Breeding behaviour of *Kunzea pomifera* (Myrtaceae): self-incompatibility, intraspecific and interspecific cross-compatibility

T. Page · G. M. Moore · J. Will · G. M. Halloran

Abstract To examine breeding system characteristics of the endemic Australian prostrate shrub *Kunzea pomifera*, artificial hybridisations were undertaken using thirteen different genotypes of *K. pomifera*, to elucidate: (1) self-incompatibility, (2) intraspecific cross-compatibility in the species and (3) interspecific cross-compatibility with each of *K. ambiguа* and *K. ericoides*. *K. pomifera* exhibited very low self-compatibility, with the barrier to self-fertilisation being prevention of pollen-tube growth in the style or ovary. Following intraspecific pollination amongst a number of different genotypes of *K. pomifera*, 38.4% of pollinated flowers developed fruit; 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* (pistillate parent) and *K. ambiguа* (staminate parent) where seed set per pollinated flower (4.47) was not significantly different from intraspecific crosses (4.66). In crosses between *K. pomifera* (pistillate parent) and *K. ericoides* as staminate plant, 0.037% of pollinated flowers produced fruit, with 0.0075 seeds per pollinated flower. Reproductive barriers between these two species were evident in the style of *K. pomifera*, where the growing tips of the *K. ericoides* pollen-tubes swelled and ceased to grow.

Keywords Hybridisation incongruity · Interspecific crosses · *Kunzea ambiguа* · *Kunzea ericoides* · *Kunzea pomifera* · Self-incompatibility

Introduction

*Kunzea pomifera* (Myrtaceae) is a prostrate Australian native shrub species that produces edible berries that were highly valued by Aboriginal people (Clarke 1998) and which are now considered to have commercial potential (Graham and Hart 1997; Ahmed and Johnson 2000). Substantial variation for important economic traits exists within this and other related species, which offers the opportunity for domestication and improvement. Therefore, knowledge of its breeding system and cross-compatibility with related species that are sources for potentially useful characters is important for the development of the species as a crop. Barriers to self-pollination within (de Nettancourt 1997) and hybridisation between species (Rieseberg and Carney 1998) have evolved in many plant species to maximise fitness in the progeny. Plant species that employ ‘strategies’ to ensure out-crossing typically exhibit a range of physical, developmental and/or chemical mechanisms to restrict inbreeding. Self-incompatibility (SI) systems are important to facilitate increased levels of outbreeding within a population, to maintain high degrees of heterozygosity and in the strictest sense may be defined...
as the ‘inability of a fertile hermaphrodite seed-plant to produce zygotes after self-pollination (Lundqvist 1965).

Self-incompatibility, in all its forms, is distributed throughout angiosperms, occurring in every major phylogenetic line (Brewbaker 1957; McCubbin and Dickinson 1997) and approximately 60% of flowering plants (Hiscock and Kües 1999).

Self-incompatibility in plants can be genetically controlled and determined by the recognition and selective inhibition of a ‘self’ pollen-tube within a receptive pistil (Takayama and Isogai 2005). Self-incompatibility reactions can be divided into two distinct groups, those that exhibit gametophytic (GSI) or sporophytic (SSI) mechanisms of control. Families such as Solanaceae, Rosaceae and Papaveraceae possess species with GSI, whereas the families Brassicaceae, Asteraceae and Convolvulaceae possess SSI (Hiscock and Kües 1999). With GSI, the compatible response is determined by the pollen (gamete) and thus haploid genome expression, whereas in SSI, it is determined by the initial response of the diploid genome of the ‘parent’ plant (sporophyte) or incompatibility phenotype (Hiscock and McInnis 2003). Molecular processes of the single locus SI systems have been divided into three broad types: (a) SSI in the Brassicaceae, (b) S-RNase-mediated GSI and (c) GSI in Papaveraceae involving an S-protein independent of S-RNases (Hiscock and Kües 1999; McCubbin and Kao 2000). Late-acting self-incompatibility (LSI) is manifest as pollen-tubes where growth is inhibited in the ovary or ovule prior to fertilisation or post-zygotic rejection of the embryo (Seavey and Bawa 1986). These authors regarded instances where inhibition of ‘self’-pollen was evident before reaching an ovule as delayed expression of GSI and possibly distinct from those where inhibition takes place following pollen-tube entry of the ovule. In contrast to GSI and SSI, the genetic control and physiology of LSI are yet to be clearly understood (Allen and Hiscock 2008). Whitehouse (1950) proposed that SI likely featured in, and was fundamental to, the evolutionary success of many of the basal angiosperms. Recent phylogenetic studies indicate that SI is likely the ancestral mating system of angiosperms, with the possibility of late-acting SI in the ovary as the ‘primitive’ condition (Allen and Hiscock 2008).

