that RAPD variation was greatest between individual within populations, with little population differentiation evident between the three natural populations evaluated. The relationships between accessions of *K.pomifera*, for the amplified region, were assessed using non-metric multidimensional scaling and UPGMA cluster analysis. The resulting two- and three-dimensional plots and dendrogram revealed that morphologically distinct individuals were differentiated across 36 polymorphic RAPD bands, with little evidence for discrete genetic differences between populations. These results indicate that it is likely that genetic introgression has occurred between these three geographically separated populations, possibly through long-distance dispersal of seeds by birds and people. Significant degrees of genetic variation found between genotypes of *K.pomifera* indicate the likelihood for substantial levels of genetic variation in the species, which should be exploited in its domestication and improvement for commercial production.

6.2 Introduction

Natural genetic variation in a plant species, most of which is believed to have arisen as a consequence of its evolutionary adaptation, provides the main genetic resource for further evolution and plant domestication and breeding (Hancock 1998). Therefore an understanding of genetic variation in natural populations of *Kunzea pomifera* is of primary concern for both its *in situ*, and perhaps *ex situ*, conservation and breeding as a commercial crop. Measuring genetic variation at the molecular level provides information on the nature and extent of genetic diversity of a plant species comparatively free of environmental influences as encountered when using phenotypic variation of plant characters for assessing such diversity. The molecular method based on the polymerase chain reaction with the use of arbitrary primers with a known sequence (RAPD), is applicable for use in species where no previous genetic study has been undertaken (Welsh and McClelland 1990; Williams *et al.* 1990), as is the case with *K.pomifera*. This aim of the present study was to measure the level of genetic variation within and between 29 wild accessions of *K.pomifera* from three morphologically-divergent natural populations (Kangaroo Island (Ki), Ki Ki (Kk) and Lake Hawdon (Lh), as detailed in Section 5) and 9 cultivated accessions of the species using the RAPDs (randomly amplified polymorphic DNA) technique.

6.3 Materials and methods

Ten plants were randomly sampled from each of three natural populations of *K.pomifera* from Kangaroo Island, Ki Ki, and Lake Hawdon in South Australia. All samples were propagated by cuttings and three replicates were maintained in a single growing environment at Burnley College. Three ramets of nine accessions (Ki-2b,-2e,-2h, Kk-1d,-1L,-3b, Lh-1i,-1b and -1e) were initially screened using RAPDs to optimise the PCR conditions and determine the reliability of producing consistent DNA banding patterns for genetically-identical individuals.

Fresh leaf material (100mg) was ground with liquid nitrogen using a mortar and pestle until it became a fine powder, and DNA was extracted using Qiagen Dneasy® method. RAPD amplification reagents were mixed in a laminar-flow cabinet (Clyde Apac HWS-180) in thin-walled PCR tubes (Life Technologies, AUS-3211-00), which contained 2.0µL 10X reaction buffer (Fisher Biotech) 1.5mM MgCl₂, 100µM each of dATP, dCTP, dGTP and dTTP), 0.25mM oligonucleotide primer, 10ng DNA template, 2 units *Taq* DNA polymerase and autoclaved Milli-Q water to a final volume of 20µL. Amplification was carried out in a Biometra PCR thermocycler and subjected to a series of temperatures as follows: initial denaturation at 94°C for 4 minutes, followed by 45 cycles of 94°C for

1 min, 36°C for 2 min, 72°C for 2 min, and a final extension step at 72°C for 10 mins. The amplified solution was stored at 4°C before amplified products were separated by electrophoresis.

The amplification products of 21 decamer oligodeoxyribonucleotide primers (Operon Technologies, Alameda, CA, USA: OPB-series and OPM-07) of arbitrary sequence were evaluated using the DNA extracts of two randomly-selected accessions from each of the three natural populations of *K.pomifera* (Ki3a, Ki1e, Lh1g, Lh2u, Kk3p, Kk3j). Three primers (Opb14, Opb-19 and Opb-20) produced clear polymorphic bands, but only Opb-19 and Opb-20 were reproducible. These two primers were used to evaluate the level of genetic diversity within and between 29 individuals from the three wild populations of *Kunzea pomifera*.

6.4 Results

Of the 24 arbitrary primers screened, two of them, Opb-19 and Opb-20 generated consistent reproducible polymorphic banding. A total of 31 polymorphic bands were scored which discriminated the three populations. From the squared Euclidean distance matrix an AMOVA (Table 4) revealed that most (76.5%) of the variation detected was attributed to that between individual populations. When individual comparisons were made between two individual populations the lowest molecular variation was found between Kk and Ki (16.25%) while that between populations Kk and Lh (28.6%) and between Ki and Lh (27.4%) were higher and somewhat similar.

Source of Variation	d.f.	Sum of squares	Variance components	Percentage of variation	P-value
Between populations	2	244.864	1.27683 Va	23.50	0.000
Within populations	277	1151.400	4.15668 Vb	76.50	0.000

Table 4: Analysis of molecular variance (AMOVA) for 29 individuals in 3 populations (Ki, Kk and Lh) of *K.pomifera* using 34 RAPD fragments. The data shows the degrees of freedom (d.f.), the sum of squared deviation, the variance component estimate, the percentage of total variance contributed by each component and the probability of obtaining a more extreme component estimate by chance (P-value).

Cluster analysis of the RAPD data using UPGMA gave a dendrogram (Figure 6) wherein the separation of individual accessions did not conform to the differentiation found between the three natural populations of *K.pomifera* for leaf morphology (Section 5). Accessions from the Lh population tended to cluster together, but accessions from the populations Ki or Kk did not cluster with their respective populations. All but two accessions, Kmt1 and Kmt2, which occurred naturally less than 5 metres apart, could be discriminated from each other. The cultivated accession Ba clustered with the Ldes‘S’ accession, despite considerable morphological differences in leaf shape and plant habit between them.

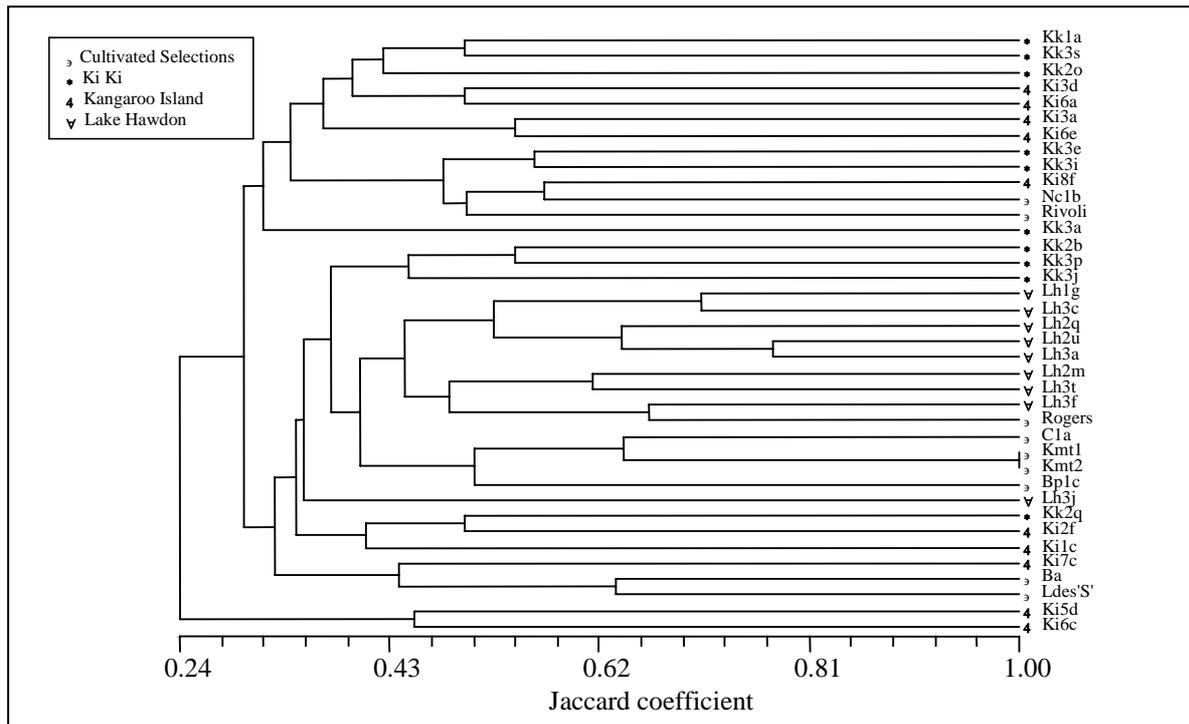


Figure 6: Dendrogram showing the estimated genetic similarity between 38 *K.pomifera* accessions using 2 decamer primers, the Jaccard coefficient, and UPGMA clustering

Non-metric multidimensional scaling gave convergence after 76 iterations with a Stress 1 value of 0.270 in two-dimensions (Figure 7) and after 82 iterations with a Stress 1 value of 0.183 in three dimensions. In both two- and three-dimensional representations no distinct differentiation was found between the three natural populations of *K.pomifera*, but all accessions from the Lh population appeared to group together.

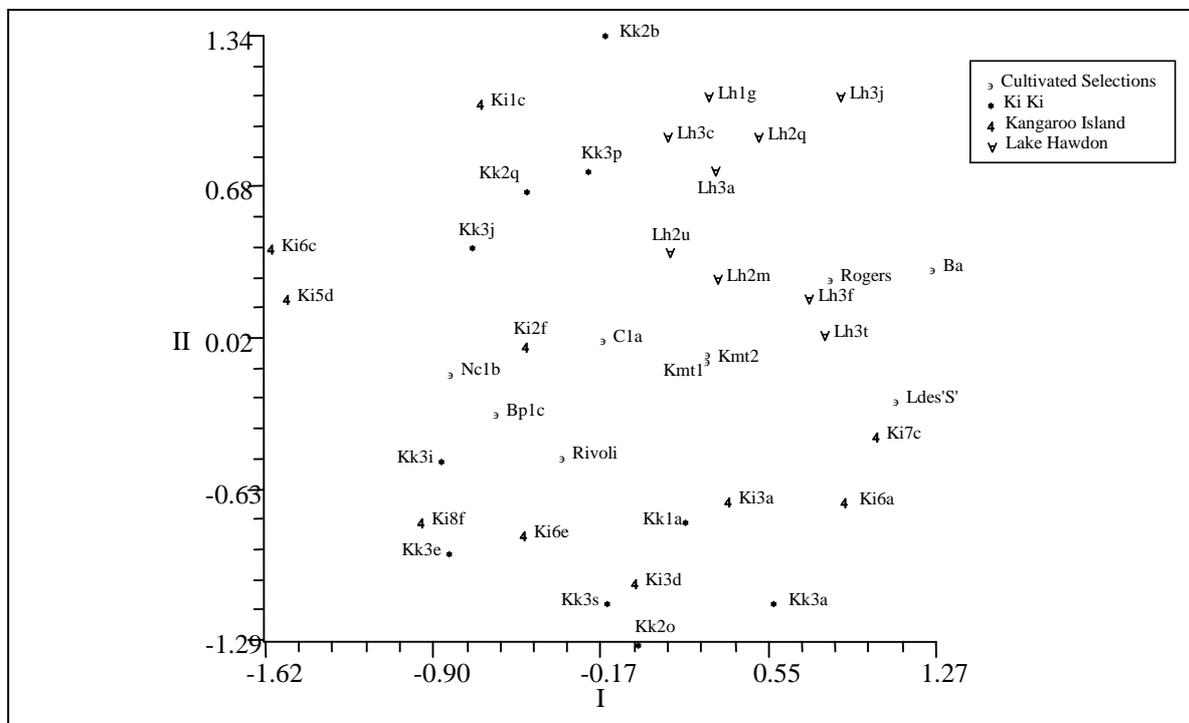


Figure 7: NMDS of 38 *K.pomifera* accessions, in two dimensions, generated from a Jaccard similarity matrix using 2 decamer primers

6.5 Discussion

6.5.1 Genetic diversity within and between populations of *K.pomifera*

The results of the cluster analysis and NMDS indicated a lack of divergence between the three populations of *K.pomifera*, with some individuals between populations more closely related, than some of those within, populations. There was essentially a uniform distribution of RAPD variation between individuals regardless of population (*i.e.* greater variation was found within, than between, populations). A similar phenomenon of RAPD variation being greater between individuals within than between populations has been found for other outbreeding species such as *Eucalyptus globulus* (Nesbitt *et al.* 1995), *E.risdonii*, *E.amygdalina* (Sale *et al.* 1996) and *Grevillea scapigera* (Rossetto *et al.* 1995). For *Grevillea scapigera* low molecular divergence between populations was reportedly in agreement with low morphological variation between such populations, although no particular characters were discussed. Molecular variation in a natural hybrid swarm of *E.amygdalina* and *E.risdonii* and nearby allopatric stands, which was assessed using RAPD amplified fragments, was found to be greatest (82%) between individuals within populations and lowest (7.7%) between the species (Sale *et al.* 1996). These authors proposed that morphological divergence between populations is not necessarily reflected as variation in RAPD markers since the majority of RAPD markers result from non-coding regions of the genome. These results are consistent with those from the outbreeding tree species, *Populus tremuloides*, where Yeh *et al.* (1995) found that 97.4% and 2.5% of RAPD variation occurred among and between populations respectively.

No significant molecular divergence was found, using AMOVA between the three natural populations evaluated in the present study. In the outbreeding species *Banksia cuneata* Maguire and Sedgley (1997) found no significant genetic divergence between natural populations using RAPDs, and, furthermore, some individuals between populations were more closely related than some within populations. These authors proposed that bird-pollinators were effective in maintaining introgression between populations of the species. Given that the three natural populations of *K.pomifera* in the present study, were not differentiated on the basis of molecular variation it is possible that gene flow has occurred between them even with a distance of approximately 380km between the two most geographically-separated populations (Ki to Lh). It is possible that Aboriginal people, as both consumers and traders of its fruit (Low 1989; Clarke 1998), may have provided the potential for seed dispersal over such distances. Furthermore birds, as consumers of its fruit (Wrigley and Fagg 1993), may also be important seed-dispersal agents of the species over long distances. Such seed dispersal mechanisms could lead to introgression between geographically-distant populations, which would be beneficial in terms of increasing the effective population size and reducing the effects of inbreeding depression and genetic drift (Ellstrand and Elam 1993). The lack of molecular differentiation between the three natural populations of *K.pomifera* may be a result of gene-flow between them and a lack of differential selection at these loci, which are likely to be adaptively neutral. Significant differentiation found between three populations of *Banksia saxicola* using AFLPs was attributed to geographic isolation between these populations with large areas of unsuitable habitat between them (Evans 2001). With *K.pomifera*, broad areas of unsuitable habitat between the populations studied does not appear to exist and before European settlement, the distribution of this species may have been continuous along the coast of south-eastern South Australia, with the current distribution being the fragmentation of such a possible continuum of distribution

The results from the both cluster analysis and ordination were consistent with those of the AMOVA, wherein a low level of overall differentiation was found between the three populations but the Lh population appeared to be the most divergent of the three natural populations. No differentiation was found between the accessions Kmt1 and Kmt2, which were sampled less than 5 metres from each other with a sealed roadway between them, indicating a close genetic relationship. In natural populations of *Eucalyptus globulus* ssp. *globulus* Skabo *et al.* (1998) evaluated molecular variation using RAPDs and found that closely related genotypes occurred within 25 metres of each other, but there was only weak association between geographic distance and genetic similarity among all the individuals sampled. While fruit/seed dispersal over wide distances is possible in *K.pomifera*, the close genetic relationship between Kmt1 and Kmt2 indicates the likelihood that many seeds are

dispersed only very short distances as found in many *Eucalyptus* populations (Potts and Wiltshire 1997)

Given that considerable genetic diversity in *K.pomifera* can be found within populations adequate collection from within populations would be required to sample genetic variation for *ex situ* genetic conservation and selection for forms that suit commercial production of the species. However, since the molecular variation in *K.pomifera*, found using RAPD loci, was not found to be representative of morphological variation (See Section 5), selection for 'horticultural' forms between populations is also recommended to access the morphological variation that is likely to exist between populations.

6.5.2 Conclusion

In the present study, molecular variation found in *K.pomifera* populations using RAPDs, provides a basis for future sampling of its natural populations for the conservation and exploitation of genetic resources of *K.pomifera* for commercial production. The greater molecular variation found among individuals within, than between, populations of *K.pomifera*, has also been found in other outbreeding species of *Eucalyptus* (Nesbitt *et al.* 1995; Sale *et al.* 1996) using RAPDs, and indicates the likelihood that genetic introgression has occurred between these three geographically-distant populations, possibly through long-distance dispersal of seeds by birds and people. Conversely the close genetic relationship between two individuals sampled within 5 metres of each other indicates the likelihood that many seeds are dispersed only very short distances as also found in many *Eucalyptus* spp. (Potts and Wiltshire 1997). Considering that significant genetic variation was detected between genotypes of *K.pomifera*, using RAPDs, such variation indicates a good potential source of genetic variation for the domestication and improvement of the species for commercial production.

7. Pollen germination in *Kunzea pomifera* as influenced by sucrose concentration and storage

7.1 Abstract

In vitro germination of freshly-collected pollen from seven accessions (Ba, Ki2d, Kk1j, Kms2, Kmt1, Kmt2 and Ph1a) of *Kunzea pomifera* was examined in a range of liquid media consisting of boric acid (2×10^{-3} M H_3BO_3), calcium nitrate (6×10^{-3} M $\text{Ca}(\text{NO}_3)_2$) and different concentrations of sucrose (0, 5, 10, 20, 30 and 40%). A maximum pollen germination percentage of 87% occurred in the medium of 10% sucrose and significantly reduced levels from this maximum were found in 0, 30, and 40% sucrose. Freshly-harvested pollen of five accessions (Ba, C1a, Ki2d, Kmt1, Ph1a) of *K.pomifera* was germinated in liquid media with 5% and 10% sucrose. There was no significant differences in the germination levels between these different sucrose concentrations for each of the five accessions. The germination level of 'Ba' pollen was 7%, significantly lower than the mean of each of the four remaining accessions, which ranged from 55% in Ki2d to 89% in Ph1a. Pollen of the accession Kmt1, stored for twelve months at 4°C at 10% RH, was evaluated for germination at intervals over this period, in media with different sucrose concentrations ranging from 1-10% in 1% increments. There were no significant differences in pollen germination between any of the sucrose concentrations. While there were significant differences in the levels of pollen germination of different storage periods, there was no correlation between germination percentage and storage time. Pollen of the *K.pomifera* accession Kmt1 stored under such conditions for 370 days, was used in artificial pollination in a compatible intraspecific cross with Kmt2, resulting in 3.4 seeds per fruit, which was considerably fewer than the 17.2 seeds developed per fruit in the hybridisation of this accession using fresh pollen.

7.2 Introduction

In the domestication of *K.pomifera* and its breeding and selection for commercial improvement, controlled pollination will be important for the incorporation of desirable characters from wild sources in new cultivars. The success of controlled pollination depends on the application of viable pollen to a receptive compatible stigma. *In vitro* pollen germination can be used to evaluate the viability of pollen for use in controlled pollination. Storing pollen to extend its longevity can be used to overcome problems in plant breeding where there is non-synchrony in the maturation of potential male and female parents of a proposed hybridisation, or in the case where, because of wide geographical separation between the potential parents of a proposed cross, there is a need to transport pollen over long distances. There were three primary aims to the present study of pollen viability in *K.pomifera* (a) to evaluate *in vitro* germination of pollen from a range of genotypes and to optimize the sucrose concentration of the germination media, (b) to evaluate *in vitro* pollen germination to determine the longevity of pollen stored at 4°C and 10% relative humidity and (c) to determine the effectiveness of such pollen storage for the artificial hybridisation of accessions of *K.pomifera* possessing non-synchronous flowering.

7.3 Materials and methods

For observations of pollen morphology under the SEM, anthers and pollen were fixed overnight in glutaraldehyde in 0.05M phosphate buffer at a pH of 6.8, prepared using the methods of (Beyrle *et al.* 1995). The fixed tissues were washed in 0.05M phosphate buffer three times for 15 minutes each. To improve conductivity of the specimens they were soaked in 1% osmium tetroxide (OsO_4) in distilled water for 2-hours followed by three 15-minute washes in distilled water. Specimens were then dehydrated in a graded ethanol series (10%, 20%, 30%, 50%, 70%, 90%, 100% (three times) EtOH) for 15 minutes at each step, except for 70%, which was left overnight. The EtOH was purged from the specimens with CO_2 , using a critical-point drier (Ladd CPD Research Industries). Once dried, specimens were mounted on conductive adhesive carbon tape and sputter-coated with gold, in a

pressurised (1061psi) argon (Ar) atmosphere (Ladd). Specimens were then viewed under a scanning electron microscope (Phillips XL30) using 20kV at a magnification range of x400-14000.

Mature anthers were harvested from 20 flowers of each of eight genotypes of *K.pomifera* (Ba, C1a, Ki2d, Kk1j, Kms2, Kmt1, Kmt2, Ph1a) growing under conditions described in Section 1, and placed into microcentrifuge tubes and taken to the laboratory. For the pollen-storage experiment a bulk sample of mature anthers of one accession (Kmt₁) was harvested on the 22nd October 2001 and placed in a petri-dish and stored in a desiccator, containing silica gel, at 4°C.

The pollen-germination stock solution consisted of a 1:1 volume mixture of 2×10^{-3} M H₃BO₃ (boric acid) and 6×10^{-3} M Ca(NO₃)₂ (calcium nitrate). Different amounts of sucrose were added to this stock solution to make concentrations of 0 - 40 % w/w (x g sucrose / 100g solution). In 2001 the response of pollen germination of seven genotypes of *K.pomifera* (Ba, Ki2d, Kk1j, Kms2, Kmt1, Kmt2 and Ph1a) to variation in sucrose concentration was evaluated in solutions of 0, 5, 10, 20, 30 and 40% sucrose with each genotype replicated 3 times in each sucrose treatment. In 2002 variation in pollen germination between five genotypes (Ba, C1a, Ki2d, Kmt1, Ph1a) was evaluated using two sucrose concentrations of 5 and 10%, wherein pollen lots of each genotype were replicated 10 times in each sucrose treatment. The pollen of Kmt1, stored at 4°C, was germinated in sucrose concentrations ranging from 1-10% in 1% increments. The sucrose concentration of each solution was checked with a hand-refractometer and adjusted to the appropriate concentration where necessary.

In the laminar flow cabinet 50µL of each sucrose solution was pipetted into a single well of a sterilised flat-bottomed ELISA tray (Falcon[®] 35-3072 Microtest[™] 96). Pollen was collected from the microcentrifuge tubes (for the sucrose concentration/ genotype experiment) or petri-dishes (for the pollen storage experiment) using a sterilised pipette tip and immersed in the well of a given sucrose concentration, which was repeated for all concentrations. The number of pollen grains to be included in each well was determined approximately by counting grains under a light microscope, and where necessary, adjusted to approximately 500 pollen grains per 50µL. The pollen and sucrose samples were covered with the ELISA tray-lids, sealed with Parafilm M[®], and placed in a growth cabinet at 23°C for 48 hours with light levels (approximately 7000lux) maintained by four 30-watt, cool white, fluorescent tubes.

Following the 48-hour germination period the samples were viewed directly in each ELISA well, using a light microscope, at a magnification of 10X. The number of pollen grains present in the field of view and the number of germinated pollen grains were counted using a Colby hand-tally counter. Pollen grains were considered germinated only when the length of the pollen-tube exceeded 50% of the diameter of the pollen grain (Weinbaum *et al.* 1984; Potts and Marsden-Smedley 1989)

Following 370 days under 4°C and 10% relative humidity storage, the pollen and anther mix was placed in a humidifier at 90% humidity for 3½ hours before being used for artificial pollination of 72 flowers of Kmt2 that had been emasculated at one day before anthesis and six days prior to pollination. Pollen, and at least one entire anther, were transferred to a receptive stigma using a 200µL pipette tip, on November 20th 2002. The fruit that developed following artificial pollination were harvested on 29th January 2003 and the seeds in each fruit were counted. Resulting seed was sown on moist filter paper in sealed petri-dishes under natural light on 20th February 2003.

7.4 Results

7.4.1 Response of pollen germination to variation in sucrose concentration

There were significant ($p < 0.001$) differences in pollen germination level between sucrose concentrations with no significant interaction between sucrose concentration and germination level for each accession. The germination level of pollen from the Ba accession was significantly ($p < 0.001$) lower than that of any of the other accessions, but with no significant differences among the remaining six accessions; the mean germination levels for these six accessions are presented in Figure 8.

Maximum pollen germination for each of the accessions occurred at 5, 10 and 20% sucrose with no

significant differences between the three concentrations, in which the mean germination percentage ranged from 12.3% in Ba to 91.3% in Kmt2. The mean levels of pollen germination for all accessions in germinating media containing 0, 30 and 40% sucrose were significantly lower than at 5,10 and 20% sucrose. The only differences in the mean levels of pollen germination among 0, 30 and 40% sucrose treatments, was a significantly greater level in 0%, compared with 40%, sucrose.

It appeared, in this study, that pollen-tubes were considerably shorter in 0, 30 and 40% sucrose solutions than in the other sucrose treatments. The bursting of pollen tubes was also observed, and appeared to be substantially greater in media without, than with, sucrose. Burst pollen was still considered germinated if the length of the burst tubes exceeded 50% of the diameter of the pollen grain.

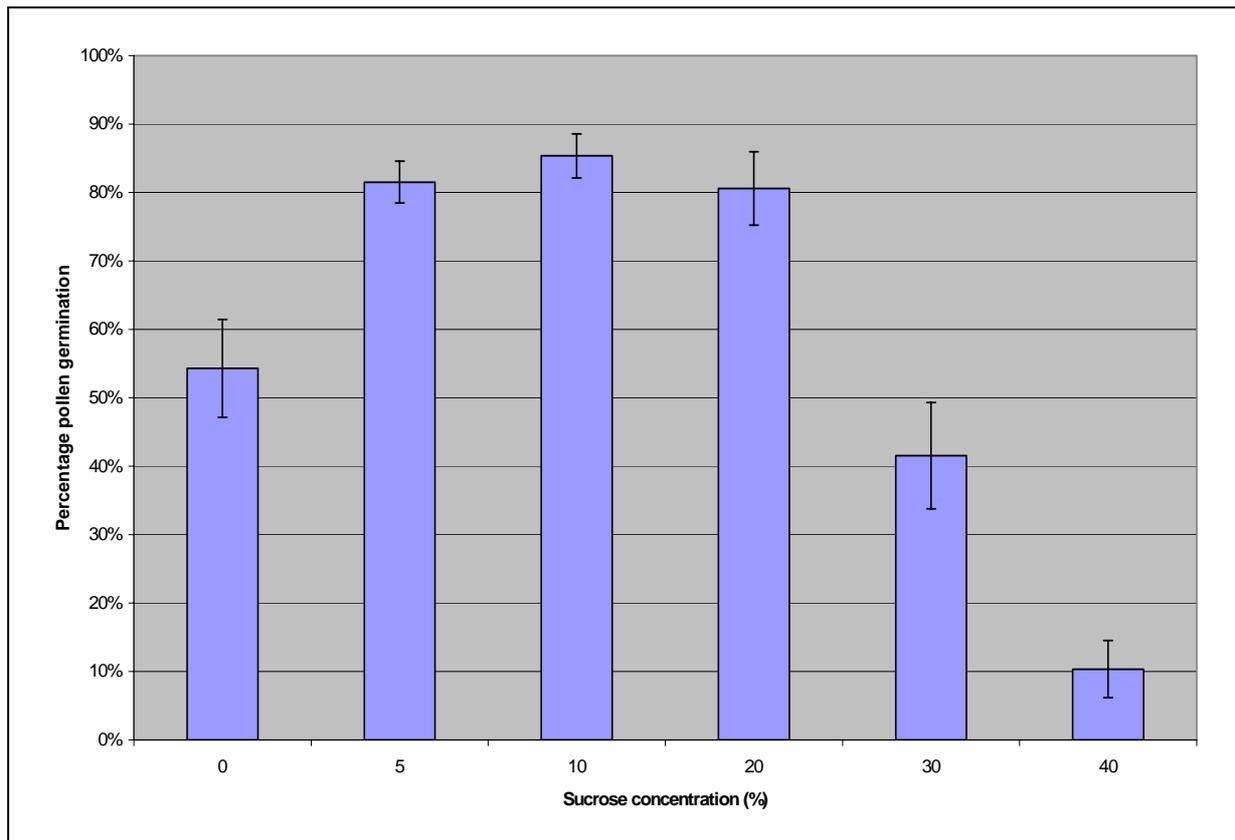


Figure 8: Mean *in vitro* germination levels of pollen for six genotypes (Ki2d, Kk1j, Kms2, Kmt1, Kmt2 and Ph1a) of *K.pomifera* under different concentrations of sucrose. Vertical bars represent standard errors

7.4.2 *In vitro* germination of fresh pollen from *K.pomifera*

No significant differences were found in the mean *in vitro* germination percentage of five accessions of *K.pomifera* (Ba, C1a, Ki2d, Kmt1, Ph1a), between germination media containing sucrose concentrations of 5 and 10%. Significant differences ($p < 0.001$) were found, however, between genotypes for mean germination level at both 5 and 10% sucrose (Figure 9). The mean germination percentage of the accession Ba, across both sucrose concentrations, was 7%, which was significantly ($p < 0.001$) lower than the four remaining accessions. There was a severe shortage of pollen on the Ba accession and in many circumstances it was not possible to achieve 500 pollen grains per 50 μ L. In the remaining accessions the mean germination level of pollen ranged from 55% in Ki2d to 89% in Ph1a, with only C1a and Kmt1 having similar mean germination percentages of 75% and 70% respectively. A wide range of variation in the level of pollen germination occurred in each accession across the two sucrose concentrations, which was as follows: Kmt1, 6.9% to 93.5%; Ki2d 12.5% to 87.7% and C1a 29.3% to 92.6%.

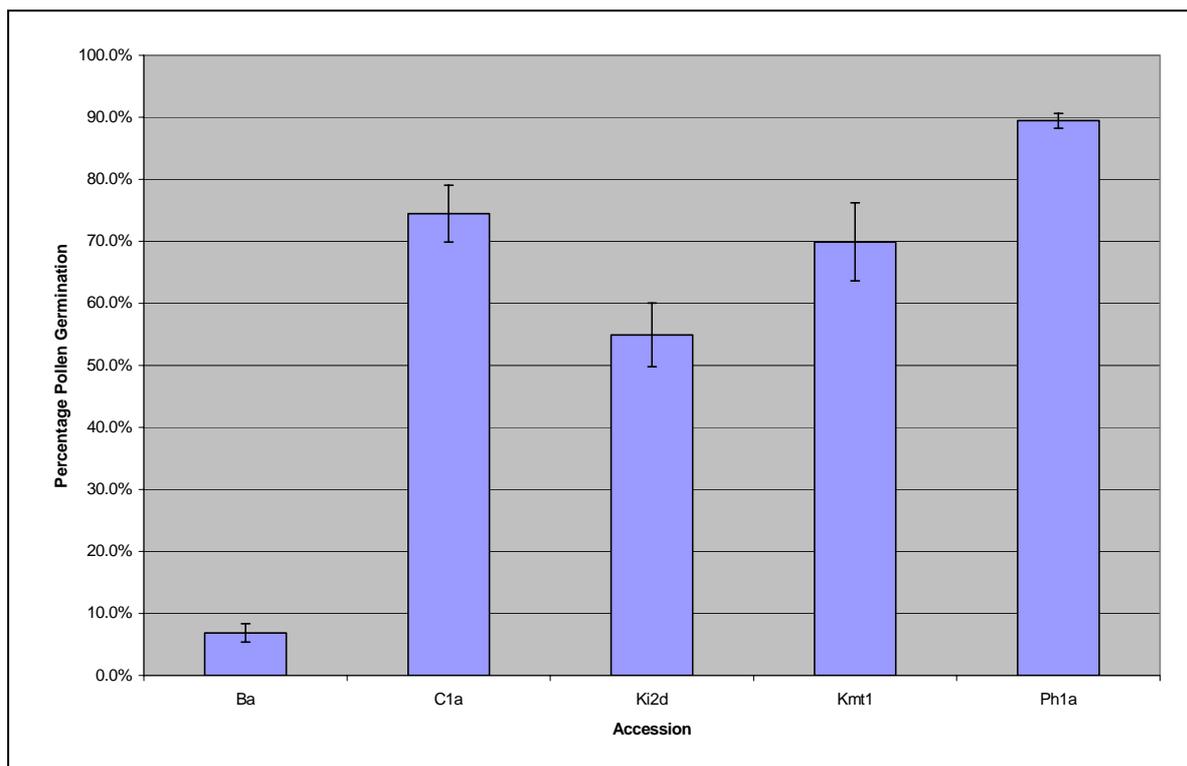


Figure 9: Mean *in vitro* germination levels of pollen from five accessions of *K.pomifera* over 5 and 10% sucrose. Vertical bars represent standard errors.

7.4.3 Low temperature pollen storage and *in vitro* germination

Pollen of the *K.pomifera* accession, Kmt₁, remained viable over the storage period of 12 months, with a viability ranging from 15 - 73% germination between different storage periods with no significant difference in pollen germination level between sucrose treatments. Under the storage conditions used, significant differences were found in levels of pollen germination between the pollen-storage times, but there was no significant difference in pollen germination between fresh pollen (zero days) and that stored for 370 days after harvest (Figure 10). Furthermore there was little correlation between percentage germination and pollen age with a Pearson correlation coefficient of -0.21.

While the number of pollen grains per sample for viability analysis was standardised, it was difficult to control the evenness of distribution of pollen in the germination medium, because it would often form pollen ‘clusters’, a characteristic that has also been reported in the germination of *Eucalyptus mannifera* ssp. *maculosa* (syn. *E.maculosa*) pollen (Boden 1958). Considerable pollen distribution effects were observed, wherein higher germination occurred in samples with dense pollen ‘clusters’, compared with more evenly-distributed samples. This distribution effect in samples with very dense clusters made it very difficult to differentiate germinated from un-germinated pollen grains. Therefore the measurement of pollen germination was undertaken in areas within a sample where individual pollen grains could be discriminated.

These distribution effects were pronounced in samples from 361 and 370 days storage because an increasing shortage of pollen under the storage conditions meant it was difficult to standardise both the pollen distribution in, and pollen number between, samples. For the pollen samples from 361 days storage, it was possible to achieve a pollen density of approximately only 200 pollen grains per 50µL, which is substantially less than the 500 grains per 50µL for all other storage treatments. To control the concentration of pollen grains in the 370 day treatment, entire anthers were included in the germination solution and agitated with a pipette tip to release the pollen that was attached. When these anthers were left in sucrose solution, high germination and pollen-tube growth was observed.

While the germination of these pollen grains was not counted they may have stimulated the germination of other pollen grains in the sample, especially those that were immediately adjacent.

Following artificial pollination of a receptive stigma with pollen stored under the prescribed conditions, a total of 73.4% of flowers developed into fruit, with a mean number of 3.4 seeds for each fruit, and a germination level of 75% after 12 weeks.

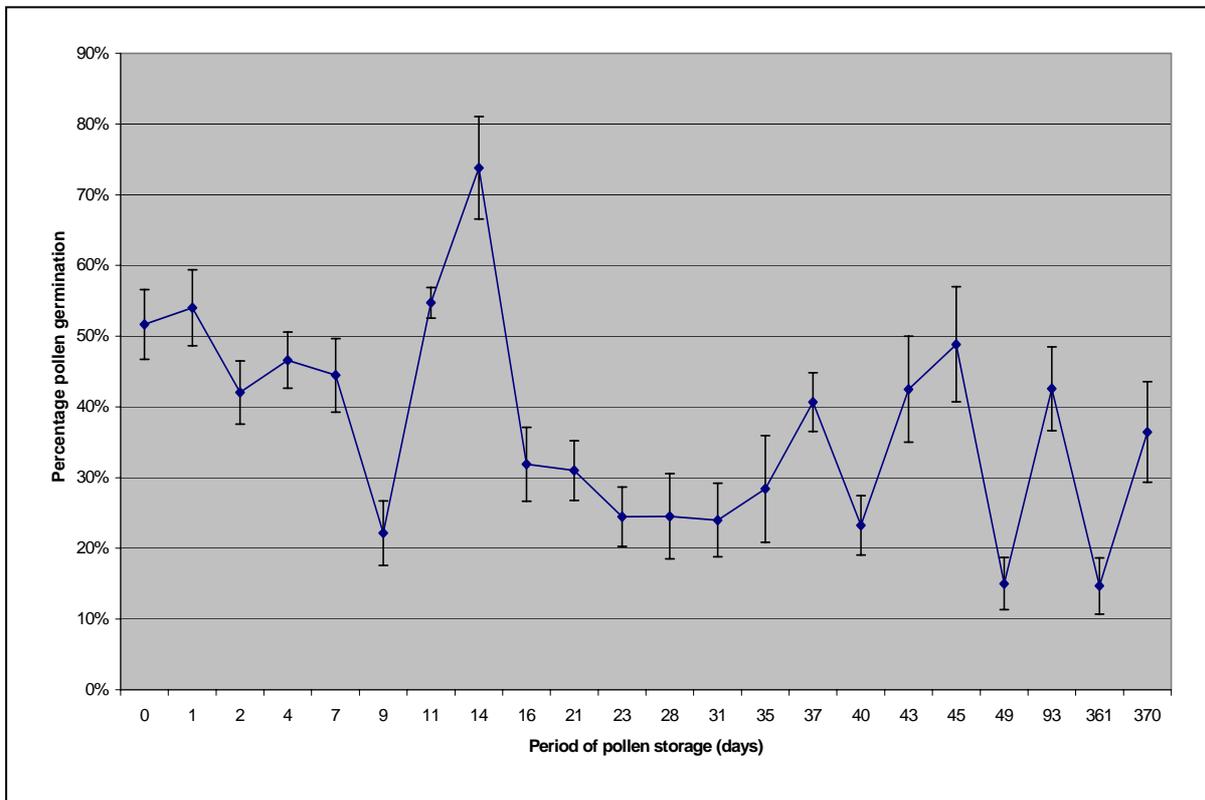


Figure 10: *In vitro* germination percentage of pollen stored at 4°C from 0 to 370 days. Vertical bars represent standard errors.

7.4.4 Pollen morphology and possible implications for its function

Scanning electron micrographs of fresh pollen grains of the *K.pomifera* accession Kmt1 indicated that, in the polar view, they are triangular, semi-lobate and approximately 18-20µm in length. The grains are isobilateral in shape with germinal apertures, appearing as oval-shaped pores at the three apices/corners of the grain (Plate 6). Each aperture is considered to be a colpus because its length is greater than its width (Fægri and Iversen 1989). Although the grain can be considered to be isobilateral the colpi are positioned closer to one of the poles and this pole is considered to be on the underside of the pollen grain. Given the grain has three colpus-type apertures/pores the grain is considered to be of the tricolporate type (Pike 1956). The furrows linking the colpi are open and meet at the poles of the pollen grain, which can be described as syncolpate (Erdtman 1952; Knox *et al.* 1994) (Plate 6). The triangular pattern of the furrows/colpi is similar to that observed in the pollen grains of *Eucalyptus phoenicea*, *E.spathulata* (Gadek and Martin 1982), *E.rhodantha* (Heslop-Harrison and Heslop-Harrison 1985), *E.spathulata*, *E.leptophylla* and *E.cladocalyx* (Ellis and Sedgley 1992). The outer exine layer of the pollen grain in *K.pomifera* is smooth with a distinct lack of ornamentation, or sculpturing, which is consistent with many species in the family Myrtaceae, which often have pollen with smooth, or faintly-patterned, surfaces (Pike 1956). Knox *et al.* (1994) proposed that the pollen-coat materials in eucalypts were generally important in the mutual adhesion of pollen in the open anther. While pollen grains of *K.pomifera* adhere to each other (Plate 7), in the present study, no material on the surface of the pollen-grain was observed which would possibly promote such adhesion, but this may be due to its possible removal in the preparation of the pollen for SEM and not necessarily to the absence of such material. Pike (1956) studied the morphology of pollen from twelve

species of *Leptospermum* and described them as being quite uniform to the point that pollen from different species within the genus were indistinguishable; the pollen of the *Leptospermum* spp. was also found to be similar in morphology to that of *Kunzea ambigua*, *K.muelleri* and *K.parvifolia*, except that pollen from the latter three species was slightly larger than that of *Leptospermum*. It therefore appears that pollen morphology is not likely to indicate taxonomic differentiation within the genus *Kunzea*.

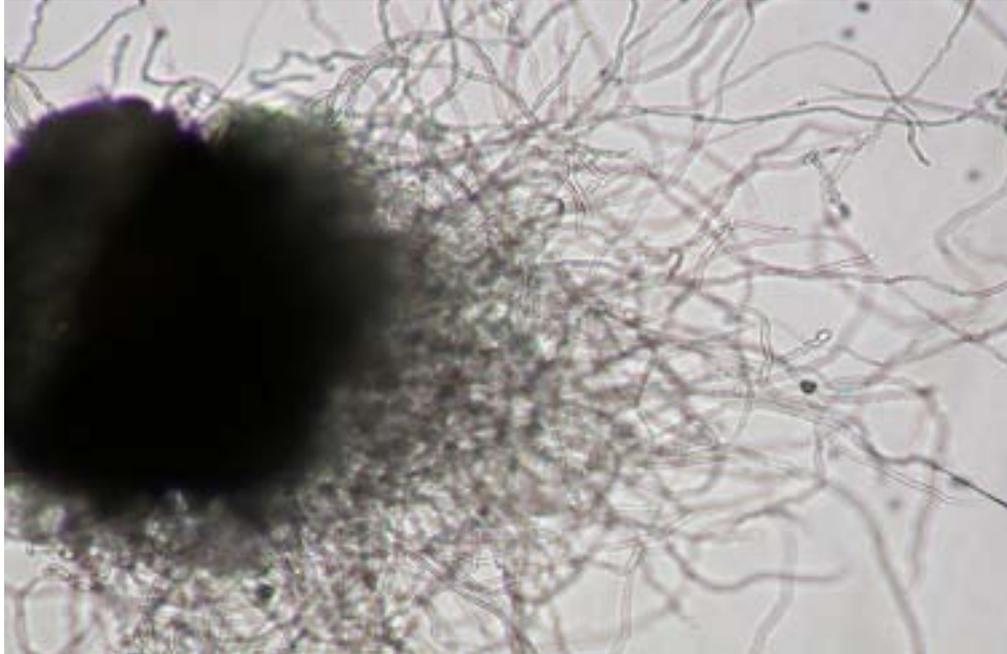


Plate 5: Pollen tubes of *K.pomifera* growing outwardly from a dense population of pollen grains around an anther that had been stored for 370 days at 4°C and 10% relative humidity.

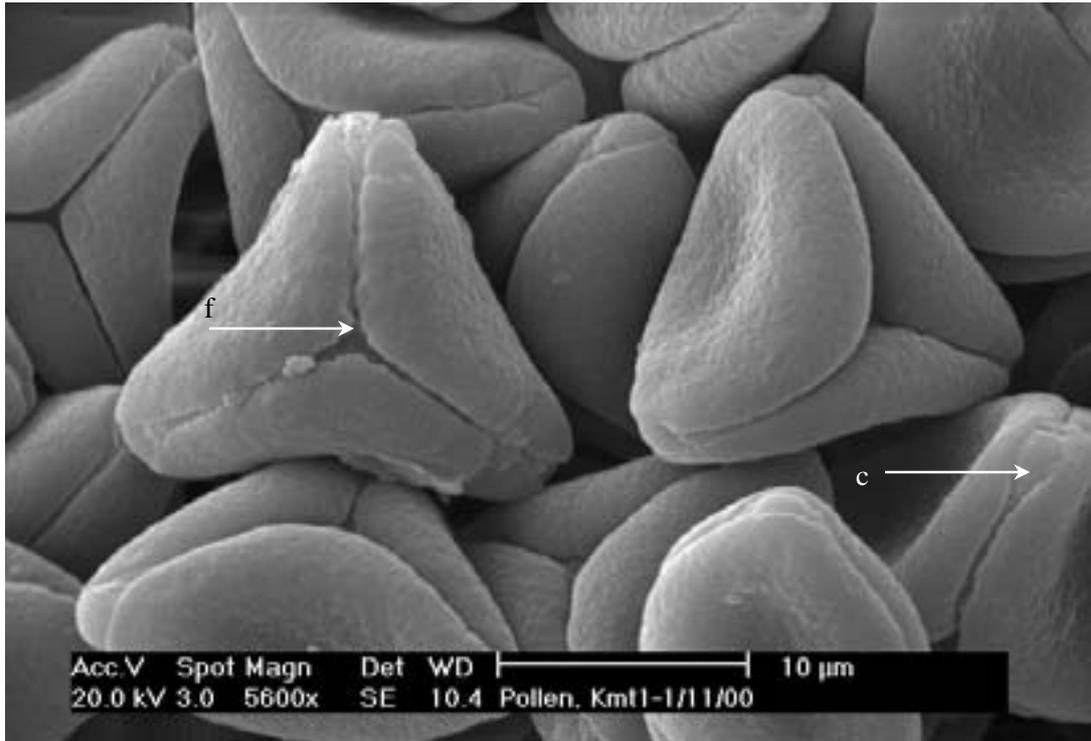


Plate 6: Scanning electron micrograph of the tricolpate pollen grains of *Kunzea pomifera*. c: colpus positioned on one of the apices/corners, but closer to one of the poles, which is considered to be the underside of the grain, f: syncolpate furrows linking the three colpi.