In regulating the types and frequencies of hybridisation, SI can influence the genetic structure of the population of a species and can also influence the strategies to be adopted in breeding programmes of commercial species. Beardsell et al. (1993b) proposed that self-incompatibility was prevalent in the family Myrtaceae. It has been established that a mixed, but primarily allogamous, or preferential out-crossing, breeding system is widespread in the genus Eucalyptus (Griffin 1982; Sedgley 1985; Sedgley and Smith 1989; Ellis and Sedgley 1992; Pound et al. 2002).

Interspecific compatibility can effectively restrict hybridisation, leading to reduced introgression between species and maintenance of species boundaries. Interspecific incompatibility may be regulated by mechanisms of alleles within, and proteins interacting with, the S-locus, which is often evident as unilateral incompatibility (Hiscock and Dickinson 1993; Hancock et al. 2003). Alternatively, interspecific incompatibility may be independent of the S-locus and manifest as incongruency where an absence of suitable genetic information in one partner may result in prezygotic barriers to effective fertilisation between two species (Hogenboom 1973, 1975, 1984; Liedl et al. 1996). Many species, however, have the capacity to hybridise with other species with no barrier to fertilisation. In the genus Kunzea, there are examples of both artificial (de Lange et al. 2005) and natural hybridisation (Tierney and Wardle 2005) between species. Natural hybrids of Kunzea capitata and K. rupestris exhibited variable reproductive fitness between populations, with sterile hybrids produced at some locations and fertile seed-bearing hybrids at others (Tierney and Wardle 2008). Because there is little published information on the breeding system of K. pomifera, the objectives of the present study were to determine levels of (1) self-compatibility in genotypes of K. pomifera, (2) cross-compatibility between different genotypes of K. pomifera and (3) cross-compatibility between K. pomifera and each of K. ambigua and K. ericoides.

Materials and methods

Thirteen accessions of Kunzea pomifera were used to assess self-compatibility within, and intraspecific compatibility between, these accessions. The plants were grown in 300-mm-diameter pots in a soil-less potting medium, in an insect-proof greenhouse with capillary irrigation. The accessions used in the present experiment were chosen from across the natural range of the species (south-eastern South Australia and Western Victoria) (Fig. 1), but each exhibited horticultural potential in terms of either semi-upright habit (accessions Ba, Ki2d, Ki7c, and Ki3h) or having yielded fruit with a diameter of at least 10 mm (accessions C1a, C1e, Gle, K14f, Ki1b, Kmt1, Kmt2, Lh1a and Nc1b). Flowers were emasculated before anthesis using surgical scissors to cut the hypanthial rim (including the anthers) from the hypanthium. The floral-tube of each flower was marked with acrylic paint and colour-coded for the date of emasculation. When every flower in an inflorescence was emasculated, fresh pollen harvested from a single accession (isolated from the pollination experiment) was applied to each stigma using a 1,000-µl sterilised pipette tip. Following pollination, inflorescences were
marked with a plastic colour-coded tag for each pollen parent and date of pollination.

Seven accessions of *K. pomifera* were used as pistillate parents (accessions Ba, C1a, Cle, Ki2d, Ki3h, Ki7c and Kmt2) to evaluate pollen germination on the stigma, and pollen-tube growth in the style and ovary following self-pollination, cross-pollination between them. Interspecific pollinations mated each accession with *K. ambigua* and *K. ericoides*. Flowers were hand emasculated 1 d prior to anthesis for 14 d; with a total of 467 stigmas pollinated (77 self-, 253 intraspecific and 137 interspecific pollinations) 6 d after emasculation, and flowers were harvested 5 d after pollination. Excised flowers were fixed in 45% glacial acetic acid: 70% ethanol (1:3 v/v) for 24 h, soaked in deionised water for 30 min before being transferred to 4 M sodium hydroxide for 3 h at room temperature (15–20°C). They were soaked again in deionised water for 12 h and placed in an aqueous solution 0.1% aniline blue with 0.1 M potassium acetate (K3PO4) for a further 12 h. Carpels were immersed in 60 μl of glycerine, cover-slip applied and light pressure exerted using forceps to slightly flatten the preparation. Pollen-tubes were observed at 100× and 200× magnification using a Nikon E400 fluorescence microscope. Pollen numbers were evaluated according to predetermined categories (0 = 0, 1 = 1–30, 2 = 31–100, 3 = 101–200, 4 = 201–1,000 and 5 = 1,001–2,000) on the stigma and halfway along the transmitting tissue of the style. The presence or absence of pollen-grain/tubes was recorded for the (a) stigma, (b) half way along the style, (c) entering the ovary and (d) penetrating the ovule for each pistil assessed. Differences in the frequencies of pollen number categories and presence/absence scores between pollination treatments were analysed using a chi-squared goodness-of-fit test.