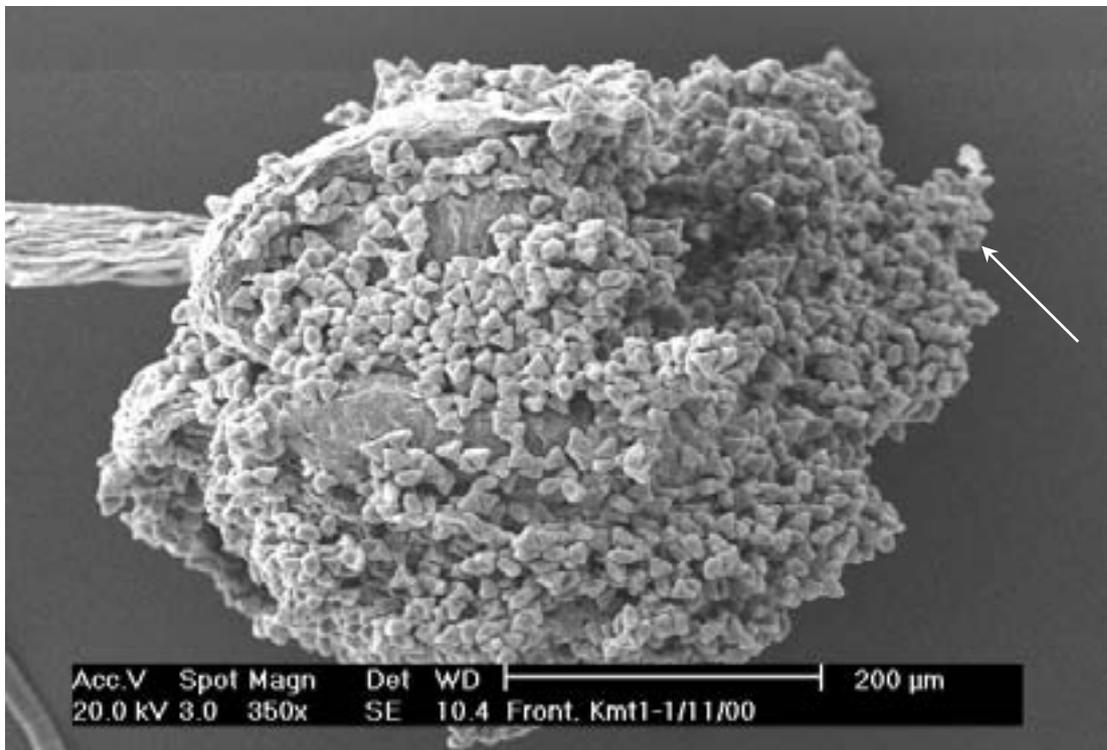


Plate 7: Scanning electron micrograph of anther of *K.pomifera* shedding pollen, with evidence of mutual adhesion of pollen grains as indicated by the arrow.

7.5 Discussion

7.5.1 Response of pollen germination to variation in sucrose concentration

In the present study a mean pollen germination level of 48.9% for seven accessions of *K.pomifera* in a solution without sucrose was considerably higher than that observed for pollen of each of *Eucalyptus globulus*, *E.urnigera*, *E.morrisbyi* and *E.ovata* (Potts and Marsden-Smedley 1989). Such differences indicate the possibility that small quantities of carbohydrate may be present in the pollen grain of *K.pomifera* to stimulate its germination. While this difference in pollen germination capacity between *K.pomifera* and species of *Eucalyptus* may be due to other physiological differences between these genera, the lack of calcium in the germination media of the Potts and Marsden-Smedley (1989) study may have contributed to a leaking of carbohydrates from the pollen grain (Vasil 1987), which may have reduced their potential for *in vitro* germination. Heslop-Harrison and Heslop-Harrison (1985) proposed that the outer refractive layer of the oncus of the pollen grain of *Eucalyptus rhodantha*, which allowed the emergence of the pollen tube, provided primary protection of the vegetative cell against immediate bursting of the pollen grain from exposure to osmotic stress.

The mean maximum *in vitro* germination and growth of pollen tubes across the seven *K.pomifera* accessions occurred in sucrose concentrations of 5, 10 and 20%. No significant differences in the level of pollen germination were found, over all accessions, between these concentrations of sucrose. Therefore any one of these sucrose concentrations could be used in evaluating viability of pollen in *K.pomifera*. Since pollen germinates and produces pollen-tubes over a wide range of sucrose concentrations it is likely that it possesses physiological flexibility to maintain normal function on the stigmas of genotypes with different osmotic potentials. Levels of pollen germination in *K.pomifera* were similar to those observed for *in vitro* germination of pollen from two *Clematis armandii* cultivars and one *Clematis* hybrid (Holmes 2000). The author observed pollen germination in solutions with sucrose concentrations ranging from 5 to 20% wherein maximum germination levels occurred at 10% for the two cultivars and 5% for the hybrid. The optimal sucrose concentration for *in vitro* pollen germination in *K.pomifera* appears to be considerably lower than in *Eucalyptus*, wherein Potts and Marsden-Smedley (1989) found maximum germination levels in sucrose solutions of 30% for *Eucalyptus globulus* and *E.ovata*, 20-30% for *E. urnigera*, and 20-40% for *E.morrisbyi*.

While measurements of pollen-tube lengths were not made in the present study, it was clear that pollen-tube growth was substantially greater in solutions containing 5,10 and 20% sucrose compared with those of 0,30 and 40% sucrose. Pollen germination levels and pollen-tube lengths in solutions without sucrose, were substantially lower than in solutions containing 20%, and lower, levels of sucrose. This indicates that sucrose is important for both the regulation of water absorption by the pollen grain for pollen germination, and as a source of carbohydrate for pollen-tube growth. Further investigations in *K.pomifera* involving the measurement of pollen germination and pollen-tube lengths in media wherein sucrose is substituted with polyethylene glycol may help determine the contribution of sucrose to osmotic regulation of germination and nutrition of the developing pollen-tube.

The observation of increased levels of pollen-tube bursting in solutions without sucrose, compared with all sucrose concentrations from 5-40%, is consistent with observations in *Eucalyptus pulverulenta* in which Boden (1958) proposed that almost all pollen tubes had burst in the germination solution with the 'lowest' sucrose concentration, but only 10% had burst at 'higher' concentrations. These observations indicate the likelihood that sucrose reduces osmotic pressure in the pollen-germination medium and most likely affects the hydration of the developing pollen-tube.

7.5.2 *In vitro* germination of fresh *K.pomifera* pollen

Significant variation in levels of pollen germination between genotypes of *K.pomifera* when germinated in a medium containing 5 and 10% sucrose, indicate that pollen viability is likely to possess a degree of genetic control, which may influence its capacity to effect fertilisation and subsequent seed set following pollination of a receptive compatible stigma. While this type of genetic variation is clearly evident in the low pollen-germination rates recorded for the accession Ba, compared with other *K.pomifera* accessions, *in vitro* pollen-density effects may have somewhat

confounded this result. The low pollen production of this accession meant that it was difficult to attain a consistent number of pollen grains in each sample. However low germination levels were observed even in high-density clusters of pollen within a sample, indicating that density effects may not be the only factor influencing the low germination rate. Regardless of the mechanisms involved in the low pollen germination of this accession, such a characteristic reduces its value as a pollen parent in a breeding programme. The apparent divergence of the accession Ba from the other accessions of *K.pomifera*, in its low pollen production and germination level in the present study is consistent with observations of its divergence from other accessions in leaf morphology and plant habit.

Substantial variation in germination levels occurred between replicates in the present study, indicating the likelihood of variability inherent in the method of assessing viability and hence the need for replication to determine levels of *in vitro* viability of such pollen. In *Acacia mangium* and *A.auriculiformis*, *in vitro* pollen germination level was also quite variable, wherein pollen germination ranged from 3-85% between replicates using the pollen from a single inflorescence (Sedgley 1992b).

Maximum germination percentages of 20 and 32.5% were found for fresh pollen of *Eucalyptus rhodantha* and *E.caesia*, respectively, after 24 hours in a liquid medium of 10^{-3} M- H_3BO_3 and 10^{-3} M- $Ca(NO_3)_2$ and 20% sucrose (Heslop-Harrison and Heslop-Harrison 1985). Although the proportion of sterile pollen in *E.rhodantha* was not presented, the authors indicated that it was high, which was most likely implicated in the substantial variation in both pollen-grain size and shape in this species. In *Mimulus guttatus* and *Collinsia verna* (Kelly *et al.* 2002) found that pollen diameter was positively associated with pollen viability. In the present experiment a high level of consistency in size and shape of freshly-harvested pollen grains of the accession Kmt1 was found using SEM (Plate 6); the pollen from this accession also demonstrated a high level of *in vitro* pollen germination (70%) (Figure 9), and capacity for storage under cool dry conditions, with little effect on viability over 12 months (Section 7.4.3). Further experimentation of relative pollen diameter in *K.pomifera*, particularly the Ba accession with its low *in vitro* viability (7%), would be beneficial in terms of using this character as an indication of pollen germination potential.

7.5.3 Low temperature pollen storage and *in vitro* germination

The present study revealed that pollen of the accession Kmt1 of *K.pomifera* could be stored for up to twelve months at 4°C at a RH of 10% with no effect on percentage germination compared with fresh pollen with a high pollen density in the germination medium. Pollen longevity in five *Verticordia* species (*V.etheliana*, *V.helichrysantha*, *V.picta*, *V.staminosa* and *V.monadelpha*), when stored at 2°C over silica gel, was found to be 40.6 weeks with approximately 43% of the pollen remaining viable, compared with fresh pollen with 85-88.5% viability (Tyagi *et al.* 1992). The general description of pollen from the species of the genera *Eucalyptus* (Pryor 1976; Heslop-Harrison and Heslop-Harrison 1985; Williams *et al.* 1999) and *Verticordia* (Tyagi *et al.* 1992), as being 'robust' may also be used to describe the pollen of *K.pomifera*, given that its pollen can be stored under low temperature and low relative humidity for twelve months with little effect on the *in vitro* germination level.

The optimal sucrose concentration for maximum levels of *in vitro* germination of fresh pollen in *K.pomifera* was found to be 5-20%. For stored pollen *in vitro* germination was evaluated in solutions with 1-10% sucrose (with 1% increments) but no significant differences in germination level were found between any of these concentrations. However, it is possible that this range of concentrations may not have been optimal for the germination of stored pollen and further research appears to be necessary to determine exactly the optimum concentration of sucrose for maximum germination level of such stored pollen. The optimal sucrose concentration for maximum levels of *in vitro* germination of fresh pollen in *Pistacia vera* was 10-20%, but after four months storage at -20°C its germination level was significantly greater in 20% sucrose (Polito and Luza 1988). These authors proposed that stored pollen may have more exacting requirements for *in vitro* germination than for fresh pollen, including its re-hydration prior to germination. Controlled re-hydration of desiccated pollen, by exposure to a humid environment of 95-100% relative humidity for one or more hours, has been shown to have a significant positive effect on *in vitro* pollen germination levels in *Corylus avellana* (hazel) (Heslop-Harrison *et al.* 1986), *Juglans* spp. (English walnut) (Luza and Polito 1987), *Pistacia*

vera (pistachio) (Polito and Luza 1988) and *Carya illinoensis* (pecan) (Yates and Sparks 1989). Controlled re-hydration of *K.pomifera* pollen was not practised in the present study of *in vitro* pollen germination. It is possible that pollen desiccation may be, at least part of, the reason for variation in the germination level of fresh pollen of the accession Kmt1 between years, with a level of 52% in 2001 and 70% in 2002. The difference in germination levels of fresh pollen in 2001 and 2002 could have been due, at least partly, to differential desiccation of the samples during the harvesting activity which, in 2001 was lengthy (4 hours) and in 2002 short (10-15 minutes). Given also that significant differences in pollen germination between each of Ki2d, Kmt1 and Ph1a occurred in 2002 but not in 2001, it is possible that different physiological and environmental conditions during pollen development in 2001, compared with 2002, may have differentially influenced the capacities for its germination.

It is quite possible that gametic selection pressures operate on the pollen of heterogeneous populations under storage (Hanna and Towill 1995). For these reasons, because of the likelihood for differential selection upon pollen in storage, it is important, for the maintenance of the full genetic integrity of pollen 'populations', that storage condition be chosen wherein possible differential selection is absent or minimal. Seeds from *Zea mays*, produced after fertilisation from pollen stored at 1°C for 72 hours, were found to germinate faster, and have a higher percent germination, than that produced from fresh pollen (Bocsi *et al.* 1990). These authors hypothesised that storage of pollen at 1°C for 72hrs prior to pollination could be used in selecting a breeding population for cold tolerance. Considering that stored pollen may experience some gametic selection, offspring from pollination using stored pollen may be quite different genetically from those generated with fresh pollen, of an identical genotype. However, given that little research has been undertaken on the possible effect of pollen storage on (male) gametic selection it is not possible to predict potential changes to the genetic make-up of offspring wrought by such selection. Considering the possible importance of pollen viability and vigour as factors influencing gametic selection in the style, *in vitro* germination procedures that do not make measures of the rate of growth of pollen tubes, may not provide an accurate indication of pollen fitness in terms of its capacity to effect fertilisation and seed set following pollination of a compatible stigma. Sedgley and Griffin (1989) proposed that the germination of pollen is controlled by the vegetative nucleus such that germination may occur in the absence of a viable generative nucleus. Therefore it may be necessary to distinguish between pollen germinating *in vitro* as being 'viable' and that which is able to effect seed set following its application to a compatible stigma, as being 'fertile'.

Pollen density effects, wherein higher germination levels were observed in samples with greater numbers of pollen-grains, were observed throughout experiments in the present study, regardless of the sucrose, genotype or storage treatment. Since pollen germination levels, in the current studies, were measured at densities in which pollen grains could be distinguished, such assessments may have underestimated potential pollen germination levels in samples of high density. Pollen-density effects have been observed in *Prunus avium* where higher germination rates were observed at higher pollen densities (La Porta and Roselli 1991). The capacity of *K.pomifera* pollen to adhere to each other to form clumps (Plate 7) is of potential benefit in maintaining high pollen density with possible enhancement of its germination level and to possibly facilitate the transfer of sufficient pollen from the anther to a receptive stigma by either natural insect pollinators or during artificial pollination.

A total of 73.4% of flowers, pollinated with stored pollen developed into fruit in the cross Kmt1(♂) x Kmt2(♀). A mean of 3.4 seeds per fruit were produced following pollination of Kmt2 with (stored) pollen of Kmt1, and the seed germination level was found to be 75%. This indicates that pollen of *K.pomifera* can remain viable when stored for 370 days at 4°C and 10% RH as indicated by its successful capacity for fertilisation in an intraspecific cross. However, the 3.4 seeds per fruit in this cross, using stored pollen, was substantially less than the 17.2 seeds produced per fruit in the same artificial cross using fresh pollen (Table 6). This difference, however, may have been confounded by a somewhat lower number of pollen grains used in artificial pollination with stored, than fresh, pollen, indicating the likelihood that the lower pollen density in the former may have caused reduced pollen germination.

7.6 Conclusion

Pollen of *K.pomifera* germinates and develops pollen-tubes readily in solutions comprised of 2×10^{-3} M H_3BO_3 , 6×10^{-3} M $Ca(NO_3)_2$ and either 5, 10 or 20% sucrose, any of which can be used as a general medium for assessing pollen viability in this species. Variation in pollen germination levels between genotypes of *K.pomifera* indicates that processes conferring its pollen viability are likely to be under a considerable degree of genetic control. Likewise it is possible that genetic variation also exists, between genotypes of the species, in the capacity of pollen to effect fertilisation and promote seed set following pollination of a receptive compatible stigma, and therefore most likely operates as a selective influence in cross-pollination in natural populations.

While differences in pollen viability may be expected between genotypes under storage at 4°C and 10% RH, the results of the present study indicate that pollen of *K.pomifera* can potentially be stored under these conditions for a period of, at least, 12 months with little effect on its *in vitro* germination capacity when it is germinated under conditions of 'high' pollen density. These results are encouraging in terms of maintaining the longevity of *K.pomifera* pollen stored under the prescribed conditions, particularly considering that pollen stored for twelve months can be successfully used in artificial hybridisation. By prolonging the fertility of pollen in *K.pomifera* through cool storage, it will be possible to circumvent possible restrictions in the capacity to hybridise certain genotypes because of their non-synchronous periods of flowering.

8. Onset and duration of stigma receptivity in *Kunzea pomifera*

8.1 Abstract

The time of onset and duration of stigma receptivity in *Kunzea pomifera* was evaluated by observing peroxidase activity in the stigmas of flowers of different ages, pollen-grain germination on the stigma, the timing and extent of pollen-tube entry into the ovary, and seed set following artificial pollination of flowers of different ages on plants grown under greenhouse conditions. The percentage area of the stigma showing peroxidase activity was negligible for the first four days after anthesis, but it increased to a peak of 65% at day 13 after anthesis. The percentage of pistils bearing germinated pollen and pollen-tubes entering the ovary were significantly different between flowers pollinated at different ages. Generally, the percentage of pistils bearing germinated pollen and that exhibiting pollen-tube entry into the ovary increased when pollen was applied to the stigma from the day of emasculation, to a maximum at days 7 and 8 after emasculation respectively, followed by a decline by day 10. Under greenhouse conditions the optimum pollination period, measured as the level of set seed after artificial pollination, ranged from 3 to 12 days after emasculation with no reduction in mean seed set resulting from different pollination times. Peroxidase activity in the stigma of *K.pomifera* appeared to be closely associated with stigma receptivity, which extended for a period of 10 days, while pollen remained viable on the stigma for 4-5 days under greenhouse conditions. These characteristics of its reproductive biology indicate that the rather long duration of stigma receptivity and pollen viability are not likely to significantly limit fruit and seed set in *K.pomifera* under field conditions.

8.2 Introduction

Knowledge of the timing and duration of stigma receptivity of a plant species can be used in devising artificial pollination programmes to optimise levels of fertilisation and seed set. Considering that artificial hybridisation will be an important activity in the development of cultivars of *Kunzea pomifera* for horticultural fruit production, it was considered that certain fundamental aspects of stigma receptivity and pollen biology in this species required elucidation. Determining the onset and duration of stigma receptivity can be undertaken by examining such characteristics as peroxidase activity of the stigma at different times after anthesis, the germination percentage of compatible pollen grains adhering to the stigma (Holmes 2000), the length of compatible pollen tubes growing down the stigma (Oddie and McComb 1998) and the number of fruit and seed set following pollination with compatible pollen (Holmes 2000). While peroxidase activity on the stigma surface can be considered to be an indicator of stigma receptivity (Lavathis and Bhalla 1995) its evaluation is usually accompanied by pollination experiments to ascertain its relationship with both pollen-tube growth and seed set. In determining the germination percentage of compatible pollen grains on the stigma, observations need to be made of both germinated and ungerminated grains as described in the methods of Cruzan (1986).

The purpose of the present study was to determine the time of initiation and duration of stigma receptivity in *Kunzea pomifera*, and the optimum time for pollen application to maximise levels of fruit and seed set from artificial hybridisation. This was undertaken employing four types of observations: (a) changes in pistil morphology with floral developmental, (b) the percentage area of the stigma exhibiting peroxidase activity, (c) *in vivo* pollen germination and pollen-tube growth following pollination of stigmas at various times after anthesis and (d) the level of fruit and seed set per fruit following pollination of stigmas at various times after anthesis.

8.3 Materials and methods

8.3.1 Peroxidase activity

Five flowers of a single accession of *K.pomifera* (Kmr₁) were emasculated daily one day before anthesis (designated as day -1) and the hypanthium (floral tube) was marked using acrylic paint, with a different colour representing a different day, for a total of 19 days. On day 19, five styles were

excised from flowers for each day of emasculation and the stigma of each was evaluated for the presence of peroxidase activity using a modified method described by Dafni and Maues (1998). A single Peroxtestmo-Ko peroxidase indicator paper (Macherey-Nagel) was immersed in 250 μ L of deionised water for 2 minutes to suspend the o-Tolidine hydrochloride powder, present on the paper, in the water. Using a pipette, 1 μ L of the suspension was applied to the stigma of a freshly-excised style. The stigma was observed from above, under a dissecting microscope (x40), to detect any reaction to the solution, which would be visible as the appearance of a blue colour on the stigma, indicating receptivity (Dafni and Maues 1998). The level of peroxidase reaction was recorded as the percentage of the stigma surface showing a blue colour regardless of the intensity. Percent peroxidase values were arcsine-transformed and fitted to a linear regression against time (days). Differences in peroxidase activity at different times (days) after anthesis were analysed using a one-way ANOVA with Tukey's pairwise comparisons.

8.3.2 *In vivo* pollen-tube growth

Five flowers from each of three accessions of *K.pomifera* (Ki2d, Kmt2, C1a) were emasculated by hand one day prior to anthesis on 7 separate days (0,2,4,6,7,8 and 10 days before pollination), and the hypanthium of these flowers was marked with acrylic paint, colour-coded to represent the date of emasculation. All 105 flowers from each accession were pollinated on the one day and the number of days from emasculation to pollination was referred to as 'flower-age'. Flowers were harvested 6 days after pollination and carpels were excised and fixed in 45% glacial acetic acid: 70% ethanol (1:3 v/v) for 24 hours. The carpels were removed from the fixative and soaked in deionised water for 30 minutes before being transferred to a 4M sodium hydroxide solution for 3 hours at room temperature (15-20°C). They were soaked again in deionised water for 12 hours and then placed in a 0.1% solution of water-soluble aniline blue with 0.1 M potassium acetate (K₃PO₄) for a further 12 hours. Carpels were placed on a microscope slide in 60 μ L of glycerin. A cover-slip was put over the carpels and glycerin and light pressure was applied using forceps to slightly flatten the preparation. Pollen-tubes were observed at 100x and 200x magnification under a Nikon E400 fluorescence microscope possessing a 450-490 nm green filter. In this examination the following characteristics were measured (a) pollen-grain number on the stigma, (b) the presence or absence of germinated pollen grains and the number of pollen-tubes that had penetrated the stigma, (c) pollen-tube number in the style at 50% of the style length and (d) the presence or absence of pollen-tubes that had entered the ovary. Prior to the present experiment pollen-grain and pollen-tube numbers were counted on 15 different pistils and classified according to their number on a scale of 1-5 which was constituted as follows: (1= 1-30, 2=31-100, 3=101-200, 4=201-1000, 5=1001-2000 pollen grains or -tubes). In the present experiment the number of pollen-grains on the stigma and pollen-tubes in the style were estimated on this scale established for the species.

8.3.3 Seed development

One flower from each of five inflorescences from a single accession of *K.pomifera* (Kmr₁) was emasculated daily by hand one day prior to anthesis for 13 days. The hypanthium of all emasculated flowers was marked with acrylic paint, colour-coded for the date of emasculation. To undertake artificial pollinations, mature shedding-anthers were removed from the filaments using pointed forceps and placed in a microcentrifuge tube. A 1mL pipette tip was used to agitate the pollen/anther mix to facilitate adhesion of pollen grains to the plastic tip. The pollen was transferred from the pipette tip to the stigma using light pressure. All 65 flowers were pollinated on the same day with a pollen mixture consisting of three different genotypes (C1a, Kmt2 and Lh1a) of *K.pomifera*. The pollen mixture was used to limit any potential influence of specific combining ability among the genotypes. Each flower was tagged according to the number of days after emasculation (flower age) when it was pollinated. Those flowers that developed into fruit were harvested four months after pollination and the seed in each fruit was counted.

8.4 Results

8.4.1 Peroxidase activity

Significant ($p < 0.001$) differences were found in the area of the stigma exhibiting peroxidase activity at different times from anthesis. Negligible peroxidase activity was observed for the first five days after emasculation, but the percentage area of the stigma exhibiting such activity increased from day four to a peak at day 13 after anthesis (Figure 11). A significant ($p < 0.01$) increase was found in the mean percentage peroxidase activity between the stigmas from flowers less than five days after anthesis to those at, or beyond, this age. For those stigmas between five and 17 days after anthesis there was a significant positive correlation ($r = 0.51$) between the area of the stigma exhibiting peroxidase activity and the number of days from anthesis. However, less than 25% of the variation in peroxidase reaction was described by the linear relationship with 'days', indicating that there was substantial variation for peroxidase activity between stigmas at each time of measurement.

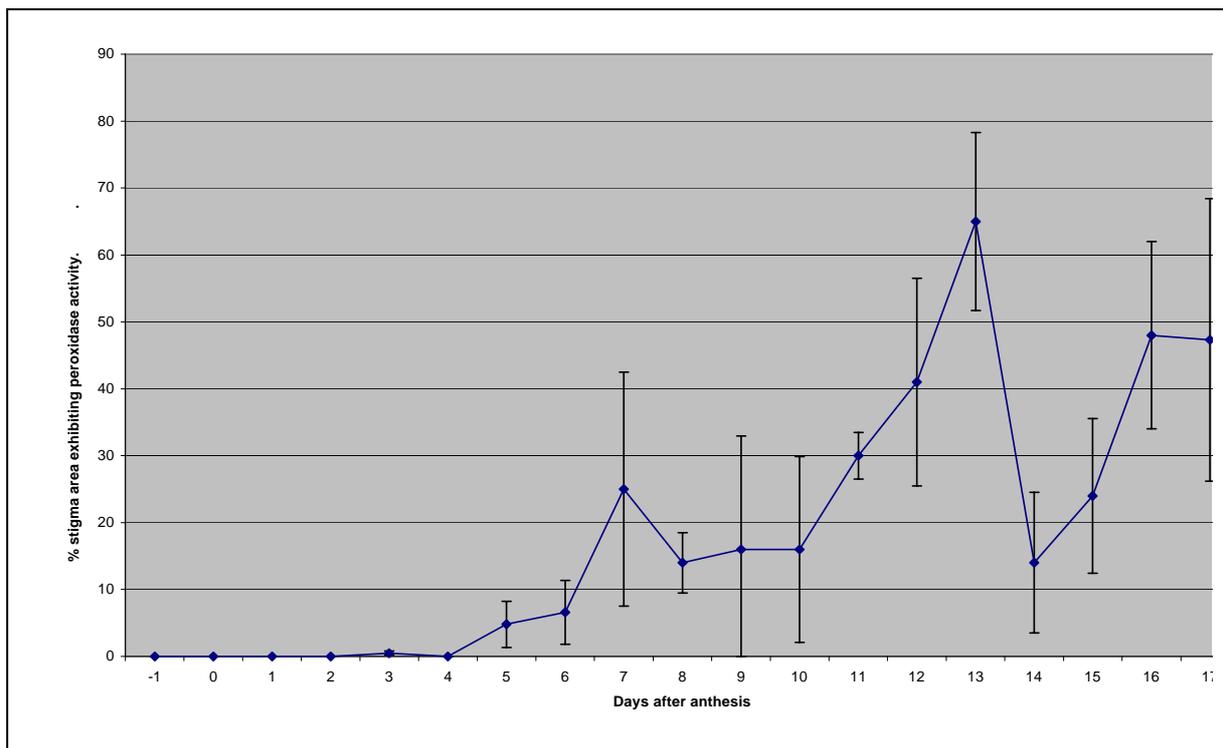


Figure 11: Percentage area of the stigma exhibiting peroxidase activity in flowers of *Kunzea pomifera* from 1 day before, to 17 days after, anthesis. Vertical bars represent standard errors.

8.4.2 *In vivo* pollen tube growth

Binary logistic regression, using a quadratic model for the presence or absence of pollen germination on the stigma, revealed an increase in the number of stigmas exhibiting pollen germination from one day before, to a maximum at 5.5 days after, anthesis, and a subsequent decrease from this peak until 9 days after anthesis. When pollen was applied to stigmas on the same day as emasculation (one day prior to anthesis), the number of stigmas exhibiting pollen germination was significantly ($p < 0.01$) lower than for those which were pollinated between three and seven days after emasculation, but there was no significant difference between the number of stigmas bearing germinated pollen, when pollen was applied at emasculation and nine days after anthesis.

In most cases pollen germinating on the stigma produced pollen-tubes that penetrated the ovary. A significant correlation was found (Pearson's correlation coefficient = 0.80) for the percentage of pistils showing pollen germination and pollen-tube entry of the ovary. Therefore the limiting factor in pollen-tubes reaching the ovary in the first three days after emasculation (-1 to 1 days from anthesis) appears to be stigma receptivity. A significant positive correlation was found also between the

number of pollen-grains on the stigma and the number of pollen-tubes in the style (Pearson's coefficient = 0.71).

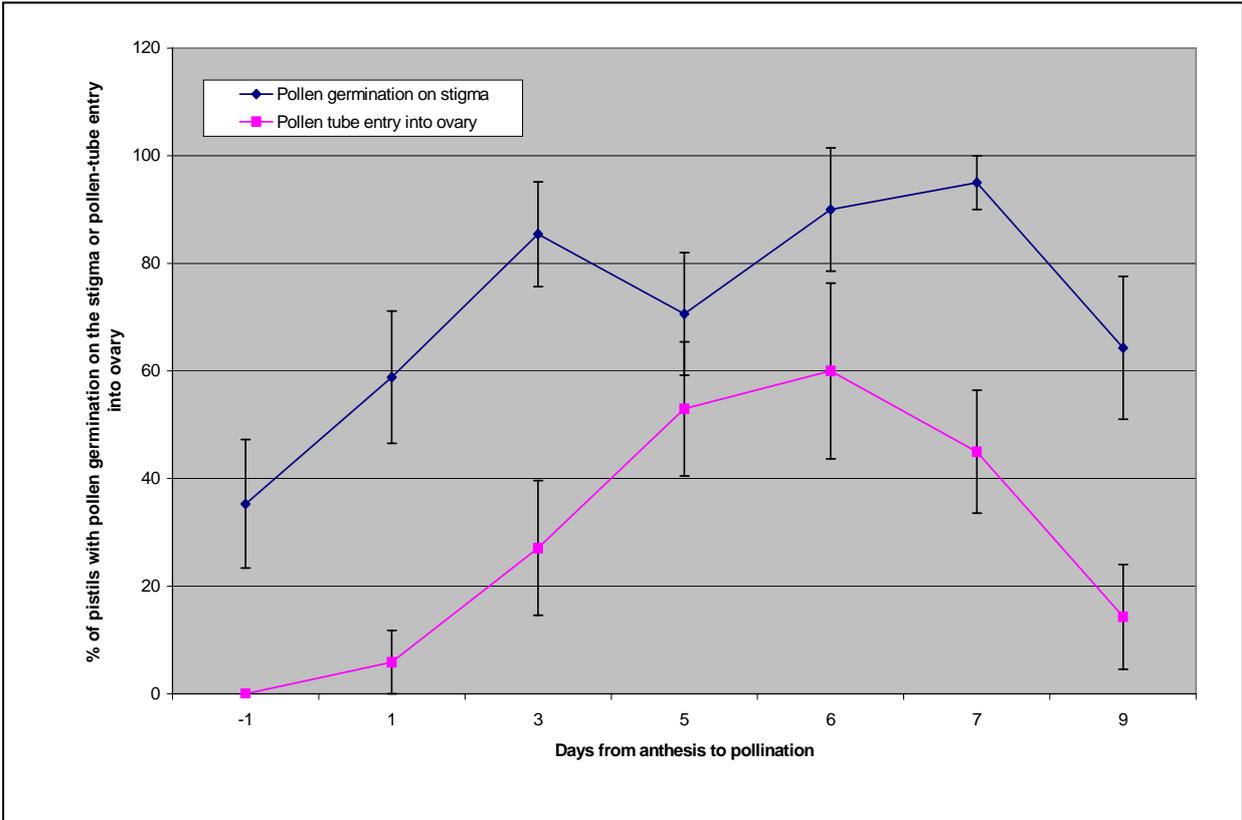


Figure 12: Percentage of pistils in *Kunzea pomifera* exhibiting (a) pollen germination on the stigma, and (b) pollen tube entry into ovary when artificially-pollinated from 1 day before, to 9 days after, anthesis. Vertical bars represent standard errors.

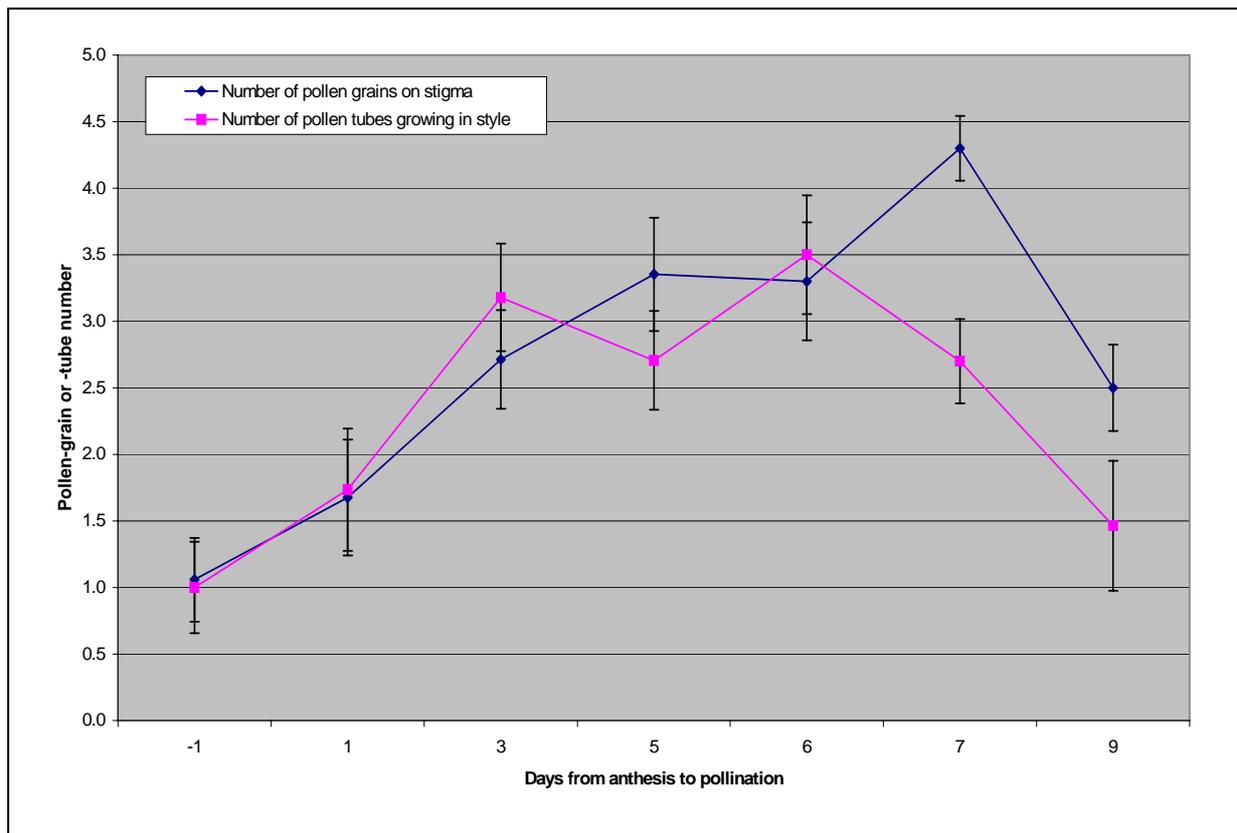


Figure 13: The number of pollen grains on the stigma and pollen-tubes in the style in flowers of *Kunzea pomifera* pollinated from 1 day before, to 9 days after, anthesis. Vertical bars represent standard errors.

8.4.2.1 Seed development

A weak positive correlation ($r=0.30$) was found between the mean number of seeds per fruit and flower age (days from anthesis to pollination). Using linear regression of these two characters only 9% of the variation in seed set was attributable to the linear relationship with flower age, and there was substantial variation in seed set within each flower age category (Figure 14). No significant differences were found between flower age and the mean number of seeds per fruit. However when the age of flowers was broken into two groups consisting of one day before to one day after anthesis and 2-11 days after anthesis, the number of seeds set were significantly ($p<0.01$) for the latter, compared with the former, group. The mean seed set per fruit was 5.9 and 21.6 for the early and late flower age groups respectively. Furthermore there was no correlation between the mean number of seeds per fruit and flower age in the 2-11 day category. This indicates that stigma receptivity was not a limiting factor for successful pollination of flowers across this range of maturity.

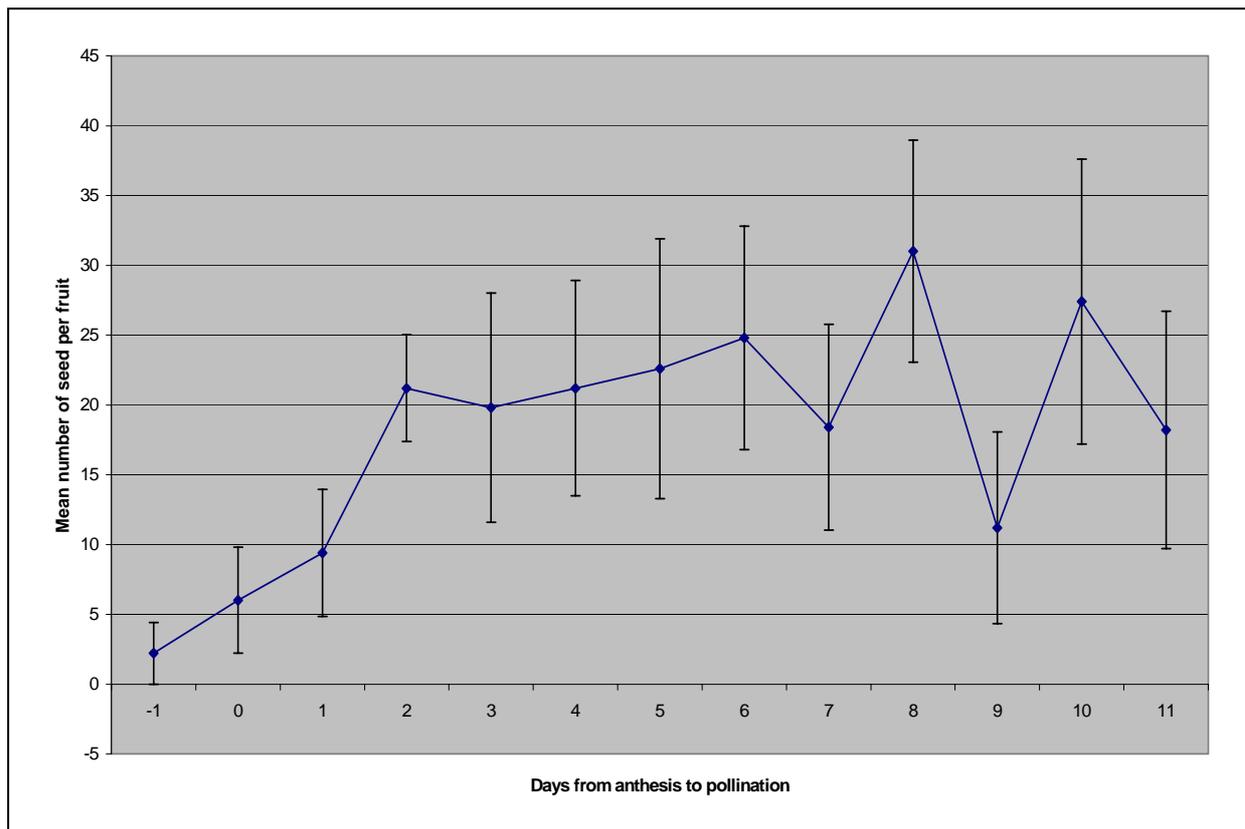


Figure 14: Mean number of seeds per fruit in *Kunzea pomifera* following application of viable pollen to compatible stigmas 1 day before, to 11 days after, anthesis. Vertical bars represent standard errors.

8.4.3 Discussion

In the present study significant positive correlation found between the percentage area of the stigma exhibiting peroxidase activity and time (days after anthesis) indicates that, in *K.pomifera*, as the stigma matures the mean peroxidase activity increases. Since no reduction in the peroxidase activity was observed over the test period of seventeen days, it is likely that a stigma remains receptive for this period and any reduction in receptivity occurs in flowers later than, at least, seventeen days after, anthesis. The substantial variation in peroxidase activity between stigmas within a given day indicates that stigma age may not be the only factor involved in causing this variation. The onset and length of stigma receptivity may depend on factors such as temperature (Hodgson 1976a; Griffin and Hand 1979; Furukawa and Bukovac 1989; Burgos *et al.* 1991) plant nutrition (Williams 1965) and maternal assimilate allocation to the stigma (Sanzol and Herrero 2001). Therefore peroxidase activity, and hence, stigma receptivity, may be closely associated with the phenological stage of the flower, whose expression may be dependent on variation in temperature and also maternal assimilate allocation to the flower.

The positive correlation found between the number of pollen grains on the stigma and the number of pollen-tubes in the style of *K.pomifera*, indicates that the pollen grains that had germinated and their tubes that penetrated the stigma remained attached throughout the preparation of the carpels for observation under the microscope. Since measurements of pollen-tube growth were made when they were half way down the style, this correlation also indicates that the transmitting tissue did not limit pollen-tube growth in the first 50% of the style. To evaluate relationships between *in vitro* and *in vivo* pollen germination, a measure of the number of both germinated and ungerminated pollen grains on the style would need to be undertaken (Cruzan 1986; Visser *et al.* 1988; Birrenkott and Stang 1989). Based on the positive association between the number of pollen grains on the stigma and pollen-tube number in the style, in the present study, it is proposed that at pollen-tube numbers of up to 2000 in the transmitting-tissue a limitation does not appear to be placed on the number of pollen-tubes in the style

for at least the first 50% of its length. However, at these densities a sharp reduction in the number of pollen-tubes was observed regularly at 80-90% of the length of the style before entry into the ovary.

No peroxidase activity was observed in the stigmas *K.pomifera* flowers up to three days after anthesis but pollen germination and seed set was observed from pollinating flowers of such maturity. In the pollen germination experiment, while flowers were pollinated 1 day before anthesis (*i.e.* at emasculation), they were not harvested for observation until six days later. On days five and six after anthesis a small percentage of the area of the stigma of such flowers exhibited peroxidase activity, indicating that the stigma was beginning to become receptive. It is therefore proposed that a proportion of pollen applied at emasculation, remained viable, and germinated at approximately five days after anthesis when the stigma became receptive. This proposal can be supported by the occurrence of seed-set in flowers pollinated at emasculation. In both cases, however, pollen germination and seed set from flowers pollinated at emasculation were both significantly lower than in those pollinated between 5 and 9 days after anthesis. No pollen-tubes were found to penetrate the ovary when pollen was applied at emasculation, but there was evidence for pollen-tube entry of the ovary when it was applied two days later. It is therefore proposed that it took approximately 48 hours for pollen-tubes to grow from the stigma and eventually penetrate the ovary.

In the present study the effective pollination period (EPP) of *K.pomifera* was found to be 13 days, since seed set was recorded as a consequence of pollen being applied to the stigma from 1 day before, to 11 days after, anthesis. Seed production from pollination one day before, to one day after, anthesis was significantly less than that from pollination at two to eleven days after anthesis. There were no significant differences in the mean number of seed set from pollination between two and eleven days after anthesis and thus, while the effective pollination period was 13 days, the optimum pollination period for *K.pomifera*, appears to extend for only approximately ten days. However, peroxidase activity was detected on the stigmas of flowers up to seventeen days after anthesis and thus the optimum pollination period may be somewhat longer than the ten days for maximum seed set found in the present study. In *Eucalyptus regnans*, the optimal time for pollination, in terms of the levels of seed set, was 12-14 days after anthesis, and following this, stigma receptivity was found to decline rapidly within one to two days (Griffin and Hand 1979). In *K.pomifera* a significant decrease in pollen germination and pollen-tube entry into the ovary was evident in flowers pollinated from seven to nine days after anthesis, indicating a reduction in stigma receptivity beyond this period. However, peroxidase activity and mean seed set were not significantly different for an additional ten and four days respectively. Further investigation is therefore required to determine the longevity of the pistil and whether desiccation of the style or ovule degeneration limits the capacity of viable pollen to effect fertilisation in the latter stages of the life of a flower.

In the breeding of *K.pomifera* maximising the number of seed per fruit is desirable to maximise the production of a range of segregants for a particular fruit. Considering the absence of a positive correlation between the level of seed set and the time of pollination between two and eleven days after anthesis, stigma receptivity does not appear to limit the level of seed set over that period, which allows considerable flexibility in the timing of artificial pollination. To attain maximum seed set in crosses in *K.pomifera* it would therefore be recommended that artificial pollination be undertaken two to eleven days following anthesis. Peak stigma receptivity was estimated to be eight days after anthesis in *Eucalyptus spathulata* and *E.leptophylla* and ten days after anthesis in *E.cladocalyx* (Ellis and Sedgley 1992). In *E.urnigera*, stigma receptivity, determined as the percentage of flowers exhibiting peroxidase activity, was estimated to occur between 13 and 30 days after anthesis, with a peak at day 20 (Savva *et al.* 1988). They proposed that the longer time from anthesis to stigma receptivity in *E.urnigera*, compared with other eucalypt species, was, to some extent, a consequence of low temperatures during flowering.