Fruit and seed production in *K. pomifera* were evaluated in 356 unpollinated, 387 self-pollinated, 2,085 cross-pollinated (between different accessions) and 1,022 interspecific-pollinated flowers (754 with *K. ambigua* and 268 with *K. ericoides*). Pollinated flowers were left on the plants for ~4 months from pollination to fruit harvest. Fruits were classified according to date of emasculation, date of pollination of its ‘parent’ flower and pollen source. Fruits were dried in an incubator at 30°C for 3 d, after which seeds were removed and counted using a Colby hand-counter for each category of hybrid fruit. Germination of seeds from artificial pollination was undertaken by placing them on filter paper (Whatman No. 1), moistened with deionised water in a sealed 55-mm Petri dish and incubated in a growth cabinet at 23°C with 12-h photoperiod and light levels of approximately 7,000 lux maintained by four 30 watt, cool white, fluorescent tubes. Seeds were considered germinated after emergence of the first true leaves. Plants of interspecific crosses were grown in pots until flowering and fruiting. Differences in seed set between pollination treatments were analysed using a one-way ANOVA with Tukey’s pairwise comparisons. A Spearman’s rank correlation coefficient was used to examine relationships between pollen-tube and seed set observations.

**Results**

No pollen grains or pollen-tubes were found in the pistils of the 44 emasculated and un-pollinated flowers (Fig. 2) in five accessions (Ba, C1a, Ki2d, Ki3h and Kmt2), indicating that chance pollination of such flowers was a rare event. In three (C1a, Ki2d and Kmt2) of the five accessions, no fruit was produced in emasculated unpollinated flowers. In the unpollinated treatment across all accessions, approximately 10% of flowers developed into fruit; the accession Ba possessed the highest percentage of fruit (44%), and 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. Across all accessions, the number of apparently successful fertilisation events per unpollinated flower exhibited a frequency of 0.003, which was considered to be the level of chance self- or cross-pollination for the experiment.

**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 all *K. pomifera* accessions occurred without any indication of abnormalities compared with intraspecific cross-pollination. The percentage of pistils possessing
germinated ‘self’ pollen (79.9%) was not significantly different from the percentage from intraspecific pollination (85.0%) (Fig. 2). The number of pollen grains germinating on the stigma and the number of pollen-tubes in the style (reaching at least 50% of the style length) following self-pollination were not significantly different from that for intraspecific cross-pollination. The growth of many ‘self’ pollen-tubes, however, terminated at approximately 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 of *K. pomifera* evaluated under fluorescence, no instances were observed of pollen-tube penetration of an ovule.

For the *K. pomifera* accessions, the mean fruit and seed set per self-pollinated flower was 0.062 and 0.11, respectively, which were significantly ($P < 0.001$) lower than the respective values of 0.384 and 4.6 resulting from intraspecific cross-pollination (Figs. 2, 3). Seed set following self-fertilisation occurred in two accessions (C1a and Ki2d), but the numbers of seeds produced per fruit were approximately 9.2 and 2.3 times lower than from cross-pollination of the respective accessions. None of the fruit set in the remaining accessions following self-pollination contained seed. The percentage fruit set of 7.8% in self-pollinated flowers of the accession Ba was significantly ($P < 0.001$) lower than for its unpollinated flowers, in which 44% set fruit without seed. Conversely, the percentage fruit set in self-pollinated flowers of C1a (4%) and Ki2d (7%) appeared to be greater than their respective from the unpollinated treatment, neither of which set seed (0% for...
both accessions), although no chi-squared statistics could be calculated.

Intraspecific cross-pollination in *K. pomifera*

For the intraspecific crosses of *K. pomifera*, pollen germination and pollen-tube growth was observed in 85% of the pistils examined (Fig. 2), but in only 35% of flowers pollinated were pollen-tubes observed entering an ovule, indicating the potential fertilisation level (Fig. 2). Ovule penetration was not significantly different from the level of fruit set per interspecific cross-pollinated flower (38.4%). An apparent disorientation of pollen-tubes growing in the style was observed in 4.7% of the flowers of intraspecific crosses. In each case, the determinate pollen-tubes were observed to grow and coalesce perpendicular to the central axis with some pollen-tubes also appearing to grow back towards the stigma (Fig. 4).

Variation was found in all in vivo pollen germination and tube growth characteristics between pistillate parents in the intraspecific crosses. Of 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 pollen-tubes, with a mean seed set of only 1.7 seeds per pollinated flower. In contrast, 47% of the 40 flowers of accession C1a showed ovule penetration by pollen-tubes, with 7.7 seeds set per pollinated flower. There was no significant correlation between the percentage pistils with evidence of ovule penetration and the number of seeds set per pollinated flower across all accessions.

Of the 2,085 intraspecific crosses made in *K. pomifera*, 38.4% resulted in the production of fruit (Fig. 3), ranging from 1.1% in Ba-(♂) × C1a-(♀) to 97.1% in Kmt1-(♂) × Ba-(♀). Variation was also observed among crosses for the mean number of seeds per fruit, from 1.4 in Ba-(♂) × Ki2d-(♀) to 33.1 in Kmt1-(♂) × C1a-(♀), with a mean of 12.1 seeds per fruit over all crosses. A total of 1,022 seeds of these intraspecific crosses were sown and exhibited a germination percentage of 80.2.