The relatively long effective pollination period (EPP) found for *K.pomifera* in the present study is potentially useful from the perspective of fruit production and plant breeding. Since it appears that stigma receptivity, pollen-tube growth and ovule longevity do not significantly limit fruit and seed set in this species, the indication is that artificial hybridisation in *K.pomifera* will not likely be beset with

complications affecting fruit and seed production. In the commercial production of *K.pomifera*, being a facultatively allogamous species (Section 9), the efficacy and activity of insect pollinators (Bierzychudek 1981; Tangmitcharoen and Owens 1997b), may be critical in the attainment of maximum yield. In *Actinidia deliciosa* (kiwifruit) low levels of pollination were found to lead to reduced fruit size and weight (Howpage *et al.* 1998). In *Eucalyptus regnans*, while capsule and seed yield were believed to be limited by levels of cross pollination, the introduction of beehives into the forest during the flowering season did not lead to increased capsule or seed yield (Eldridge 1963). It was therefore proposed that nutrient supply was most likely a significant limiting factor to capsule and seed production.

8.4.4 Conclusion

Significant increases in (a) the percentage area of the stigma exhibiting peroxidase activity (b) levels both of pollen-grain germination on the stigma and pollen-tube entry into the ovary and (c) levels of seed set, were found in flowers pollinated two days after anthesis than those pollinated one day before, to one day after, anthesis. Pollen applied one day before anthesis remained viable on the stigma until the stigma became receptive, to ultimately effect fertilisation and seed set, which however, was less than when applied to *K.pomifera* two days after anthesis. It therefore appears necessary to isolate the flowers of *K.pomifera* at least 24 hours prior to anthesis to limit the possibility of contamination with foreign pollen.

In terms of maximising the level of seed set per fruit, the optimum pollination period for the *K.pomifera* accession Kmr₁ was found to be ten days (a period from two to eleven days following anthesis). While it is possible that there is some variation in the optimum pollination period in different genotypes of *K.pomifera*, the relatively long period found in this accession, and the longevity of pollen applied to its stigma, under greenhouse conditions, indicates that obtaining seeded fruit from artificial pollination would be generally successful within the species, assuming no genetic differences in cross-compatibility between genotypes.

9. Breeding behaviour of *Kunzea pomifera*: self-incompatibility, intra- and interspecific cross-compatibility

9.1 Abstract

Artificial hybridization was undertaken, using ten different genotypes of *Kunzea pomifera* to elucidate (i) self-incompatibility (ii) intraspecific compatibility in this species, and between *K.pomifera* with each of *K.ericoides* and *K.ambigua* to elucidate (iii) interspecific compatibility. *K.pomifera* exhibited very low self-compatibility with 0.11 seeds per pollination, with the barrier to self-fertilisation being prevention of growth of pollen tubes in the style or ovary. *K.pomifera* can therefore be considered to be a preferential facultative allogamous species. Following intraspecific pollination in *K.pomifera* only 38.3% of flowers developed fruit and it appears that the arrest of compatible pollen tubes in the style, preventing fertilisation, contributes to the low fruit set in this species. Interspecific compatibility was examined between *K.pomifera*, when used as the pistillate parent, in crosses with *K.ambigua*, wherein seed set per pollinated flower (4.47) was somewhat comparable with the mean value (4.66) of crosses amongst different accessions of *K.pomifera*. In which a comparable number of seeds were produced per pollinated flower in *K.pomifera*(♀) x *K.ambigua*(♂) (4.47) and intraspecific *K.pomifera* pollinations (4.66). Conversely, incongruity was observed between *K.pomifera*, when used as the pistillate parent in hybridisation with *K.ericoides*. Reproductive barriers between these two species were evident within the style of *K.pomifera* wherein the tips of the *K.ericoides* pollen-tubes swelled and ceased to grow. The evolutionary affinities between the three species of *Kunzea*, and the implications of such relationships for the commercial breeding of *K.pomifera* (muntries) are discussed.

9.2 Introduction

The development of muntries as a significant horticultural crop will necessitate the initiation of a breeding programme to produce forms suited to commercial production that have potentially high yield and high fruit quality. The development of a breeding programme for any plant species depends upon knowledge of its breeding system so that appropriate, efficient breeding methods can be used to rapidly and objectively incorporate potentially useful variation in its commercial forms. Greater objectivity will be afforded breeding activities for optimum yield, product quality and biotic resistance if a detailed knowledge is available of genetic variation for potentially useful characters, both within the species, and other species with which it is cross-compatible. Two important considerations in the development of a breeding programme in *K.pomifera* for its commercial production are firstly the nature of the breeding system in the species and secondly its cross-compatibility with related species that are a sources for potentially useful characters for such a breeding programme. The objectives of the present study were to ascertain levels of (i) self-incompatibility in genotypes of *K.pomifera*, (ii) cross-compatibility between different genotypes of *K.pomifera* and (iii) cross-compatibility between *K.pomifera* and each of *K.ambigua* and *K.ericoides*.

9.3 Materials and methods

9.3.1 Controlled pollination

Thirteen accessions of *Kunzea pomifera* were used to study self-incompatibility within, and intraspecific compatibility between, these accessions. The plants were grown in 300mm-diameter pots in a pine-bark based potting medium and in an insect-proof greenhouse with capillary irrigation. Flowers were emasculated before anthesis using surgical scissors to cut the hypanthial rim (which included anthers) from the hypanthium. The floral tube of each flower was marked with acrylic paint, colour-coded for the date of emasculation. When every flower in an inflorescence was emasculated fresh pollen, harvested from a single accession (which was isolated from the pollination experiment), was applied to each stigma using a 1000µL sterilised pipette tip. Following pollination, the inflorescence was marked with a plastic tag colour-coded for the male parent, with the date of pollination recorded on the tag.

Seven accession of *K.pomifera* were used as female parents to evaluate pollen germination on the stigma and pollen-tube growth in the style and ovary following self-, outcross, and interspecific (with each of *K.ambigua* and *K.ericoides*) pollination. Flowers were emasculated daily by hand one day prior to anthesis for 14 days, and the floral tube of these flowers was marked with acrylic paint, colour-coded for the date of emasculation. A total of 467 stigmas were pollinated (77 self-, 253 intraspecific and 137 interspecific pollinations) 6 days after emasculation and flowers were harvested 5 days after pollination and then fixed in 45% glacial acetic acid: 70% ethanol (1:3 v/v) for 24 hours and prepared for examination under the fluorescence microscope using the procedure outlined in Section 8.3.2.

Fruit and seed production in *K.pomifera* was evaluated in 356 unpollinated, 387 self-, 2085 outcross and 1022 interspecific (754 with *K.ambigua* and 268 with *K.ericoides*) pollinated flowers. Pollinated flowers were left on the plants for approximately 4 months from pollination (late September 2001) to fruit harvest (2nd February 2002). Fruit was classified according to the date of emasculation, the date of pollination of its 'parent' flower and the male parent source. The fruit were dried in an incubator at 30°C for 3 days, after which the seeds were removed from them and counted, using a Colby hand-counter, for each hybrid category of fruit. The accessions used in the present experiment were selected from across the natural range of the species, but each exhibited horticultural potential in terms of either semi-upright habit (Ba., Ki2d, Ki7c, Ki3h) or having previously yielded fruit with a diameter at least 10mm (C1a, C1e, Gle, Ki4f, , Ki1b, Kmt1, Kmt2, Lh1a, Nc1b) (See Section 1 for collection site of these accessions). Germination of seed from artificial pollination was undertaken by placing them on filter paper (Whatmann N^o.1), moistened with deionised water, in a sealed-petri dish (55mm full plate), and then transferring them to a growth cabinet at 23°C and a 12hr photoperiod with light levels of approximately 7000lux maintained by four 30 watt, cool white, fluorescence tubes. Seeds were considered germinated after emergence of the first true leaves.

9.4 Results

No pollen grains or pollen tubes were found in the pistils of the 44 emasculated and un-pollinated flowers when observed under the fluorescence microscope, indicating that chance pollination of such flowers was a rare event. In three of the five accessions no fruit was produced in emasculated unpollinated flowers. In the unpollinated treatment, approximately 10% of flowers developed into fruit; the accession Ba possessed the highest percentage of fruit, but no fruit of this accession contained viable seed. Three fruits developed from a single unpollinated inflorescence of the accession Ki3h, and in one of these fruits a single seed developed, suggesting that pollen had inadvertently reached the stigma of one flower. The number of apparently successful fertilisation events per un-pollinated flower exhibited a frequency of 0.003, which was considered to be the level of chance self- or cross- pollination for the experiment.

9.4.1 Self-incompatibility in *K.pomifera*

The germination of pollen grains on the stigma and the growth of pollen-tubes in the style following self-pollination of *K.pomifera* occurred without any indication of abnormalities compared with intraspecific cross-pollination. The percentage of pistils possessing germinated 'self' pollen (79.9%) was slightly lower than that from intraspecific pollination (85%). The number of pollen grains germinating on the stigma and the number of pollen tubes in the style (at 50% of the style length) following self-pollination, which ranged from 31-100, appeared to be lower than that for intraspecific cross-pollination, which ranged from 101-200. Many 'self' pollen-tubes did not continue to grow beyond 90% of the length of the style and those that grew beyond this length discontinued growth in the ovary. Of the 77 self-pollinated flowers evaluated under fluorescence no instances were observed of pollen-tube penetration of an ovule.

Accession (<i>K.pomifera</i>)	N ^o . of pollinated flowers	Total n ^o . of fruit set	Fruit set per pollinated flower	Total n ^o . of seed set	Seed set per fruit	Seed set per pollinated flower
Ba	153	12	7.8	0	0	0.00
C1a	149	6	4.0	20	3.33	0.13
Ki2d	85	6	7.1	21	3.50	0.25
Total	387	24	6.2	41	1.71	0.11

Table 5: Fruit and seed set following artificial self-pollination in three accessions of *K.pomifera*.

Mean fruit and seed set per self-pollinated flower was 6.2% and 0.11 respectively (Table 5), which was substantially lower than the respective values of 39% and 4.6 resulting from intraspecific cross-pollination (Table 6). Seed set following self-fertilisation occurred in accessions C1a and Ki2d, but the number of seeds produced per fruit was approximately 9.2 and 2.3 times lower than from cross-pollination of the respective accessions. None of the fruit that was set in the accession Ba following self-pollination possessed seed. The percentage fruit set of 7.8% in this accession was substantially lower than from its unpollinated flowers, in which 44% of them set fruit without seed.

9.4.2 Intraspecific cross-compatibility in *K.pomifera*

Pollen germination and pollen-tube growth in the intraspecific crosses was found in 85% of the pistils examined. Of the pistils where pollen germination was observed, only 85% had evidence of pollen germination and 41% of ovules exhibited pollen-tube entry. Therefore only 35% of flowers pollinated showed evidence of fertilisation, which is comparable to the level of fruit set per pollinated flower of 38.8%. An apparent disorientation of pollen-tubes growing in the style was observed in 4.7% of flowers of intraspecific crosses.

Of the 2085 intraspecific crosses made in *K.pomifera* only 38.4% resulted in the production of fruit, ranging from 1.1% in C1a-(♀) x Ba-(♂) to 97.1% in Ba-(♀) x Ki4f-(♂) (Table 6). Variation was also observed among crosses for the number of seeds set per fruit from 1.4 in Ki2d-(♀) x Ba-(♂) to 33.1 in C1a-(♀) x Kmt1-(♂), with a mean of 12.1 seeds per fruit over all crosses. A total of 1022 seeds of these intraspecific crosses were sown, 80.2% of which germinated.

Female parent (<i>K.pomifera</i>)	Male parent (<i>K.pomifera</i>)	N ^o . of flowers pollinated	Total n ^o . of fruit set	Fruit set per pollinated flower (%)	Total n ^o . of seed	Seed set per fruit	Seed set per pollinated flower
Ba	C1a	81	73	90.1	164	2.2	2.0
Ba	Gle	76	73	96.1	354	4.8	4.7
Ba	Ki1b	23	20	87.0	103	5.2	4.5
Ba	Ki2d	59	49	83.1	172	3.5	2.9
Ba	Ki3h	24	21	87.5	156	7.4	6.5
Ba	Ki4f	34	33	97.1	257	7.8	7.6
Ba	Kmt1	70	49	70.0	256	5.2	3.7
Ba	Lh1a	87	66	75.9	232	3.5	2.7
C1a	Ba	183	2	1.1	11	5.5	0.1
C1a	Ki2d	195	39	20.0	1012	25.9	5.2
C1a	Kmt1	210	106	50.5	3504	33.1	16.7
C1e	Ba	40	8	20.0	54	6.8	1.4
C1e	Nc1b	10	3	30.0	41	13.7	4.1
Ki2d	Ba	112	8	7.1	11	1.4	0.1
Ki2d	C1a	56	17	30.4	117	6.9	2.1
Ki2d	Kmt1	72	25	34.7	270	10.8	3.8
Ki3h	Ba	120	23	19.2	187	8.1	1.6
Ki3h	Gle	87	34	39.1	453	13.3	5.2
Ki3h	Ki1b	32	6	18.8	106	17.7	3.3
Ki3h	Ki4f	24	6	25.0	87	14.5	3.6
Ki3h	Lh1a	94	49	52.1	569	11.6	6.1
Ki7c	Ba	156	8	5.1	87	10.9	0.6
Ki7c	Lh1a	65	29	44.6	348	12.0	5.4
Kmt2	Ba	57	2	3.5	11	5.5	0.2
Kmt2	C1a	37	21	56.8	475	22.6	12.8
Kmt2	Ki2d	53	23	43.4	544	23.7	10.3
Kmt2	Kmt1	28	5	17.9	86	17.2	3.1
	Total	2085	798	38.4	9667	12.1	4.6

Table 6: Fruit and seed set in various crosses among 13 accessions of *Kunzea pomifera*

Variation was found in all *in vivo* pollen germination and pollen-tube growth characteristics between female parents in the intraspecific crosses. In the 64 flowers of Ki2d that were pollinated with pollen from three different accessions (Ba, C1a and Kmt2), none showed evidence of ovule penetration by the pollen-tubes, with a mean of only 1.7 seeds set per pollinated flower from such pollinations.

Considerable variation was found in the capacity of the female parent to develop fruit and seed over the intraspecific crosses. Among the seven female parents of *K.pomifera* the percentage of fruit set per pollination ranged from 16.7 in Ki7c to 84.6 in Ba, and the number of seed set per pollinated flower ranged from 1.7 in Ki2d to 7.70 in C1a. Following intraspecific pollination the accession Ba set 4.4 seeds per fruit, the lowest level for all accessions evaluated, but 84.6% of its flowers of its intraspecific crosses set fruit, which was the greatest for any of the accessions.

The mean percentage of fruit set per pollination of 84.6 when Ba was used as the pistillate parent, was far greater than the mean of 25.4 for all remaining accessions. This difference, however, would be confounded by the lower fruit and seed set when Ba was used as a pollen-parent in intraspecific crosses, since the poor performance of the Ba pollen influenced the fruit and seed set in its crosses

with all accessions. The mean fruiting percentage of the remaining six accessions, with the data from Ba pollination excluded, was 35, with a range of 21-49.

The performance of the pollen parent, in terms of its capacity to effect fertilisation and influence the level of seed set in the female parent, was compared among different genotypes of *K.pomifera*. Amongst the crosses of the 10 different male genotypes of *K.pomifera* with seven female genotypes the percentage fruit set in the female parent per pollination ranged from 7.6-88.0% and the number of seed set per pollination ranged from 0.54-11.45, indicating that there was considerable variation in the capacity of the pollen from different genotypes to effect fertilisation and influence seed development in intraspecific crosses.

In the intraspecific crosses 7.6% of flowers developed fruit when Ba was used as the pollen source, which was markedly lower than the combined mean for all remaining intraspecific crosses wherein 52.7% of pollinated flowers developed into fruit. The mean number of seeds set per fruit for the intraspecific crosses using Ba pollen of 7.1, was substantially lower than many of these crosses, and much lower than the mean of all, crosses.

9.4.3 Interspecific cross-compatibility between *K.pomifera* with each of *K.ambigua* and *K.ericoides*

The germination of pollen and growth of pollen-tubes of *K.ambigua* on the stigma and in the style of seven accessions of *K.pomifera* was similar to that in the intraspecific crosses with no evidence of abnormalities. Pollen germination was observed in 69% of the crosses, and penetration of ovules (Plate 8) was observed in 24% of the pistils pollinated. In three crosses of *K.ambigua* (♂) with each of Ba, Ki2d and Kmt2 no evidence of ovule penetration by pollen-tubes was found in any of the pistils sampled, but seed set occurred in all three of these crosses (Table 7).

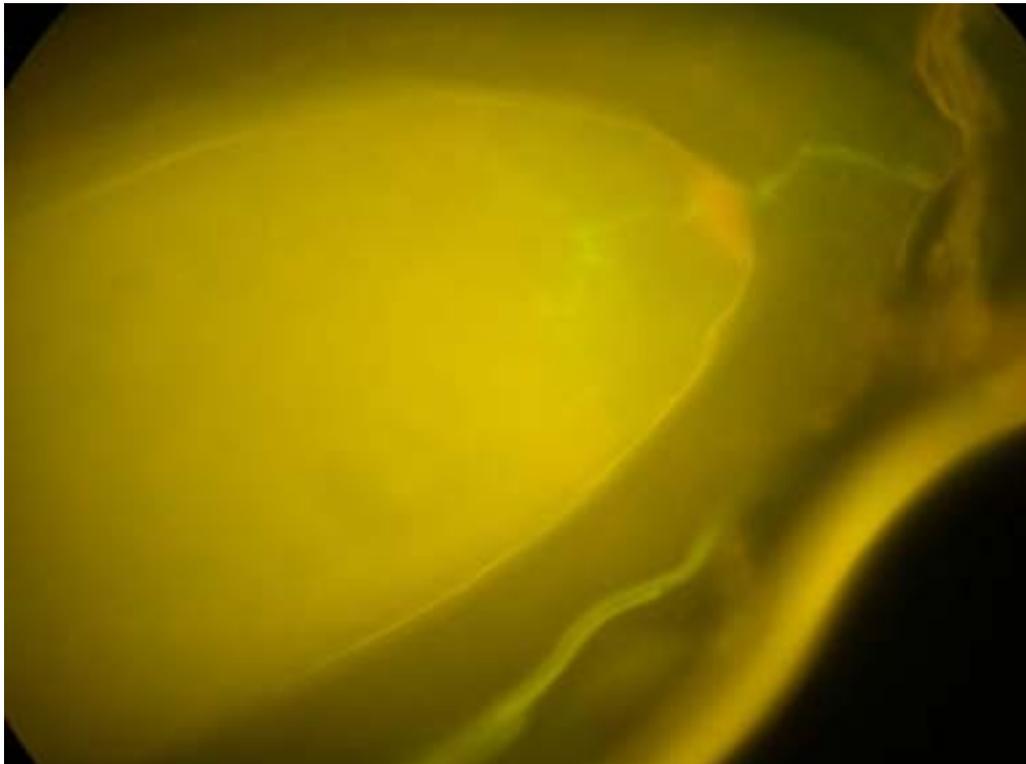


Plate 8: Penetration of an ovule (ov) of *K.pomifera* by a pollen-tube (pt) of *K.ambigua*

Variation among the F₁ crosses between *K.pomifera* (♀) and *K.ambigua* (♂) was found in the number of seeds set per pollinated flower, ranging from 1.6–7.4 (Table 7). The mean number of fruit and seed set per pollinated flower in these F₁ crosses was 12.6 and 4.5 respectively, which were comparable

with the respective means in the intraspecific crosses of 12.1 and 4.6 (Table 6). The germination percentage of the 837 F₁ (*K.pomifera* x *K.ambigua*) seed was 80.6%, which was very similar to that of seed from the intraspecific crosses (80.2%).

Female parent (<i>K.pomifera</i>)	Male parent (<i>K.ambigua</i>)	N ^o . of flowers pollinated	Total n ^o . of fruit set	Fruit set per pollinated flower (%)	Total n ^o . of seed	Seed set per fruit	Seed set per pollinated flower
Ba	Ka	129	89	69.0	824	9.3	6.4
C1a	Ka	181	48	26.5	1346	28.0	7.4
C1e	Ka	46	17	37.0	171	10.1	3.7
Ki2d	Ka	98	26	26.5	159	6.1	1.6
Ki3h	Ka	142	40	28.2	382	9.6	2.7
Ki7c	Ka	106	31	29.2	310	10.0	2.9
Kmt2	Ka	52	17	32.7	178	10.5	3.4
	Total	754	268	35.5	3370	12.6	4.5

Table 7: Fruit and seed set per fruit in F₁ hybrids of seven accessions of *K.pomifera* (♀), each with *K.ambigua* (Ka) (♂).

In the present experiment the flowering of *K.ericoides* was later than that of the majority of the accessions of *K.pomifera* and subsequently it was possible to achieve crosses between only four accessions of *K.pomifera* (Ba C1e, Ki3h and Ki7c) with *K.ericoides*. Pollen of *K.ericoides* germinated readily on the stigma of the *K.pomifera* accessions and pollen tubes were found to grow in the transmitting tissue following pollination with two accessions of *K.pomifera* (Ba and C1e). However, the tips of many pollen-tubes were found to swell, at various pollen-tube lengths, in the transmitting tissue of *K.pomifera* (Plate 9) which was not observed in any artificial self-pollination or intraspecific crosses in *K.pomifera* or in the *K.pomifera* (♀) x *K.ambigua* (♂) crosses. There was no evidence for *K.ericoides* pollen-tubes entering the ovary or ovule of the two accessions of *K.pomifera* (Ba and C1e), and, moreover, no seed set occurred from such pollinations.

Female parent (<i>K.pomifera</i>)	Male parent (<i>K.ericoides</i>)	N ^o . of flowers pollinated	Total n ^o . of fruit set	Fruit set per pollinated flower	Total n ^o . of seed	Seed set per fruit	Seed set per pollinated flower
Ba	Ke	47	45	95.7%	0	0	0.00
C1e	Ke	29	0	0.0%	0	-	0.00
Ki3h	Ke	97	0	0.0%	0	-	0.00
Ki7c	Ke	95	1	1.1%	2	2	0.0211
	Total	268	46	17.2%	2	0.043	0.0075

Table 8: Fruit and seed set per fruit in crosses of four accessions of *K.pomifera* (♀), each with *K.ericoides* (Ke) (♂).