Significant variation was found in the capacity of the pistillate parent to develop fruit and seed within the intraspecific crosses. Among the seven pistillate 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 7.7 in C1a (Table 1). Following intraspecific pollination, accession Ba set a mean of 4.4 seeds per fruit, the lowest level for all accessions evaluated, but 84.6% of the flowers of its intraspecific crosses set fruit, which was the greatest for any of the accessions (Table 1).

The mean percentage fruit set per pollination when Ba was used as the pistillate parent (84.6%) was significantly (*P < 0.001*) greater than the mean percentage (25.4) for all remaining accessions (Table 1). This difference, however, could be affected by the lower fruit (7.6%) and seed set per pollinated flower (0.5) when Ba was used as a pollen parent in intraspecific crosses (Table 2), because the poor performance of the Ba pollen appeared to influence low 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%, which was still significantly (*P < 0.001*) lower than the 86.4% of fruit set per intraspecific cross-pollinated flower of the accession Ba.

The performance of the pollen parent, in terms of its capacity to effect fertilisation and influence the level of seed set in the pistillate parent, was compared among different genotypes of *K. pomifera*. Amongst the crosses, the percentage fruit set in the pistillate parent per pollination ranged from 7.6–87.5%, and the number of seed set per pollination ranged from 0.5–10.8 (Table 2), 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 significantly (*P < 0.001*) lower than the combined mean for all remaining intraspecific crosses, wherein 52.7% of
pollinated flowers developed into fruit (Table 2). The mean number of seeds set per fruit for intraspecific crosses using Ba pollen of 7.1 was significantly lower ($P < 0.05$) than seed set for several other pollen sources (Ki2d-15.6, Kmt1-22.2 and Nc1b-13.7) and the overall mean of 12.1 (Table 2).

Interspecific cross-compatibility between *K. pomifera* and *K. ambigua*

Pollen germination and tube growth of *K. ambigua* on the stigma and in the style of seven accessions of *K. pomifera* was anatomically similar to intraspecific crosses, with no evidence of abnormalities. Pollen germination was observed in 69% of the crosses, and penetration of ovules (Fig. 5) was observed in 24% of flowers pollinated. In three crosses of *K. ambigua* ($\varnothing$) 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 each of these crosses.

Variation among the crosses between *K. ambigua* ($\varnothing$) and *K. pomifera* ($\varpi$) was found in the number of seeds set per pollinated flower, ranging from 1.6–7.4 (Table 3). The percentage of fruit set and mean seed number per pollinated flower in these crosses were 35.5 and 4.5, respectively, which were not significantly different from the

![Fig. 5](image_url) Penetration of an ovule (ov) of *K. pomifera* by a pollen-tube (pt) of *K. ambigua*
corresponding means of 38.4 and 4.6 in the intraspecific crosses. The germination percentage of the $F_1$ ($K. \textit{ambigua}$) seed was 82.9%, which was not significantly different from that of 1,022 seed from the intraspecific crosses (79.4%).

Interspecific cross-compatibility between $K. \textit{pomifera}$ and $K. \textit{ericoides}$

In the present study, the flowering time of $K. \textit{ericoides}$ was later than that of the majority of the accessions of $K. \textit{pomifera}$, and consequently it was possible to achieve crosses between only four accessions of $K. \textit{pomifera}$ (Ba, C1e, Ki3h and Ki7c) with $K. \textit{ericoides}$. Pollen of $K. \textit{ericoides}$ germinated readily on the stigma of the $K. \textit{pomifera}$ accessions, and pollen-tubes were found to grow in the transmitting tissue of the style following pollination. However, the tips of many pollen-tubes were found to swell, at various distances of pollen-tube growth, in the lower the part of the style (Fig. 6). Such swelling of pollen-tube tips was not observed in any artificial self-pollination, intraspecific crosses in $K. \textit{pomifera}$ or in the $K. \textit{ambigua}$ ($\delta$) × $K. \textit{pomifera}$ ($\varphi$) crosses. There was no evidence for $K. \textit{ericoides}$ pollen-tubes entering the ovary or ovule of the $K. \textit{pomifera}$ accessions.

The hybridisation of $K. \textit{ericoides}$ ($\delta$) with $K. \textit{pomifera}$ ($\varphi$) resulted in only two seeds developing from a total of 268 crosses, which occurred in a single fruit of the cross between Ki7c × $K. \textit{ericoides}$. 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. Seed set per pollinated flower was not significantly different between $K. \textit{ericoides}$- and self-pollinated treatments. The two hybrid seeds between $K. \textit{ericoides}$ ($\delta$) and $K. \textit{pomifera}$ ($\varphi$) failed to germinate. In the cross between $K. \textit{ericoides}$ and the $K. \textit{pomifera}$ accession Ba, 95.7% of the flowers pollinated developed into fruit without any seed, which was significantly ($P < 0.01$) greater than that of unpollinated flowers of Ba (44.0%) and that from self-pollination (7.8%) of this accession. The fruit that developed in Ba after interspecific hybridisation with $K. \textit{ericoides}$ was of a similar size to those of intraspecific crosses involving Ba, but greater in size than that which developed from unpollinated flowers of this accession.