Plate 9: Interspecific incompatibility, manifest as swollen pollen-tube tips (s-pt) of *K.ericoides* in the transmitting tissue (tt) of the style of *K.pomifera*.

The hybridisation of *K.pomifera* (♀) and *K.ericoides* (♂) resulted in only two seeds developing from a total of 268 crosses, which occurred in a single fruit of the cross between Ki7c x *K.ericoides* (Table 8). The success rate of this pollination can therefore be expressed as 1 fertilisation event per 134 pollinations (a frequency of 0.0075) indicating very low cross-compatibility between these species. In the cross between *K.ericoides* and the *K.pomifera* accession Ba, 95.7% of the flowers pollinated developed into fruit without any seed, which was markedly greater than that of unpollinated flowers of Ba (44%) and that from self-pollination (7.8%) of this accession. The fruit that developed in Ba after interspecific hybridisation with *K.ericoides* were of a similar size to those of intraspecific crosses involving Ba, but greater in size from that which developed from unpollinated flowers of this accession.

9.5 Discussion

9.5.1 Unpollinated flowers of *K.pomifera*

In the present study, in both the absence of pollination and in cross-pollination, at both the intra- and interspecific levels, a certain propensity for parthenocarpy was revealed in *K.pomifera*. Of the emasculated unpollinated flowers in the accession Ba, 44% developed fruit indicating the likelihood that this accession possesses a strong capacity for parthenocarpy. While no measurements on the size of these fruit were made it was apparent that they were substantially smaller than those which developed following intraspecific cross-pollination. In the *K.pomifera* accession Ki3h one of the three fruits that developed produced a single seed, suggesting the likelihood that this fruit arose from inadvertent pollination from ‘foreign’ pollen. However, the development of two seedless fruits in this accession, without pollination, is of interest and suggests the possibility that a biochemical stimulus from the pollination of adjacent flowers and their subsequent development of fruit may cause unpollinated flowers in the same inflorescence to also develop fruit.

When only those inflorescences without inadvertent pollination were considered in the accession Ki3h, no fruit development resulted from unpollinated flowers, which is consistent with observations in three of the five accessions (C1a, Ki2d & Kmt2). Given a lack of fruit development from unpollinated

flowers in four of the five *K.pomifera* accessions, and in the accession Ba, considerably smaller parthenocarpic fruit relative to those developed after pollination, it is proposed that under commercial production reduced fruit set would occur in the absence of successful cross-pollination this species. This is also found in other perennial fruit crops, such as apple and pear, where fruit growth and size are positively associated with seed development and number respectively (Sedgley and Griffin 1989). In the commercial production of muntries low levels of cross-pollination would result in substantially reduced fruit yields. In the present study, in the absence of cross-pollination, the percentage of fruit set in five genotypes of *K.pomifera* was 10%, but without one of the genotypes, Ba, which exhibits parthenocarpy, it was 4%. Hence the importance, in commercial plantings, of maximising cross-pollination through the use of insect vectors, or growing the crop in areas where natural vectors ensure high levels of cross-pollination. An interesting feature of accession Ba was its apparently high level of parthenocarpy and fruit set following compatible pollination. Further investigation is required to determine the relative size of parthenocarpic fruit on individuals wherein little competition for assimilate exists between pollinated and unpollinated fruits. A breeding strategy to exploit the fruiting characteristics of Ba *i.e.* (i) a high level of parthenocarpy plus (ii) a high level of fruit set from cross-pollination could lead to genotypes with high fruit yield in situations when insect pollinators are not fully effective in ensuring cross-pollination.

Flowers in the *K.pomifera* accession Ba, which were artificially hybridized with *K.ericoides* pollen, gave 95.7% fruit set, but all without seeds. There was no apparent difference in the size of such fruit compared with those from intraspecific crosses to Ba as the female parent. Given that parthenocarpic fruit in Ba were considerably smaller than that following pollination with *K.ericoides*, it appears that its pollen tubes in the style of the Ba accession exerted an effect on the development of fruit regardless of successful fertilisation. In *Vaccinium macrocarpon* (cranberry) Birrenkott and Stang (1989) found flowers were more likely to abort before fruit development when the stigma received less than ten pollen tetrads and proposed that an inadequate number of pollen tubes growing in the style, and a subsequent lack of fertilisation, is a contributing factor to the abortion of fruit in this species. The influence of pollination with incompatible, or un-viable, pollen on fruit development, and the interaction between developing fruits within an inflorescence in *K.pomifera* are matters in need of further investigation in the domestication of this species.

9.5.2 Self-incompatibility in *K.pomifera*

Resolving the nature of the breeding system in *Kunzea pomifera* is important for planning appropriate breeding strategies for its commercial improvement. Such knowledge is also important in interpreting the nature and extent of genetic variability in natural populations of the species and, in turn, it would lead to greater efficiency in the evaluation and use of this natural variation for plant breeding. The mean fruit and seed set per pollinated flower in *K.pomifera* were consistently greater following cross-, compared with self-pollination, wherein fruit set from cross-pollination was 6-times, and seed set 44-times, greater respectively than from selfing. Many 'self' pollen-tubes grew to approximately 90% of the length of the style and those that grew beyond this length discontinued growth in the ovary. In *Vaccinium corymbosum* 'self' pollen grains were found to germinate readily and the pollen-tubes grew rapidly to the base of the style, but very little seed set occurred from selfing (El-Agamy *et al.* 1981). In *Eucalyptus globulus* ssp. *globulus* the mechanism of self-incompatibility was found to act in both the late pre- and early postzygotic phases, evident by the slight reduction in the level of pollen-tube penetration of ovules and increase in post-zygotic abortion when flowers were pollinated with 'self' pollen compared with 'cross' pollen (Pound *et al.* 2002a)

In *K.pomifera* it is proposed that an effective self-incompatibility mechanism(s) operates in the lower part of the style to limit the production of 'self' seed. These observations add further to other studies indicating that self-incompatibility is prevalent in the family Myrtaceae (Beardsell *et al.* 1993b). The genus *Eucalyptus* is described as having a primarily allogamous, or preferential outcrossing, breeding system (Griffin 1982; Sedgley *et al.* 1989; Sedgley and Smith 1989; Ellis and Sedgley 1992; Pound *et al.* 2002a) and none of these studies found a barrier for 'self' - pollen tube growth in the style. Self-incompatibility has also been recorded in *Thryptomene calycina* (Beardsell *et al.* 1993a), *Melaleuca capitata* and *M.nematophylla* (Barlow and Forrester 1984).

Considering that fruit set in *K.pomifera* was lower from self-pollination than intraspecific pollination, commercial plantations based on clonal material from just the one genotype source would be expected to cause low levels of successful fertilisation because such clones would behave as 'selfs' and SI would prevent fruit/ seed set. Vander Kloet and Lyrene (1987) proposed that reduced fruit set in *Vaccinium corymbosum* would occur in plantations consisting of closely related clones than those planted with two or more unrelated individuals. Maintaining high levels of genetic variation in seed propagation populations (seed orchards) of eucalypts is important to promote outbreeding and heterozygosity and avoid inbreeding depression in the progeny used for commercial plantations (Moran *et al.* 1989; Eldridge *et al.* 1993). However clonal propagation can be used to maintain uniformity in the quality of the harvested product and to maximise heterosis in plantations, as found for those established in Aracruz, Brazil (Brandao *et al.* 1984). Such clonal eucalypt plantations can be commercially productive, since the final products (pulpwood, saw-logs, charcoal, essential oils) are, in effect, vegetative characters that are independent of the requirement for successful reproduction. Clonal plantations have also been used in the commercial production of *Melaleuca alternifolia* for tea-tree oil and *Chamelaucium uncinatum* for flower production, since there is no requirement for sexual reproduction in the commercial production of these plant products. Considering that the production of muntries is dependent on successful sexual reproduction, the breeding strategies required for its domestication will rely strongly on those developed for other fruit crops such as blueberries.

9.5.3 Intraspecific hybridisation in *K.pomifera*

The conduct of artificial intraspecific hybridisation in *K.pomifera*, because of its high level of self-incompatibility, simulates somewhat its breeding behaviour in its natural state, in that the parents of each cross will be genetically different for most characters, including, most likely, dissimilarity for the genes conditioning self-incompatibility, such that all crosses would be compatible matings. Thus levels of fruit and seed set in such crosses can reveal, with reasonable accuracy, the levels likely to be obtained in fully cross-compatible matings in its natural populations.

The mean level of fruit set amongst crosses of nine accessions of *K.pomifera* was 38%. Of the 253 flowers pollinated only 85% of them exhibited evidence of pollen germination and 41% of which gave evidence of pollen-tubes entering, at least, one ovule. Thus, evidence of successful pollination was found in only 35% of the flowers pollinated, which was similar to the level of fruit set (38%). Since it was found that pollen-tubes of *K.pomifera* in these crosses took approximately 48-hours to reach the ovary, it is assumed that observations of pollen-tube growth six days after pollination gave accurate assessments of the ultimate growth of pollen-tubes. In *Eucalyptus calophylla* Egerton-Warburton *et al.* (1993) found that only 25% of pollen tubes present in the stigma reached the base of the style. In the present study the level of pollen-tube penetration of an ovule could be used only as a guide because no evidence of ovule penetration was found for Ki2d(♀) pollinated with intraspecific pollen but 1.7 seeds were set for each flower pollinated with such pollen. Further investigation is therefore necessary to determine the factors limiting the level of fruit set per pollinated flower in intraspecific crosses.

In crosses amongst different accessions of *K.pomifera* greater numbers of fruit were set per cross to the Ba(♀) accession (84.6%) compared with the mean of the remaining accessions (35%). The variation observed in the level of fruit development (16.7-84.6%) between accessions used as female parents, and the substantially elevated levels in Ba, represents an opportunity to breed and select for this character to maximise potential yields. While having a greater fruiting percentage than other genotypes of *K.pomifera* the accession Ba produced only 4.4 seeds per fruit, with a mean of 19.3 seeds over the other accessions combined. The highest number of seeds per fruit were 30.8 and 21.9 in C1a and Kmt2 respectively. While these levels of seed production are desirable for generating large numbers of hybrid progeny for selection, examination of the effect of seed number on fruit size and quality is required before the implications of this character on breeding strategies can be assessed.

There was considerable variation in the levels of fruit and seed set amongst the intraspecific crosses between different genotypes of *K.pomifera*. In Ki3h, for instance, the mean level of fruit set in its

crosses varied from 18.8 to 52.1% depending on the pollen parent used in the cross. A mean of 0.54 seeds were set per pollinated flower when pollen from accession Ba was used to pollinate six other genotypes of *K.pomifera*, which was markedly lower than the mean of 5.7 seeds per pollinated flower for the remaining nine male parents. Given that a significantly lower *in vitro* pollen germination level was found in the Ba accession (7%) than that of four other accessions (C1a, Ki2d, Kmt1 and Ph1a: ranging from 55% to 89%)(See Section 7.4.2), it is proposed that the low level of seed set, when Ba was used as a pollen source, was the result of the low level of pollen germination found on the stigmas of its intraspecific crosses.

The fruit and seed set per pollinated flower in Kmt1(♂) x Kmt2(♀), whose ortet plants were separated by only 5 metres in their natural population, was considerably less than the mean for the combined intraspecific crosses in Kmt2(♀) and Kmt1(♂). However fruit and seed set per pollinated flower in this cross was appreciably greater than those from self-pollination of Ba, C1a and Ki2d. It is possible Kmt2 and Kmt1 may have a degree of genetic similarity, possibly as sibs derived from either one or two of the same parents. This result is consistent with the findings for *Vaccinium corymbosum* (Vander Kloet and Lyrene 1987) and *V.ashei* (Darnell and Lyrene 1989) wherein fewer seeds developed from artificial crosses between siblings, half-sibs or related cultivars than in crosses between unrelated individuals. Given that intraspecific crosses between related individuals is likely to result in low fruit set, it would appear necessary that commercial plantings of *K.pomifera* contain two or more unrelated genotypes with synchronous flowering times to maximise cross-fertilisation and, consequently, fruit production.

9.5.4 Interspecific crosses between *K.pomifera* and each of *K.ambigua* and *K.ericoides*

When pollen of a single accession of *K.ambigua* was used to pollinate seven accessions of *K.pomifera* the fruit and seed set per pollinated flower was 35.5% and 4.5 respectively, which were somewhat similar to the respective values of 38.3% and 4.6 for intraspecific crosses in *K.pomifera*. Three accessions of *K.pomifera* (Ba, C1e and Ki7c) exhibited higher mean seed set when crossed as the female parent with *K.ambigua* than for intraspecific crosses between them. Furthermore, the germination percentage of the seeds derived from the crossing of *K.pomifera* with *K.ambigua* was similar to that of seed obtained from intraspecific crosses in *K.pomifera*. These results indicate the likelihood that little, or no, reproductive barrier exists between *K.pomifera* and *K.ambigua*.

The incorporation of the upright plant habit from *K.ambigua* in *K.pomifera* may be possible by hybridisation between the two species and subsequent selection providing additive genetic effects predominate in the characters of interest (general combining ability). However a more involved procedure of reciprocal recurrent selection would be necessary to combine the desirable traits from both species in cultivars if non-additive gene effects predominated in the F₁ hybrids (specific combining ability) (Eldridge *et al.* 1993). Given that the gene pool of *K.ambigua* appears to be readily available for the breeding of commercial cultivars of muntries, some assessment of its genetic variability for potentially useful commercial characters, and the nature of their genetic control, would help determine the extent of the potential usefulness of this species for breeding *K.pomifera* for improved performance.

Barriers to successful introgression were found to exist between *Eucalyptus crebra* and *E.melanophloia*, wherein Drake (1981) found the hybrid population produced only 10% of the capsule yield of either parental species, which under natural selection, would put the hybrids at a competitive disadvantage. While segregating populations can be generated through artificial hybridisation of *Chamelaucium uncinatum* with each of *C.megalopetalum*, *Verticordia plumosa* and *V.grandis*, the resulting progeny of all crosses were infertile (Gowns *et al.* 2002) and therefore it was not feasible to carry out further breeding with them. While the high cross- compatibility between *K.pomifera* and *K.ambigua* indicates the likelihood that they are not widely divergent genetically, it would be necessary also to evaluate the fertility and seed production level of both their F₁ hybrid and F₂ progeny, because it is possible that genetic divergence between two species may not be significantly manifest until these post-hybridisation stages.

While there are no documented studies of artificial interspecific hybridisation between *Kunzea* species, reports of putative hybrids between *K.capitata* x *K.rupestris* (Wilson 2002) *K.capitata* x *K.ambigua* (Elliot and Jones 1993; Wilson 2002) *K.affinis* x *K.preissiana*, *K.micrantha* x *K.recurva*, *K.pulchella* x *K.baxteri* (Elliot and Jones 1993), and *K.ericoides* x *K.sinclarii* (Harris *et al.* 1992) indicate that the designation of species rank within the genus does not necessarily imply no cross-compatibility between certain species of the genus. Toelken (pers. comm. 2002) indicated hybridisation was possible between many species of *Kunzea* except for those involving *K.ericoides*. In ornamental horticulture and forestry the potential economic value of interspecific hybridisation often surpasses hybridisation within the commercial species (Sedgley *et al.* 1993). However interspecific hybridisation can lead to much variation in the resultant progeny that is undesirable. For instance, highly variable populations with progeny ranging from stunted to vigorous individuals were recorded from hybridisation of *Eucalyptus camaldulensis* x *E.globulus* (McComb *et al.* 2002a) and *Acacia mangium* x *A.auriculiformis* (Sedgley *et al.* 1992a). While there were no reproductive barriers between the two pairs of species, an intensive search amongst their hybrid progeny was required to identify phenotypes of commercial value. Thus the possible contribution of potentially useful genetic variation in *K.ambigua* for the commercial improvement of *K.pomifera* may require rigorous evaluation and selection amongst their hybrid progeny. In this endeavour, however, the high level of cross-compatibility between the two species indicates the likelihood that they are not widely divergent genetically and chromosomally (*i.e.* few chromosome structural differences) and thus the transfer of characters, even those under quantitative genetic control, would appear to be feasible from interspecific crosses.

9.6 Conclusion

Except for the accession Ba of *K.pomifera*, which appears to possess a high level of parthenocarpy, a prerequisite for substantial levels of fruit development in the species is successful pollination. The parthenocarpic fruit of Ba, however, was found to be substantially smaller than that resulting from intraspecific pollination. *K.pomifera* was found to be a facultative outcrossing species, where a barrier to successful self-fertilisation was evident as pollen-tube arrest in the style or ovary. Self-incompatibility in the species was manifest as levels of fruit and seed set that were 6- and 44 times lower respectively than found in intraspecific crosses. In the commercial production of muntries it is therefore proposed that a number of different genotypes are required within a plantation that ensures outbreeding to maximise fruit size and yield.

While the mean percentage fruit set from crosses amongst different genotypes of *K.pomifera* was somewhat low (38.4%), considerable variation was found between individual crosses. This indicates that there is scope in breeding to exploit this variation to maximise yields of commercial cultivars.

No barriers to hybridisation were found between *K.pomifera* and *K.ambigua*, with viable F₁ hybrid seed being readily produced between the two species. The high level of genetic compatibility between these species is highly significant for the breeding of *K.pomifera* in that potentially useful variation of *K.ambigua* appears to be readily accessible for breeding of improved cultivars of the former species.

Strong pre-zygotic reproductive barriers were found to operate between *K.pomifera* and *K.ericoides*, which was manifest in the swelling of the tips of pollen tubes, and the arrest of their growth in the style in crosses of *K.pomifera* (as female parent), with *K.ericoides*. Such incongruity between these species prevented successful hybridisation and thus development of F₁ hybrid seed. Genetic variation in *K.ericoides* therefore does not appear to be readily accessible for the breeding of improved cultivars of *K.pomifera*.

10. *Kunzea pomifera* ideotype

10.1 Abstract

Detailed studies of variation within and between natural populations of *Kunzea pomifera* (muntries) and its reproductive biology are discussed in terms of its domestication for commercial production, culminating in the proposal of an ideotype or plant type considered ideal for its commercial production. The proposed ideotype for this species grown in southern Australia has a phenotype primarily of upright habit, tolerant of a wide range of soil types, reproductively precocious, regular bearing, with a capacity for high yields of glabrous fruit of consistent size and colour with few seeds and a high pulp:seed ratio, sugar and moisture content. The proposed ideotype for *K.pomifera* also considers appropriate breeding and selection procedures, both to produce and further refine the characteristics proposed for the ideotype.

10.2 Introduction

The concept of the ideotype was proposed by (Donald 1968) for genetically increasing the yield potential of wheat crops. The philosophy behind proposing an ideotype was that the breeding programme would be orientated towards a model phenotype which would contribute, both directly and indirectly, towards increasing the genetic yield potential of the crop and the efficiency of breeding to attain this ideal phenotype particularly with regard to proposing selection strategies for certain ideotype characters. Breeding strategies using crop ideotypes have been used primarily for field crops such as *Hordeum vulgare* (barley) (Rasmusson 1987), *Cicer arietinum* (chickpea) (Siddique and Sedgley 1985; Siddique and Sedgley 1986; Siddique and Sedgley 1987), and oilseed *Brassica* spp. (Thurling 1991). However ideotypes have also been proposed for the forest tree species *Picea abies* and *Pinus sylvestris* (Kärki and Tigerstedt 1985), the agroforestry tree species *Gleditsia triachanthos* (Dickman *et al.* 1994) and for fruit trees such as *Mangifera indica* (mango) (Tyagi 1986) and *Malus* spp. (apple) (Dickman *et al.* 1994)

The potential value of the ideotype for breeding commercial cultivars can be maximised by morphological characters and knowledge of their mode of inheritance, that can influence physiological processes determining the yield of the economic product (Thurling 1991). Therefore knowledge of the particular characters that contribute positively to the physiological processes that determine fruit size, yield and quality is essential for the development of an achievable ideotype (Belford and Sedgley 1991). Such knowledge is lacking in *K.pomifera* and therefore devising a comprehensive ideotype for this species will involve a certain amount of speculation. However the ideotype for this species, even if incomplete, provides a useful set of goals for the breeding programme, in terms of germplasm required and for devising strategies for its evaluation in the breeding of the ideotype. Furthermore, the ideotype provides direction for further physiological research into plant form and function towards modifications that the breeder might effect to further increase yield potential of the crop (Dickman *et al.* 1994). Given that the ideotype generally comprises a number of characters that contribute to the final economic product, breeding of the ideotype would involve genetic improvement at a number of loci and therefore genetic gains may be achieved without severely restricting genetic variability within the breeding population at any one locus (Belford and Sedgley 1991; Dickman *et al.* 1994). This is particularly important for an outbreeding species such as *K.pomifera*, to avoid inbreeding depression and to ensure maintenance of genetic diversity in the breeding population, which is essential for both short and long term improvement.

Over the last century, a number of fruits have been domesticated, including cranberry (*Vaccinium macrocarpon*), blueberry (*Vaccinium* sp.), kiwi fruit (*Actinidia deliciosa*) and macadamia (*Macadamia integrifolia*). In deriving breeding and selection strategies in the domestication of muntries, some of the experience gained with these species is most likely relevant. Of these species, experience with blueberry domestication appears to be most relevant to muntries because of similarities in fruit size, plant habit and market niche opportunities between the two species. The domestication process of a wild plant species can involve experimentation over a considerable time span. Initial domestication

for blueberries took place over approximately 14 years from 1906 to 1920 (Galletta and Ballington 1996). The commercial development of the Australian native species quandong (*Santalum acuminatum*) took approximately 12 years from 1973 to 1985, during which time the CSIRO made assessments of growth characters, phenotypic variation and propagation methods from three plantations established in 1981 (Sedgley 1984; Possingham 1986). Similarly for muntries, the assessment of desirable production traits in genotypes selected from wild populations and the subsequent breeding to attain the ideal commercial plant form (ideotype), can be expected to involve a protracted period.

An important rationale for the domestication of a plant species must be a perceived market demand, both locally and worldwide, for its commercial product. Small local and overseas markets currently exist for both the fresh and processed product of muntries. Current demand for muntries is met, primarily through harvest from wild populations but the fruit is generally of variable quality. Future market growth depends upon a supply of fruit that is reliable and of consistent quality (Beal, pers. comm. 1999). Muntries have a unique taste that could be of appeal in a market that entertains novel food flavours. With horticultural production and market development, it is believed that the demand has the potential to expand substantially to possibly occupy a level of consumption approaching that of blueberry. In Australia, it took approximately ten years of sustained marketing for blueberries to gain recognition in the marketplace (Patel 1996). Muntries currently occupy a small niche market in Australia, and because it is a largely unknown foodstuff, even in Australia, it will take time before there is wide public awareness and acceptance of its place as another berry fruit, both in Australia and worldwide.