### Discussion

**Unpollinated flowers of $K. \textit{pomifera}$**

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 $K. \textit{pomifera}$. 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 fruits were made, it was apparent that they were substantially smaller than those that developed following intraspecific cross-pollination.

**Table 3** Fruit and seed set per fruit in interspecific crosses of seven accessions of $K. \textit{pomifera}$, each with $K. \textit{ambigua}$ (Ka) pollen

<table>
<thead>
<tr>
<th>Female parent ($K. \textit{pomifera}$)</th>
<th>Male parent ($K. \textit{ambigua}$)</th>
<th>No. of flowers pollinated</th>
<th>Total no. of fruit set</th>
<th>Fruit set per pollinated flower (%)</th>
<th>Total no. of seed</th>
<th>Seed set per fruit</th>
<th>Seed set per pollinated flower</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ba</td>
<td>Ka</td>
<td>129</td>
<td>89</td>
<td>69.0</td>
<td>824</td>
<td>9.3</td>
<td>6.4</td>
</tr>
<tr>
<td>C1a</td>
<td>Ka</td>
<td>181</td>
<td>48</td>
<td>26.5</td>
<td>1,346</td>
<td>28.0</td>
<td>7.4</td>
</tr>
<tr>
<td>C1e</td>
<td>Ka</td>
<td>46</td>
<td>17</td>
<td>37.0</td>
<td>171</td>
<td>10.1</td>
<td>3.7</td>
</tr>
<tr>
<td>Ki2d</td>
<td>Ka</td>
<td>98</td>
<td>26</td>
<td>26.5</td>
<td>159</td>
<td>6.1</td>
<td>1.6</td>
</tr>
<tr>
<td>Ki3h</td>
<td>Ka</td>
<td>142</td>
<td>40</td>
<td>28.2</td>
<td>382</td>
<td>9.6</td>
<td>2.7</td>
</tr>
<tr>
<td>Ki7c</td>
<td>Ka</td>
<td>106</td>
<td>31</td>
<td>29.2</td>
<td>310</td>
<td>10.0</td>
<td>2.9</td>
</tr>
<tr>
<td>Kmt2</td>
<td>Ka</td>
<td>52</td>
<td>17</td>
<td>32.7</td>
<td>178</td>
<td>10.5</td>
<td>3.4</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>754</td>
<td>268</td>
<td>35.5</td>
<td>3,370</td>
<td>12.6</td>
<td>4.5</td>
</tr>
</tbody>
</table>
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 develop fruit. Further investigation is required to determine the relative sizes of ‘pollinated’ and ‘unpollinated’ parthenocarpic fruits.

When only those inflorescences without inadvertent pollination were considered for the accession Ki3h, no fruit development resulted from unpollinated flowers, which is consistent with observations in three of the five accessions (C1a, K12d and Kmt2). In the absence of pollination, the percentage of fruit set in five genotypes of *K. pomifera* was 10%; excluding the genotype Ba, which exhibits a degree of apparent parthenocarpy, the level was 4%, both of which are significantly (P < 0.01) lower than cross-pollinated fruit (38%). It is proposed that reduced fruit set would occur in the absence of successful pollination in the species, indicating the importance of facilitating cross-pollination in commercial plantings. Fruit growth and size/shape are also positively associated with seed development and number, respectively, in *Malus domestica* (apple) (Dražeta et al. 2004) and *Pyrus* spp. (pear) (Callan and Lombard 1978; Rohitha and Klinac 1990).

The *K. pomifera* accession Ba hybridised with *K. ericoides* pollen gave 95.7% fruit set without any seeds. Such fruits were similar in size to intraspecific crosses with Ba as the pistillate parent. The small and most likely parthenocarpic fruit in Ba, which developed without pollination, was considerably smaller than fruit developed after pollination with *K. ericoides*. This suggests that pollen-tubes in the style of the Ba accession influenced fruit development regardless of successful fertilisation. Dong et al. (1998) found that pollen deposition on the stigma or early pollen-tube growth in the style of *Malus domestica* (apple) induced or enhanced gene action in the ovariates, resulting in the initiation of fruit development. In *Vaccinium macr-carpon* (cranberry), Birrenkott and Stang (1989) proposed that inadequate pollen-tube growth and fertilisation contributes to the abortion of fruit. The influence of pollination with incompatible or non-viable pollen on fruit development and the interaction between developing fruits within an inflorescence in *K. pomifera* need further investigation.

Self-incompatibility in *K. pomifera*

A self-incompatibility mechanism(s) operating in the lower part of the style of *K. pomifera* was evident as a reduction in pollen-tube growth at the base of the style and ovary and may be an example of late-acting SI (LSI). This mechanism(s) effectively limited the production of `self` seed, where the mean number of fruit and seed set per pollinated flower in *K. pomifera* following controlled intraspecific cross-pollination was 6-times and 44-times greater, respectively, than that from controlled self-pollination. LSI may improve the capacity of the maternal plant to exert control over gamete and zygote selection and ‘flexibility in the choice of paternal parent’, compared with both SSI and GSI (Seavey and Bawa 1986). The plasticity in pollen-tube selection associated with late-acting SI may offer considerable advantage to allocate resources regardless of the pollen source in conditions where there is scarce pollen (in fragmented populations) and ensure allocation of resources to those most vigorous under conditions of resource limitations (Lloyd 1980; Bawa and Webb 1984; Seavey and Bawa 1986). In plants with late-acting SI, the successful development of a zygote into a seed may be dependent upon maternal resource availability, and a greater proportion of self-fertilisation, which is actively modified by the maternal parent, may occur when resources are abundant (Seavey and Bawa 1986).

This phenomenon may also explain why many *Eucalyptus* species are described as having a mixed, but primarily allogamous, or preferential outcrossing, breeding system (Griffin 1982; Sedgley 1985; Griffin et al. 1987; Sedgley and Smith 1989; Ellis and Sedgley 1992; Eldridge et al. 1993; Pound et al. 2002). In *Eucalyptus regnans*, no difference was observed in levels of self- and out-crossing, with both modes of reproduction being frequently observed within individual open pollinated flowers (Griffin et al. 1987). These authors found, however, that controlled pollination of *E. regnans*, using a 1:1 ratio of ‘self’ to outcross’ pollen resulted in a seed set, wherein 81% were identified through isozyme markers, as resulting from outcrossing. When present, the apparently more robust embryos arising from outcrossing would most likely have been more vigorous, and compete more successfully for limited maternal resources, than those from selfing (Griffin et al. 1987). Preferential out-crossing also appears to operate in *E. cladocalyx* and *E. leptophylla*, since no differences were found in pollen-tube growth and ovule penetration following ‘self’ and ‘outcross’ pollination, but self-pollination resulted in extremely low seed set (Ellis and Sedgley 1992). As in many perennial plants (Lloyd 1980; Lloyd et al. 1980), *K. pomifera* produces substantially greater numbers of flowers than fruits. In this study, controlled ‘self’, intraspecific and interspecific pollinations were carried out on inflorescences within an individual ramet. While much consideration has been given to male gametophyte selection within a pistil (Mulcahy 1979; Hormaza and Herrero 1992; Herrero and Hormaza 1996; Bernasconi et al. 2004), it may also be possible that the
pollen–pistil interaction operates to maximise male gametophyte selection between pistils. The pistillate parent may limit and allocate resources to flowers and/or inflorescences with only self and outcross pollen, respectively, since it is well recognised that maternal resource allocation is often regulated for individual flowers and fruits (Lloyd 1980). If late-SI in *K. pomifera* provides a mechanism for gamete and zygote selection at the level of a flower then a reduced level of ‘self’ pollen-tube inhibition may be expected if all pollinations on an individual maternal ramet were from ‘self’ pollen. Such a mating system would be of advantage in highly fragmented natural populations, where seed production may still be possible in the total absence of ‘outcross’ pollen. Instances of late-acting pre-zygotic SI, similar to that found in *K. pomifera*, include tight coiling of self-pollen-tubes at the base of the style as observed in *Melaleuca capitata* and *M. nematophylla* (Barlow and Forrester 1984), pollen-tube rejection near the micropyle as in *Thryptomene calycina* (Beardsell et al. 1993a), and reduction in growth towards the base of the style or in the ovary before ovule penetration, as seen in * Vaccinium corymbosum* (El-Agamy et al. 1981) and several species of Eucalyptus (Pound et al. 2002; Horsley and Johnson 2007).

Late-acting self-incompatibility has been identified in apparently phylogenetically distant families such as Apocynaceae, Bignoniaceae, Myrtaceae, Vochysiaceae and Winteraceae, and in several families of the orders Fabales, Liliales and Malvales (Gibbs and Bianchi 1999). The presence of LSI in such a diverse range of families may be indicative of multiple independent evolution during angiosperm diversification (Allen and Hiscock 2008). The presence of late-acting SI in some basal angiosperms is believed to be evidence that it appeared very early in the evolution of angiosperms (Godley and Smith 1981; Endress and Igersheim 2000; Endress 2001; Sage and Sampson 2003) and possibly predated the evolution of the closed carpel (Kenrick et al. 1986). In some species, LSI may have further evolved into or was replaced by more complex self-incompatibility systems in the stigma or style (Barrett 1988; Allen and Hiscock 2008).