Important preliminary research on the domestication of a wild plant species is that which aims to evaluate genetic variation for a range of plant characters in its natural gene pool (*i.e.* variation in wild populations of the species and related, reproductively compatible, species) considered best suited for its eventual commercial production. To maintain objectivity in this research, it is useful to conceptualise an ideal plant form (ideotype) that embodies desirable characteristics, both for the plant and its commercial product. The aim of the current study was to review plant characters in *K.pomifera* that are likely to be advantageous for its commercial production in terms of maximising fruit quality, consistency and yield.

10.3 Ideotype characters in *Kunzea pomifera*

Over the last two years, representative collections from natural *Kunzea pomifera* populations were grown at the Burnley Horticultural College and assessed for a number of traits considered to be important for (i) evaluating overall genetic variation in the species and (ii) the identification of plant and fruit morphological characters for breeding forms for its domestication.

10.3.1 Plant growth and development

10.3.1.1 Plant growth habit

The most important issue facing the efficient commercial production of muntries is that of modifying its plant growth habit towards a more erect, and more readily harvestable, form. The species is generally of procumbent habit, which presents difficulties in terms of ease of harvest, reducing weed competition and the providing a microenvironment that is somewhat conducive to foliar disease. Trellising of *K.pomifera* and grafting of the species, on to the upright species *Kunzea ambigua* and *K.ericoides*, are possible avenues for introducing erect plant habit into the commercial production of *K.pomifera*. Breeding and recurrent selection of upright forms from genetic variation in *K.pomifera* is a third, but longer-term, option for incorporating erect plant habit in muntries for its commercial production. Although primarily prostrate, there is some variation within the species for degrees of upright habit that could be used for breeding for more erect habit. Recurrent selection for upright habit in *K.pomifera* is a worthwhile consideration in attempting to incorporate upright habit in its commercial forms. Given the allogamous breeding system in the species it may be expected that recurrent selection at a high intensity for upright stature would show positive responses towards more erect forms. Such a proposal should entail establishment of estimates of heritability of the prostrate vs.

erect plant habit both from crosses between existing prostrate and semi-erect forms of the species to further evaluate the feasibility for success the recurrent selection approach for producing erect habit. A further consideration is the possible incorporation of erect plant habit from *K.ambigua*, a related species through the use of interspecific hybridisation and selection. The present study indicates that such an approach may be feasible considering the existence of a high level of cross-compatibility between *K.pomifera* and *K.ambigua* and the high level of viability of their F₁ seed. The general combining ability (GCA) variance for plant height in *V.corymbosum*, *V.angustifolium* and their hybrids was found to be greater than specific combining ability (SCA) variance (Luby and Finn 1986). The narrow sense heritability for plant height was 0.68, which demonstrated that additive genetic variance was significant for this character and that the parental phenotype would be a good indicator of plant height in their progeny.

10.3.1.2 Biotic and abiotic tolerances

A well recognized pest of *K.pomifera* is the looping caterpillar of the *Macalla* spp. (Lepidoptera) which consumes its leaves, particularly juvenile ones during spring and summer, and can be easily identified in the canopy by the characteristic webs it forms. Current growers of muntries indicate that such pests can be easily controlled by physical removal before they exact major damage to the crop. While this pest is not currently a major problem in cultivation, it is critical that future improvement in *K.pomifera* does not render it more susceptible to such a pest and thus increase the potential for crop damage from this leaf herbivore. Further investigation of potential resistance to *Macalla* spp. is required to identify (i) accessions for in the breeding resistant cultivars and (ii) the morphological and/or physiological mechanisms of resistance that would be useful for selection.

Elliot and Jones (1993) proposed that the *Kunzea* genus was broadly susceptible to the soil-borne pathogen *Phytophthora cinnamomi*. Consequently screening accessions of *K.pomifera* and *K.ambigua* for resistance to such a pathogen would be useful for determining potential resistance in these species for use in the breeding of cultivars resistant to the pathogen. Clark *et al.* (1986) evaluated resistance to *P.cinnamomi* in *Vaccinium corymbosum* and found that approximately 9% of seedlings were resistant and given that a partial recessive and quantitative inheritance was identified in the genetic control of resistance and it was proposed that genetic gain for resistance could be achieved through phenotypic selection after inoculating young seedlings with the pathogen.

Kunzea pomifera occurs naturally on alkaline calcareous sandy soils along the coast of south-eastern South Australia and western Victoria and on remnant dunes further inland. Therefore increasing the tolerance of the species to a wider range of soil types is a practical breeding goal to increase the potential range of its commercial cultivation in southern Australia. Grafting *K.pomifera* as a scion on to *K.ambigua* and *K.ericoides* provides the immediate potential for achieving such improvement. *K.ambigua* has been reported to occur naturally on siliceous and granitic soils and *K.ericoides* on upper Silurian sandstone, shale and alluvial loam soils (Judd 1990). Compared to the calcareous alkaline sandy soils in which occur across the natural distribution of *K.pomifera*, the soil types described for *K.ambigua* are much lower in the level of calcium carbonate and the soils types for *K.ericoides* would be of substantially finer texture. Breeding muntries cultivars with tolerances to a wide range of soil texture and pH level would be assisted by selection from interspecific hybrids between *K.pomifera* and *K.ambigua*.

10.3.1.3 Adventitious roots

Because in the present study, *K.pomifera* was found to be an allogamous species, it is anticipated that clonal propagation be an essential activity for fixing desirable allelic combinations in commercial cultivars particularly for characters that contain considerable non-additive genetic variance (Dickman *et al.* 1994). In the routine use of clonal propagation for these purposes the capacity for improved cultivars to initiate adventitious roots when propagated by cuttings is essential for facilitating these procedures. Significant variation was found for this capacity between accessions in *K.pomifera*, and under optimal conditions for vegetative propagation the percentage of cuttings to initiate roots ranged from 68% to 96%. In *Eucalyptus globulus* (Lemos *et al.* 1997) and *E.nitens* (Tibbits *et al.* 1997) the percentage of rooted cuttings exhibited a narrow sense heritability (h^2) of approximately 0.54 and

0.47-0.50 respectively, both with low specific combining ability (SCA) effects. These results indicate that substantial genetic gain could be achieved for rooting capacity through recurrent selection for this character.

10.3.1.4 Harvest accessibility of fruit

Floral bud initiation in muntries typically occurs just behind the apical meristem of each shoot during late autumn to early winter. These buds develop into flowers during spring, and following successful fertilisation, the fruit and seed begin to enlarge. In some accessions the vegetative apical meristem remains inactive and the fruit is consequently displayed towards the ends of the shoots on the 'outside' of the bush. In other accessions, however, the vegetative shoots grow beyond the inflorescence and thus 'enclose' the fruit within the bush making the fruit less accessible to harvest. Selection of genotypes with little, or no, vegetative re-growth from the apical meristems after flowering would improve bush 'structure' for more efficient harvesting, both for hand-picking and mechanical harvesting.

10.3.1.5 Drought resistance

The effect of drought stress on a plant can range from temporary suppression of growth to permanent injury and death (Quamme and Stushnoff 1983). Plants with a degree of drought-resistance can be classified as those that either avoid, or tolerate, low internal water potential. When selecting cultivars that are drought resistant the physiological mechanisms that confer such resistance, such as reduction in leaf area and depression of metabolic activity, can be incorporated at the expense of productivity and, ultimately, yield (Jones 1993). Therefore in breeding for drought resistance, individuals with a maximum yield in a particular water-limited environment are selected. With this approach, however, advantageous features imparting drought resistance can be obscured by other unfavourable characters, such as a low harvest index, and consequently such genotypes are discarded. Therefore direct selection of the physiological processes that confer drought resistance may lead to more rapid improvement than selecting for yield under water stress (Milburn 1979; Quizenberry 1982). Genotypes with the highest yield under soil moisture deficit conditions may not be the highest yielding under conditions of favourable soil moisture, and the most desirable cultivars would be those that have a high to moderate yield that is stable between growing seasons (Parsons 1979).

Variation in the mean density of leaf stomata in *K.pomifera* may be evaluated to determine the correlation between it and water use efficiency and drought tolerance. (Dobrenz *et al.* 1969) found that clones of *Panicum antidotale* (blue panicgrass) with a lower mean leaf stomata density were more drought resistant than those with a greater density. Water-use efficiency can be defined as ratio of CO₂ assimilated per unit of water transpired (Hsiao and Acevedo 1974). The transpiration of water and assimilation of CO₂ are often positively associated since they share many of the same transport pathways (Mansfield and Davies 1981). Improving the water-use efficiency in plants can be conferred by lowering the total resistance to CO₂ relative to the total resistance to water vapour.

In *Hordeum vulgare* (barley) mean leaf stomata density was negatively correlated with stomata resistance, and positively correlated with transpiration wherein a 25% decrease in density reduced the rate of transpiration by 24% (Miskin *et al.* 1972). Given that the heritability of stomata density was up to 28% and 74% in the F₂ and F₃ generations and the rate of photosynthesis was not influenced by mean leaf stomata density, these authors proposed that it was feasible to breed barley cultivars with a greater water-use efficiency. (Heichel 1971) proposed that both the high frequency and large size of stomata reduced stomatal resistance to transpiration and only when stomata size are similar between genotypes can stomata resistance be measured to determine its relationship with stomatal density. *K.ambigua* may also be a potential source of drought resistance characteristics for incorporating into cultivars of *K.pomifera*, since (Ashton and Webb 1977) demonstrated the capacity of *K.ambigua* to rapidly reduce its water potential, after a lowering of the relative water content in its shoots. Judd (1990) found it to accumulate osmotically-active solutes under low water potential (osmoregulation), and the capacity of its leaves to tolerate desiccation. Significant variation between genotypes of wheat was found for water deficit resistance by osmoregulation (Morgan 1977) and selection for higher levels of this character in the leaves of inbred lines has lead to increases in yield under environmental

conditions that reduce leaf water potential (Morgan 1983; Morgan 1995).

Erb *et al.* (1988) demonstrated that genetic variation for drought resistance occurred within and between *Vaccinium* species and that those occurring naturally in the southern United states (*V.darrowii*, *V.elliottii* and *V.ashei*) were typically more resistant to drought stress than those from the northern areas (*V.corymbosum*, *V.vacillans* and *V.myrtilloides*). They found that drought resistance was heritable and hybrids with half their germplasm from southern species exhibited varying degrees of drought resistance. The development of cultivars of *K.pomifera* with high drought tolerance, while maintaining high yields, would be advantageous for its continued production during years with low precipitation and for its improving its potential for dryland cultivation. High leaf diffusive resistance associated with the presence of epicuticular waxes in and around the stomatal pore *Vaccinium ashei* (Andersen *et al.* 1979; Freeman *et al.* 1979) is likely to account for its high water use efficiency (96-108g H₂O/1g CO₂) (Teramura *et al.* 1979). The incorporation of high water-use efficiency in cultivars of *K.pomifera* could help limit the volume of water required to achieve maximum yields.

10.3.2 Reproductive behaviour

10.3.2.1 Reproductive precocity

In the breeding of perennial woody species such as *K.pomifera* the time take to produce new cultivars and have them in commercial production can be protracted due to the time taken for the plant to become reproductively mature and thus come to full-bearing. Therefore any method for reducing generation periods is likely to increase the rate of improvement in woody perennials from plant breeding (Hansche 1986). Improving precocity in the commercial cultivars of *K.pomifera* would also benefit producers of the crop, because it would lead to a more rapid return on investment and, over a fixed period, greater cumulative yields than less precocious cultivars with a similar yield.

For *Olea europea* Lavee (1990) proposed that the onset of reproductive maturity can take more than 15 years following germination of the seedling. In *Eucalyptus grandis* the first flowers appear on the tree two to three years after germination and a breeding cycle could be completed within four years (Hodgson 1976c). The attainment of full reproductive potential in *E.regnans* and *E.nitens* take six or more years following the planting of seed in the field (Griffin 1989). Considerable variation in the period required for seedlings to attain full sexual maturity was found in *Prunus persica* (Hansche 1986) *Corylus avellana* (hazelnut) (Mehlenbacher and Smith 1992) and *E.globulus* (Hasan and Reid 1995) indicating the likelihood that increased precociousness in these species could be achieved through recurrent selection.

The narrow sense heritability (h^2) estimate for the number of fruit in 2-year-old seedlings *Prunus persica* was 0.33 ± 0.03 (Hansche 1986). These authors proposed that the length of the juvenile period could be reduced from 3 to 2 years over 3 cycles of “mass selection” with a selection intensity of 10% for the most precocious progeny. The general combining ability for the length of the juvenile period was significant in *Pyrus communis* and *Pyrus* hybrid cultivars, and therefore under additive genetic control, and thus selection of parents for a reduced juvenile period was likely to produce more precocious progeny (Bell and DeMarini 1991). Visser (1976) found that the juvenile period in *Malus* (apple) and *Pyrus* (pear) varieties was controlled by multiple genes and exhibited additive inheritance and consequently could be modified by selection. However, no direct association was found between precocity and yield in these varieties and therefore simultaneous selection for yield and precocity should be undertaken to develop cultivars with such characteristics (Visser *et al.* 1976). Thus in a breeding programme in *K.pomifera* selection to possibly shorten the time for genotypes to reach full sexual maturity could feasibly provide the benefits of improved cultivars to growers sooner than those with a more protracted period of juvenility. However, in such selection it would need to be established that there was not an inverse relationship between high yield potential of a genotype and rapid attainment of sexual maturity

Alternatively, in breeding *K.pomifera* it is feasible that the time to reach full sexual maturity and full yield potential, could be reduced by the exogenous application of chemical compounds reported to promote flowering. The proportion of seedlings producing flower buds and the number of flower buds

per flowering seedling of 19 month old potted seedlings of *E.globulus* were significantly greater in plants to which paclobutrazol was applied as a foliar spray at the concentration of 1000ppm w/v than those without such treatment (Hasan and Reid 1995). Flowers produced following such treatment produced viable pollen and, seed from cross-pollination germinated and gave apparently normal progeny. In *Vaccinium macrocarpon* (cranberry) Serres *et al.* (1993) found that paclobutrazol hastened the induction of flowering in seedlings and, consequently, facilitated earlier evaluation of fruit traits than untreated seedlings. In three cultivars of *V.corymbosum* ('Bluetta', 'Bluecrop' and 'Jersey') foliar application of paclobutrazol (50 and 100mg/L) increased the number of flower buds produced in plants 2 to 3 years old, but it appeared that plants needed to be vigorous for it to induce a maximum response (Ehlenfeldt 1998).

10.3.2.2 Onset and duration of flowering

K.pomifera flowers during spring from, mid-September to late-November in south-eastern Australia. Considerable variation was found in the day to first flower within and between collections from its natural populations when grown in the one environment at Burnley College. The mean day to first flower across 345 accessions from 30 populations in 2001 ranged from 16th September to 3rd November. Assuming a close correspondence between the day to first flower and the day of fruit ripening for the accessions, the extent of such variation provides the potential to develop early-, mid- and late-fruiting commercial cultivars. The mean day to first flower for individuals in the Ki Ki (Kk) population was significantly later than the Kangaroo Island (Ki) and Lake Hawdon (Lh) populations by 5 and 7 days respectively, but the latter two populations were not significantly different for the mean day to first flower. This indicates that population divergence exists for this character in *K.pomifera* and over its natural range particular populations may be a good source of germplasm for characteristics such as early- or late-flowering. Flowering duration on an individual plant appears to extend over approximately four weeks, and, in consequence, variation in the time of ripening of its fruit. While as yet little is known about the genetics of flowering and duration of fruit ripening, the possibility of selecting for condensation in the flowering period of a genotype could possibly lead to commercial cultivars with more uniform fruit-ripening time. Alternatively the development of cultivars, which have the capacity to retain ripe fruit of a high quality during the maturation of remaining fruit could be useful in producing a crop that could be harvested mechanically in a single operation.

Despite the evidence for divergence between three populations (Ki, Lh and Kk) of *K.pomifera* in the day to first flower 84.4% of the variation was found within the populations. Wide variation in the flowering times of individual trees was found within natural populations of *Eucalyptus pilularis* (Florence 1964). (Ashton 1975) also found substantial variation within a natural population of *E.regnans* (Wallaby Creek, Victoria) for the time of flowering and the period of maximum flowering. In *Leptospermum myrsinoides* and *L.continentale* no significant difference occurred in the order of first flower or peak flowering between individuals over two years of observation, indicating that flowering time in these species was under some degree of genetic control. (O'Brien and Calder 1993). Gore and Potts (1995) estimated that the narrow-sense heritability for peak flowering time in *Eucalyptus globulus* ssp. *globulus* was 0.65 and proposed that this character was under additive inheritance for intraspecific hybrids. When hybridisation between *E.globulus* ssp. *globulus* and *E.nitens* were undertaken flowering time in the F₁ hybrids were most similar to the late-flowering *E.nitens*.

Irregular bearing has been found in many tree crops and is typically the result of low levels of floral initiation (Sedgley and Griffin 1989). In *Malus* cultivars Cripps (1981) found that excessive irrigation can lead to pronounced vegetative shoot growth and trunk expansion at the expense of fruit development, leading to biennial bearing. While flower and fruit yields were substantially greater in *K.pomifera* for individuals where roots were apparently restricted in potted plants, than when grown with no root restriction in field-grown plants further investigation is required to determine if any correlation exists between vegetative vigour and the number of flower bud induced in this species. Under irrigation and adequate nutrition, it has been proposed that *K.pomifera* will maintain vegetative growth at the expense of reproductive growth (Glowinski 1991; Elliot and Jones 1993). Thus in its

production under irrigation it appears that careful management of moisture availability, just prior to floral induction will most likely be critical in optimising yield in muntries. In the commercial production of *K.pomifera* resource availability for the developing fruit may also be critical in the attainment of maximum yield. Grierson *et al.* (1982) proposed that consistent reproduction in pineapple was determined by prolonged, but moderate, drought stress. In *Mangifera indica* (mango) plant stress induced by low temperature (Whiley *et al.* 1991), drought (Chacko 1989; Crane *et al.* 1997) and branch/trunk cincturing (Leonardi *et al.* 1999) have been found to reduce vegetative growth and promote flowering

Further investigation is required to identify the mechanisms involved in the initiation of reproduction in *K.pomifera*, for the objective of developing management strategies to maximise flower production and, ultimately fruit production. In *K.ericoides* Judd (1990) found that flowering and fruiting were irregular in occurrence between years, with several populations failing to flower during five years of observation, but in other populations abundant flowering was observed in one year but poorly in other years, but no cause was proposed for such variation. This author also found that levels of flower and fruit production, across several natural populations of *K.ambigua*, were consistent between years. This characteristic in *K.ambigua* warrants further investigation to determine its potential contribution to the development of muntries cultivars with high stability in the year to year induction of reproductive buds possibly leading to higher stability of fruit yield between seasons.

10.3.2.3 Parthenocarpy, pollen fertility and cross-compatibility

The development of parthenocarpic fruit in *K.pomifera* was found in only one accession (Ba), indicating that this character is not widespread in the species. Parthenocarpy could be of potential value for maximising crop yields during periods when conditions are not favourable for sufficient cross-pollination. Parthenocarpic fruit may also be of advantage in terms of fruit quality since seeds would be absent from such fruit, leading to a reduction in the gritty texture that is associated with excessive numbers of seed.

An apparent reduction in fruit diameter of parthenocarpic fruit relative to those resulting from intraspecific cross-pollination was found in the Ba accession. Given that, in the present study, both parthenocarpic and fertilised fruit developed on the same individual, the somewhat reduced size of the former may have resulted from competition for assimilate between these fruit types. However, further investigation of parthenocarpy and its association with fruit size is required to determine if this rare, but potentially useful characteristic, can be used in the development of parthenocarpic cultivars of *K.pomifera* with high yields of large fruit. While natural parthenocarpy was found in *Mangifera indica* (mango) such fruit was substantially reduced in size compared with seeded fruits (Thimmappaiah and Singh 1982), but a hybrid cultivar ('Sindhu') was produced with medium-sized parthenocarpic fruit with a small non-viable cotyledon-free stone and low seed-to-flesh ratio (Gunjate and Burondkar 1993). The development of self-compatible, high-yielding cultivars of *Prunus dulcis* (almond) is a major breeding objective, to circumvent the requirement for cross-pollination for nut set and ultimately permitting single cultivar plantings (Dicenta *et al.* 2002).

Given that fruit production in *K.pomifera* is dependent on successful cross-pollination and seed development, yield in commercial muntries plantations can be enhanced by ensuring that cultivars produce pollen with a high level of fertility to improve their capacity to effect fertilisation and subsequent fruit set following pollination of a receptive compatible stigma. In *Vaccinium corymbosum* Vander Kloet (1983) demonstrated a significant positive correlation between pollen viability of the pollen parent and the mean number of filled seed and berries. Considerable variation in fertility of fresh pollen was found between accessions in the present study, ranging from 7% to 89%, indicating that pollen viability is likely to possess a strong degree of genetic control, and therefore may be used as a selection parameter for breeding cultivars that have the potential to maximise yields insofar as pollen viability might be a limiting factor.

Considerable variation in the level of fruit set in intraspecific crosses between different genotypes of *K.pomifera* was found wherein it varied from 18.8% to 52.1% in crosses with the accession Ki3h (♀)

depending on the pollen parent used in the cross. In five *Macadamia* cultivars nut set was lower in self-, compared with cross-, pollinated flowers and significant general- and specific combining ability with significant reciprocal effects were demonstrated amongst the cultivars for nut set (Sedgley *et al.* 1990). These authors therefore proposed that nut yield in *Macadamia* plantations is likely to be influenced by both the combination and arrangement of cultivars in the plantation. While variation in the level of fruit set between intraspecific crosses in *K.pomifera* may be confounded somewhat by the level of pollen viability in the pollen parent, it indicates that specific combining ability may operate for fruit set and thus commercial cultivars should comprise different genotypes with known high specific combining ability for high fruit yield.

In the southern highbush blueberry cultivar 'Sharpblue' fruit set percentages following self pollination were found to be 77% (El-Agamy *et al.* 1981), 37% (Lyrene 1989) and 66% (Lang and Danka 1991a). Cross-pollination was found to increase yields in the first two studies but not in the latter when compared with predominantly self-pollination. Lang and Danka (1991a) found that fruit weight and seed count was increased by 13.6% and 27% respectively following cross-pollination compared with self-pollination. Furthermore the number of days from pollination to fruit maturation was negatively correlated with seed number per fruit (Lyrene 1989). Given that cross-pollination was found to increase seed number per fruit relative to self-pollination, significantly higher proportions of early-ripening fruit and lower proportions of late-ripening fruit resulted from cross-pollination relative to self-pollination (Lang and Danka 1991a). Lyrene (1989) found that pollinations with a mixture of 'self'- and 'cross'-pollen increased the fruit ripening period by an average of 7 days compared to cross-pollination, and self-pollination by an average of 20 days over cross-pollination. (Lang and Danka 1991a) therefore proposed that optimization of cross-pollination may be a valuable for growers of blueberries that produce early-ripening fruit to attract a market premium.