In many species, it is unclear whether the reduced seed set following self pollination is the result of late-acting SI, inbreeding depression or a combination of both (Sage et al. 1994). Gibbs and Bianchi (1999) reasoned that LSI was an active mechanism since its occurrence in many species within individual plant families would not be expected if accumulated lethal equivalents associated with inbreeding depression were the latent cause of self pistil rejection. These authors recognised that the apparent family clustering may be an artefact of biased sampling among existing research publications, and a better understanding of the taxonomic distribution of LSI is needed. While the expected differences in zygotic behaviour between species with LSI and inbreeding depression have been summarised (Barrett 1988; Sage et al. 1994), it is difficult to attribute the arrest of the ‘self’ pollen-tubes in the style and ovary of *K. pomifera* to either phenomenon. The level of outcrossing in species with cryptic self-incompatibility can be dependent on the size and composition of the pollen load on stigma (Cruzan and Barrett 1996). These authors proposed that outcrossing rates are positively associated with size of the pollen load on the stigma in mating systems that rely on differences in pollen competitive ability, which contrasts with those systems controlled by variation in gamete attrition, where no such association exists. The little variability in ‘self’ seed set between genotypes observed in this study suggests the existence of late-acting SI, since greater variability in the site of pollen-tube arrest and the level of ‘self’ seed set may be expected if inbreeding depression was the sole mechanism. Comparative evaluations on the rate of pollen-tube growth between ‘self’ and outcross pollen-tubes may provide greater insight into the mechanisms operating in *K. pomifera*, a slower rate of ‘self’ pollen-tube growth suggests that SI is of greater significance than inbreeding depression (Bittencourt and Semir 2004).

The prevalence of late-acting SI is greater among woody perennial species and those with hollow or incomplete styles, compared with herbaceous and ‘solid-styled’ species (Brewbaker 1957; Kenrick et al. 1986; Barrett 1998; Allen and Hiscock 2008). While a hollow style is not significant than inbreeding depression (Bittencourt and Semir 2004). The presence of LSI in such a diverse range of families may be indicative of multiple independent evolution during angiosperm diversification (Allen and Hiscock 2008). The prevalence of late-acting SI in some basal angiosperms is believed to be evidence that it appeared very early in the evolution of angiosperms (Godley and Smith 1981; Endress and Igersheim 2000; Endress 2001; Sage and Sampson 2003) and possibly predated the evolution of the closed carpel (Kenrick et al. 1986). In some species, LSI may have further evolved into or was replaced by more complex self-incompatibility systems in the stigma or style (Barrett 1988; Allen and Hiscock 2008).

In many species, it is unclear whether the reduced seed set following self pollination is the result of late-acting SI, inbreeding depression or a combination of both (Sage et al. 1994). Gibbs and Bianchi (1999) reasoned that LSI was an active mechanism since its occurrence in many species within individual plant families would not be expected if accumulated lethal equivalents associated with inbreeding depression were the latent cause of self pistil rejection. These authors recognised that the apparent family clustering may be an artefact of biased sampling among existing research publications, and a better understanding of the taxonomic distribution of LSI is needed. While the expected differences in zygotic behaviour between species with LSI and inbreeding depression have been summarised (Barrett 1988; Sage et al. 1994), it is difficult to attribute the arrest of the ‘self’ pollen-tubes in the style and ovary of *K. pomifera* to either phenomenon. The level of outcrossing in species with cryptic self-incompatibility can be dependent on the size and composition of the pollen load on stigma (Cruzan and Barrett 1996). These authors proposed that outcrossing rates are positively associated with size of the pollen load on the stigma in mating systems that rely on differences in pollen competitive ability, which contrasts with those systems controlled by variation in gamete attrition, where no such association exists. The little variability in ‘self’ seed set between genotypes observed in this study suggests the existence of late-acting SI, since greater variability in the site of pollen-tube arrest and the level of ‘self’ seed set may be expected if inbreeding depression was the sole mechanism. Comparative evaluations on the rate of pollen-tube growth between ‘self’ and outcross pollen-tubes may provide greater insight into the mechanisms operating in *K. pomifera*, a slower rate of ‘self’ pollen-tube growth suggests that SI is of greater significance than inbreeding depression (Bittencourt and Semir 2004).

The prevalence of late-acting SI is greater among woody perennial species and those with hollow or incomplete styles, compared with herbaceous and ‘solid-styled’ species (Brewbaker 1957; Kenrick et al. 1986; Barrett 1998; Allen and Hiscock 2008). While a hollow style is not
viable seed in bagged flowers does indicate a degree of self-compatibility. de Lange et al. (2005) found self-compatibility was prevalent in the New Zealand K. ericoides complex. Variation in self-incompatibility between individuals within a species has been found in Eucalyptus cladocalyx for which Ellis and Sedgley (1992) proposed that the species was largely self-incompatible, but individual trees ranged from self-compatible to self-incompatible. In E. globulus ssp. globulus, four of five genotypes possessed greater than 99% self-incompatibility, but one individual was only 76% self-incompatible (Pound et al. 2002). There appeared to be very little variation in the degree of self-incompatibility between accessions of K. pomifera in the present study, with all accessions producing fewer than 0.3 seeds per pollinated flower from self-, compared with 4.6 seeds from cross-pollinated flowers (Fig. 3). The small number of accessions examined in this study, however, does not provide evidence for the possible extent of this variation in natural populations.