10.3.3 Fruit characters

Fruit traits of muntries are highly variable but commercial production of the species will most likely demand greater uniformity, in both the appearance and quality of its fruit.

10.3.3.1 Size

The size of muntries are highly variable but consistency in fruit size is important for its visual appeal to the consumer. The range in fruit weight and mean diameter across 98 accessions across 30 populations was 0.05 to 1.3g and 4.4mm to 11.3mm. The overall size of fruit may be of minor significance if traits like taste texture and colour are of primary importance to consumer preference and if there is incompatibility between fruit size and any of these characters. In blueberries large fruit has been more acceptable to the consumer and producer and has been found to be much easier to harvest and handle than smaller fruit when manual labour is used. Draper and Scott (1969) found that small fruit size in *Vaccinium australe* was dominant over that of large berries, but was quantitatively inherited.

The size of blueberries has been positively correlated with the number of seeds per fruit (Eaton 1967; Kushima and Austin 1979), with the latter being somewhat influenced by the genotype of the pollen parent (Garvey and Lyrene 1987). The latter authors found that mean seeds per fruit and fruit weight was lower after self-pollination (4 seeds and weight of 0.7g) than cross-pollination (11 seeds per 1.1g) in *Vaccinium ashei*. Therefore it may be possible to maximise fruit size under cultivation by ensuring that plantings consist of genotypes that have a high combining ability for seed set and consequently fruit size and its application in the domestication of *K.pomifera* warrants further investigation. The positive relationship between seed number and fruit size, however, was found to diminish as the number of seeds increase (Brewer and Dobson 1969; Lang and Danka 1991a) *i.e.* the beneficial effect of 10 seeds per fruit than five on fruit size/weight is more substantial than the benefit of having 15 rather than 10 seeds per fruit (Lyrene 1989).

Fruit size may also affect the seed-to-pulp ratio, and thus texture, which may be important in shaping the broad appeal of fruit to the consumer. It would also appear that with an increase in the diameter of

K.pomifera the simultaneous selection for a reduced number of berries per cluster may be necessary, since large fruit developed in tight clusters are prone to be misshapen.

10.3.3.2 Yield

Increasing yields for muntries is a primary breeding goal to ensure a maximum commercial return for the producer, and fruit at competitive prices to the market. The maximisation of yield may involve selection for greater partitioning of assimilate from vegetative parts of the plant and/or from photosynthesis concurrent with fruit development. Sutherland (1987) indicated that low fruit-flower ratios were quite common in hermaphroditic, outcrossing angiosperms and that significant improvements in yield can be made by maximising the percentage of flowers that develop into mature fruit. In the Ba accession of *K.pomifera* a high level of fruit set was found wherein approximately 85% of 'outcross' pollinated flowers developed into fruit, which was significantly greater than the mean of 35% in the remaining accessions. Such a high fruit-flower ratio in this accession offers the potential for its use in breeding cultivars with similar characteristics to maximise fruit yield. Variation in the fruit/flower yield in the species indicates the likelihood that there is scope to further increase this ratio from breeding and selection.

Maximising the level of yield stability in cultivars of *K.pomifera* between both years and environments is desirable to mitigate variations in the growing conditions and thus provide a stable source of income for producers of the crop. The capacity for achieving this breeding goal requires considerable resources for its evaluation in germplasm in a number of locations over several years. (Gupton *et al.* 1996) evaluated the stability of yield in *Vaccinium ashei* and *V.corymbosum* at nine sites over two years and proposed that stability appeared to be negatively associated with the level of yield *i.e.* the lowest yielding were the most stable and the highest yielding were the least stable.

10.3.3.3 Fruit-stalk attachment

An important fruit quality trait is the strength of its stalk attachment to the fruit. Muntries can become partially or completely detached from their stalks. With strong stalk attachment the fruit tears at the base, with a small amount of fruit on the stalk. Defective abscission detracts from the appearance of the individual fruit, because the exposed fruit tissue turns brown. The opening in the fruit left by the tear leaves the ovary exposed and allows the seed to drop from the fruit. The presence of free seed in the harvested fruit adversely affects the overall appearance of the product. In blueberry, a shallow dry fruit stem-scar is considered desirable as such a scar is considered to be the primary site for the entry of organisms implicated in rapid post-harvest decay (Galletta 1975). The pedicel breaking force in *Fragaria x ananassa* (strawberry) was primarily inherited by additive genetic variance, but this characteristic was highly influenced by environmental variance and therefore reducing the breaking force in these commercial cultivars through breeding may be problematic (Brown and Moore 1975).

10.3.3.4 Fruit surface

The colour of muntries fruit is typically a mosaic of green, red, purple and white. It is a highly variable trait and the proportions of these colours results in a somewhat non-uniform fruit colour. Some plants can exhibit single-coloured fruit in its fruiting clusters, but fruit often possess patches of white skin resulting from berries being closely adjacent in the bunches. This indicates that, as with many fruits, colour development depends on exposure to sunlight. The acceptability of this mottled fruit colour in muntries, to the consumer, is largely unknown and requires further investigation. As with highbush blueberries, there may be a relationship between fruit colour and taste (Galletta and Ballington 1996), which may be an important consideration in selecting for a particular fruit colour, for consistent fruit quality in muntries.

The density of hairs on the fruit surface is another important trait that needs to be reduced in the breeding and selection of muntries for improved fruit quality. Most fruit have some pubescence, which is variable between accessions, ranging from dense to very sparse. The sparse hairs are generally not noticeable to the naked eye and glabrous, or near-glabrous, fruit is more visually appealing than moderately, or strongly, pubescent fruit. Bonney's arborescent selection (Ba) of

muntries, bears fruit without hairs and thus could be a potentially useful parent in breeding for the glabrous-type fruit character.

10.3.3.5 Culinary traits

The texture of muntries is quite a uniform trait, as most genotypes appear to have a firm and crisp texture. Considering that *K.pomifera* is a relatively dry fruited species when anecdotally compared with other berry species such as *Vaccinium corymbosum* (blueberry), *Rubus* spp. (raspberry) and *Vitis vinifera* (grape), the evaluation of variation in fruit moisture content between genotypes is necessary to determine the feasibility of using it as a selection parameter, for producing cultivars with high fruit moisture content, which are likely help improve the market acceptability of the fruit.

Another important trait affecting fruit quality of muntries is seed:flesh ratio because high ratios can reduce the palatability of the fruit. Some accessions having a high a proportion of small dry seeds can have an unacceptably gritty texture. A reduction in seed size could lead to a reduction in the seed:flesh ratio, and a potentially concomitant improvement in the quality of such fruit. Therefore the selection criteria for achieving a low seed-to-pulp ratio would be for a reduction in seed number and an increase in fruit size. A significant positive correlation between seed number and fruit size/weight was found among open-pollinated genotypes of *Vaccinium corymbosum* (Eaton 1967) and *V.ashei* (Kushima and Austin 1979) but, the in the latter species this correlation was not constant across fruit size classes and was non-significant in fruit heavier than 2.3g.

Austin and Bondari (1993) proposed that the gritty texture of early rabbiteye blueberry (*Vaccinium ashei*) cultivars was caused by the large conspicuous seeds in its fruit, but the small seed size in the germplasm evaluated in their study meant that high seed number did not reduce the palatability of the fruit. Variation in the number of seeds per fruit has been found in *V.ashei* (Ehlenfeldt and Hall 1996) and selections with particularly low seed number have been cited as potential germplasm for the development of blueberry cultivars with such characteristics. These authors proposed that low seed numbers the rabbiteye cultivars T286 and ‘Tifblue’ may have resulted from the capacity of these cultivars to initiate normal fruit development after outcrossing with small amounts of pollen. In the present study substantial variation was found in the number of seeds per fruit in artificial crosses between different accessions of *K.pomifera*, which ranged from 1.4 to 33.1 (Table 6) and among open pollinated accessions ranged from 1.5 to 33.4. While it is possible that seed number per fruit may interact with fruit size, the substantial amount of variation in seed number exhibited in *K.pomifera* indicates the possibility to develop cultivars with a low seed:flesh ratio, which could potentially improve berry quality.

Fruit flavour is likely to be an important factor in marketing muntries and there is considerable variation within the species, with a range in taste from sweet and ‘fruity’ to bitter and astringent. Therefore attention to fruit taste will be required in breeding of muntries for fruit quality improvement. Sugar content of fruit could possibly serve as a potentially useful early generation test to indicate its sweetness level, with such an evaluation serving to reduce the population size in breeding, before the eventual use of taste tests, in later-generation material. In the present study, the total soluble solids in the fruit of 24 accessions of *K.pomifera* growing in a single environment, measured using a hand refractometer (Atago N1), varied from 7.8 to 16 °brix. While these data were collected over one season only, the range indicates the presence of substantial variation for such a character. The sugar-acid ratio in blueberries was found to be positively associated with the level of fruit palatability, and Woodruff *et al.* (1960) proposed that the minimum ratio for acceptable flavour and sweetness was 12.4 to 1 (Sugar:acid). However this ratio has been found to be negatively associated with storage quality of fruit (Galletta *et al.* 1971). Further research into the biochemical changes associated with palatability, and storage capacity in *K.pomifera* fruit is required to develop selection criteria that can be readily implemented in the field. Furthermore biochemical changes associated with fruit ripening (Woodruff *et al.* 1960) in *K.pomifera*, that can be easily measured in the field, could also be used by producers to determine the appropriate time for harvest to ensure the marketing of muntries of optimum fruit quality.

In hybridising *K.pomifera* with *K.ambigua* for breeding upright forms for the cultivation of muntries, it is likely that many of the fruit characteristics of both species would be combined. While (Judd 1990) described the fruits of *K.ambigua* as non-woody, there has been no published evidence that they are edible. In blueberry breeding programs aimed at incorporating germplasm from native species into the cultivated tetraploid highbush blueberry at least one generation of backcrossing to highbush was proposed necessary to achieve progeny with acceptable fruit characteristics (Draper *et al.* 1982). Given that the fruit quality in F₁ hybrids of *K.pomifera* with *K.ambigua* is likely to be inferior to that of *K.pomifera*, one or more backcross generations of backcrossing with *K.pomifera* would be required to produce upright forms of muntries with high quality fruit.

10.3.4 Proposed ideotype for muntries

Considering the detailed evaluation of variation in plant and fruit characters conducted over the last three years at Burnley, an ideotype is proposed for muntries for its commercial production, the components of which are detailed in Table 9. The proposed ideotype is derived from detailed investigation in the present study of morphological variation in *K.pomifera* that is likely to be modified as more information becomes available.

Plant growth and development	Perceived Benefits
Upright growth habit, with straight firm stems which form a self-supporting plant growing to a height of approximately 2 metres.	Simplification of crop maintenance and harvest including hygiene and possible mechanised harvest
Formation of numerous adventitious roots rapidly when propagated by cuttings	Simplify the routine clonal propagation of cultivars for commercial plantings
Rapid vegetative growth for the first 2 to 3 years	To provide sufficient sized canopy to enable high cropping once reproductively mature.
Fruit borne on the outside of the bush.	Facilitate ease of both manual and mechanical harvesting
Biotic/ abiotic tolerances	
High water use efficiency (<i>i.e.</i> unit of CO ₂ assimilation per unit of H ₂ O transpiration)	Minimise the irrigation inputs necessary to attain maximum yield
Capacity for tolerance to a wide range of soil pH and textures	Maximise the potential range of the species cultivation
Resistance to the soil-borne pathogen <i>Phytophthora cinnamomi</i>	Reduce the potential losses to production from infestation of this disease and allow it to be produced in areas where the disease is known to occur without the use of chemical intervention
Resistance for the <i>Macalla</i> spp. looping caterpillar leaf herbivore	Minimisation of the potential loss of productivity caused by defoliation
Capacity for maintenance of metabolic processes over a wide range of internal water potentials	Ensure continued photosynthesis during water-deficit and the possibility of maximising yield stability when grown in different environments with a wide range of soil moisture.
Reproductive behaviour	
Rapid attainment of reproductive maturity within 2 years	Increase the number of generations possible in a breeding programme and the onset of early cropping in commercial plantings
Consistent initiation of reproduction that is independent of 'stress' related factors.	Simplification of attaining maximum yield in both growth trials and production
Condensed duration of flowering period and fruit maturation.	Ensure a maximum volume of mature fruit for a single mechanical harvest
High pollen fertility	Maximise cross-pollination under cultivation and the associated benefits in yield
Capacity for parthenocarpic fruit development	Reduce the reliance on cross-pollination for fruit set under cultivation and to lower the seed:flesh ratio to improve fruit texture
Fruit Characters	
Consistency in size with a length to width ratio of approximately 1.	Improve the visual appeal of the fruit by consistent size and spherical shape
Uniform development of fruit colour (<i>i.e.</i> reduced area with no fruit colour development)	Improve colour consistency of the fruit surface and its visual appeal
Clean abscission from stalk at maturity, with insignificant stem scar	Improve the visual appeal of the fruit through an undamaged surface layer and maximise the quality of the harvest by reducing the level of free seed dispersing from such stem scars
High moisture and sugar content with appropriate flavour (acid/sugar ratio)	Improve the flavour, sweetness and juiciness of the fruit
Crisp fruit texture without the presence of astringency.	Improve fruit texture and palatability
Glabrous fruit surface.	Improve the appearance and texture of the fruit
Low seed-to-pulp ratio	Improve the texture of the fruit

Table 9: Proposed components of the ideotype in *Kunzea pomifera*

10.4 Potential for producing the ideotype

10.4.1 Phenotypic and genetic variation within and among natural populations

The successful development of *Kunzea pomifera* as a commercial fruit crop will depend on the exploitation of the natural variation for a number of characters within the gene pool of both this species, and most likely, the related species *K.ambigua*. Significant variation was found for mean leaf area, and stomata, oil-gland and trichome density both within and between three natural populations of *K.pomifera*. While these characters may not be used directly as selection criteria, the presence of such variation indicates that significant variation may also exist for other morphological characters of potential value for the domestication of the species. In blueberry breeding, leaf size and shape were considered to be neutral selection characters provided that the total leaf area of the canopy is sufficient to support high yields (Galletta and Mainland 1971). In *K.pomifera* the degree of divergence between populations appeared to depend on the particular character being evaluated, for instance the inland Ki Ki (Kk) population of *K.pomifera* was differentiated from both the coastal populations of Lake Hawdon and Kangaroo Island (Ki) for both leaf area and trichome density. However for mean oil gland density the Kangaroo Island (Ki) population was divergent from both the Lake Hawdon (Lh) and Ki Ki (Kk) populations.

Substantial variation in *K.pomifera* for the day to first flower, the total soluble solid content, mean weight and diameter in its fruit was found between genotypes growing in a single outside environment at Burnley College indicating that such characters were under some degree of genetic control. Given that all of these characters are highly important for the successful domestication of the species, the variation found during these initial analyses indicate a potential for improvement of future generations for commercial cultivation. While there is also likely to be substantial genetic variation in the species for yield characters further evaluation over several seasons of growth is required to make an accurate assessment of such a character. Many traits proposed for the ideotype have been based on observations of natural variation in the species. A further assessment period is necessary to evaluate the genetics (*e.g.* heritability, inheritance *etc.*) of these characters and their interaction in providing optimal levels of yield and fruit quality.

Genetic variation, tested using RAPDs, was greater within than between the three natural populations evaluated, with no evidence of differentiation between any of the populations. Given that the difference between phenotype characters and molecular characters in the level of partitioning for variation within and between three natural populations each character, important to the domestication of the species, needs to be evaluated to determine the level of partitioning.

10.4.2 Breeding and domestication

The largely self-incompatible breeding system of *K.pomifera* would appear to limit the opportunity for the production of homozygous breeding lines for the fixation of desirable characters in inbred lines in a hybrid breeding programme as used for the production of F₁ hybrid maize. The development of commercial cultivars in *K.pomifera* could be undertaken by hybridising superior genotypes to generate variability, from which superior progeny could be selected and propagated vegetatively (Allard 1999). Inbreeding does not form a significant component of cultivated blueberry improvement programmes since its self-incompatibility is manifest as reduced seed set and/or germination and inbreeding depression in the progeny. Therefore breeding strategies used to develop commercial cultivars of blueberry may also be applicable in the domestication and improvement of *K.pomifera* for fruit production. Increases in fruit size have been achieved in *Vaccinium corymbosum* (highbush blueberry) and *V.angustifolium* (lowbush blueberry) by the use of phenotypic assortive mating (large- x large- fruited crosses) with intensive selection for transgressive segregants over four to five generations, indicating that a significant additive component of the inheritance of fruit size occurs in this species (Galletta and Ballington 1996). Development of pest-resistant cultivars, however, has been undertaken using phenotypic disassortive mating (crosses between two phenotypically divergent individuals) with intensive selection for specific characters from each parent. In both breeding strategies, however, high heritability of commercial characters in the progeny is necessary to ensure effective selection for them. In *K.pomifera* elucidation of the levels of heritability of commercial

characters, such as fruit size, yield and taste is important to assess the potential usefulness of the above strategies for fruit yield and quality improvement.

The development of upright forms of *K.pomifera* could be most likely achieved by grafting it as a scion on to the upright species *K.ambigua* or *K.ericoides* grown as single-stemmed, two-metre high standards. Given that the development of upright cultivars of *K.pomifera* through the selection of hybrids between *K.pomifera* and *K.ambigua* is likely to take some time to achieve, the capacity to generate upright forms through grafting and trellising offers an interim solution to the problems of managing a prostrate plant in commercial production.

Despite the prostrate to semi-erect plant habit of *K.pomifera*, no topophysis for the presence of either prostrate or erect habit was found which indicates that it cannot be used to improve the species in terms of creating more upright forms for commercial production. The Ba accession of *K.pomifera* contains an appropriate combination of alleles required for upright growth and will be important in the development of upright forms of *K.pomifera*.

Successful inter-specific hybridisation has been achieved between *K.pomifera* and *K.ambigua*, which increases the genetic resources available to a breeding program for improvement of muntries. *Kunzea ambigua* has a natural distribution extending from eastern northern New South Wales to Tasmania. It is a variable upright ornamental shrub (1-5m high) and grows vigorously in cultivation in a wide range of soil types (Elliot and Jones 1993; Wrigley and Fagg 1993). The overall seed set from controlled pollinations between *K.pomifera* and *K.ambigua* was greater than some crosses within *K.pomifera*. Seed from the inter-specific crosses has been germinated and is now being subjected to preliminary selection based on the height-width ratio. It is feasible that crosses of *K.pomifera* x *K.ambigua* will provide the potential to combine the upright habit and apparent greater tolerance of different soil types of *K.ambigua*, with the fruit characters of *K.pomifera*. There is considerable variation in stem strength and habit in *K.ambigua*, but the juvenile stems are particularly 'droopy' and may be unable to support developing fruit. However, the straight rigid stem characters of some *K.pomifera* accessions, combined with the erect habit of *K.ambigua* should provide genotypes with erect and rigid stem conformation.

Further investigation of heritability estimates are required to determine the potential genetic gains for any of the ideotype characters currently proposed. In *Vaccinium corymbosum* (Edwards 1974) demonstrated that a traits such as fruit size, firmness, colour and plant vigour exhibited a high degree of additive genetic inheritance. High heritability estimates for fruit size and colour indicated that somewhat rapid genetic gain could be expected under phenotypic selection for such characters. Conversely low heritability estimates for fruit scar, firmness and plant vigour indicated that the improvement of these characters in subsequent generations would be slow. While naturally occurring blueberry species such as *Vaccinium stamineum* and *V.elliottii* can be utilised in breeding for the improvement of fruit qualities in the cultivated blueberry such as high total soluble solids and large fruit size in the former and small fruit stem scar and favourable soluble solid/acid balance in the latter (Ballington *et al.* 1984b), it is evident that the hybridisation of *K.ambigua* and *K.pomifera* for the development of muntries cultivars with an upright habit will result in the reduction of fruit quality and therefore further backcrossing and selection for fruit quality is likely to be necessary in such hybrids.

Given the high heterozygosity in *K.pomifera*, the possible quantitative inheritance for many of the selection traits and the broad number of characters under selection in the species, large seedling populations are likely to be required for each breeding cycle in the domestication of muntries. An efficient and expedient means of evaluating such progeny is therefore required for each selection criterion to make accurate selections for minimal cost. Selection of characters in the juvenile period, correlated with a mature trait may be used to reduce the size of the population, provided that no negative correlations exist with other characteristics. In *Vaccinium ashei* a significant positive correlation was identified between the glaucousness of the surface of leaves and fruit, and its expression in juvenile leaves could be used as an early selection criteria for the selection of glaucous fruit (Morrow *et al.* 1949). (Galletta 1975) proposed however, that the presence of glaucous fruit was

often negatively associated with plant vigour and fruit yield in natural populations and therefore seedlings with these latter two characteristics could be eliminated under intense early selection for leaf glaucousness.

While the use of multiple selection criteria can potentially maximise the level of genetic gain in achieving breeding objectives the cost of evaluation is typically increased, for instance (Borralho *et al.* 1993) found that the cost of selection for pulp production in *Eucalyptus globulus* by stem volume cost 33-50% of that when stem volume, wood density and pulp yield were used. Therefore each selection criteria needs to contribute directly to the profitability of the crop plant so that the added costs in selection of a particular character are compensated by an improvement of the profitability in planting the cultivar due to the inclusion of the character under consideration. The selection of parents in a cross can be critical in ensuring genetic gain for the characters under selection, wherein (Galletta and Mainland 1971) proposed that a potentially useful parent for breeding blueberry would show phenotypic superiority for a minimum of two to three characters and not inferior for any other characters than the average cultivar. This author suggested that it can be difficult to remove phenotypic deficiencies introduced into seedling populations by the use of parents that are inferior in characters under selection.

10.5 Conclusion

Based on the variation found for leaf morphology, molecular markers, flowering time, fruit size, total soluble solid content of the fruit of *K.pomifera*, and the techniques developed for its routine propagation and breeding to exploit such variation, the proposed ideotype appears achievable for the improvement of the species for commercial production for high yield and consistent quality for the marketplace. It is expected that a protracted period of breeding and selection would be required to achieve the ideotype, and to subsequently establish it in cultivation and expand, both local and world markets for its products. Both the potential appeal of its fruit and the promise for productive forms that would suit the needs of its commercial production indicate the likelihood that muntries could experience a successful future as a new food crop.

11. References

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