The results of this study indicate that monoclonal plantations of muntries would essentially behave as ‘selfs’ and thus be expected to have low levels of successful fertilisation and fruit set. Therefore, clonal mixtures would be recommended for commercial plantings as reported fertilisation and fruit set. Therefore, clonal mixtures would be expected to have low levels of successful in vivo fertilisation. The levels of fruit and seed set in artificial intraspecific crosses of K. pomifera (38%) was not significantly different from the level of successful in vivo fertilisation (35%). Pollen-tube penetration of an ovule, however, could be used only as a guide for potential fertility, because no evidence of ovule penetration was found for Ki2d (♀) after artificial cross-pollination, but 1.7 seeds were set for each flower pollinated with such pollen. Given that only 38% of all artificial cross-pollinated flowers set fruit in this experiment, further investigation is necessary to determine the factors limiting the level of fruit set per pollinated flower in intraspecific crosses in K. pomifera.

In crosses amongst different accessions of K. pomifera, there were greater numbers of fruit set per cross involving the pistillate 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 when used as pistillate 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. This apparently anomalous fruiting behaviour of accession Ba is most likely associated its very high propensity for parthenocarpy. The highest mean numbers 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 possible influence of seed number on fruit size and quality is required before the significance of this character in breeding strategies can be assessed.

There was considerable variation in the levels of fruit and seed set amongst the intraspecific crosses between different pollen 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. Such variation could be exploited in plant breeding activities to develop individual, or combinations of genotypes to maximise fruit yields in production. A mean of 0.54 seeds was set per pollinated flower when pollen from accession Ba was used to pollinate six other genotypes of K. pomifera, which was significantly lower than the mean of 5.7 across the remaining nine pollen 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%) (Page et al. 2006b), it is proposed that the low level of seed set, with Ba as a male parent, was due to a low level of pollen germination found on the stigmas of its intraspecific crosses.
The percentage fruit set per pollinated flower and seed set per fruit in the cross Kmt1 (♀) × Kmt2 (♂) (17.9%, 17.2, respectively), were significantly (P < 0.05) lower than the overall means of the intraspecific crosses of Kmt1 (♀) (48.7%, 22.2) and Kmt2 (♂) (29.1%, 21.9) with each of the remaining accessions. When these measures for the Kmt1 (♀) × Kmt2 (♂) cross were compared with artificial self-pollinated treatments, they were found to be significantly (P < 0.05) greater than the means for Ba (7.8%, 0.0), C1a (4.0%, 3.3) and Ki2d (7.1%, 3.5). Given that Kmt2 and Kmt1 were separated by only 5 m in their natural population, it is possible these individuals have a degree of genetic similarity, possibly as sibs derived from either one or two of the same parents in the natural population from which they were obtained. 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 to maximise cross-fertilisation, and consequently, levels of fruit production.

Interspecific crosses between K. pomifera and each of K. ambiguа and K. ericoides

When pollen of K. ambiguа was used to pollinate seven accessions of K. pomifera, the fruit and seed set per pollinated flower were 35.5% and 4.5, respectively, which were not significantly different from the respective values of 38.4% and 4.6 for all of the intraspecific crosses in K. pomifera. The crosses involving two accessions of K. pomifera (Ba and C1e) exhibited a significantly (P < 0.05) higher mean seed set when crossed as the pistillate parent with K. ambiguа (6.4 and 3.7, respectively, Table 3) compared with intraspecific accessions (3.7 and 1.9, respectively, Table 3). Furthermore, the germination percentage of the seeds derived from the crossing of K. pomifera with K. ambiguа was not statistically different to that of seed obtained from intraspecific crosses in K. pomifera. The progeny from these hybridisations were grown in pots and flowered after 3–4 years and set fruit in 4–5 years. The high level of cross-compatibility between these two species indicates the likelihood that they are not genetically very divergent, and thus the transfer of characters, even those under quantitative genetic control, would appear to be feasible from interspecific crosses. This, however, depends on the relative fitness of advanced generation hybrids, since reduced reproductive vigour in the progeny in a number of interspecific Eucalypt hybrids has been reported with no reproductive barriers in the generation of the F1 (Drake 1981; Potts 1986; Lopez et al. 2000).

Artificial interspecific hybridisation has been achieved between species within the New Zealand Kunzea ericoides complex (de Lange et al. 2005), between K. ericoides and K. sinclarii (Harris et al. 1992; de Lange and Norton 2004; de Lange et al. 2005) and K. rupestris and K. capitata (Tierney and Wardle 2008). Other putative Kunzea hybrids have been reported between K. capitata × K. ambiguа (Elliot and Jones 1993; Wilson 2002) K. affinis × K. pressiana, K. micrantha × K. recurva and K. pulchella × K. baxteri (Elliot and Jones 1993). These studies indicate that the designation of species rank within the Kunzea genus does not imply cross-incompatibility between certain species of the genus. However, de Lange et al. (2005) were unable to produce interspecific hybrids through artificial pollination between K. ericoides complex with each of K. baxteri, K. parvifolia and K. peduncularis.

The site of rejection for K. ericoides pollen-tubes, in the lower part of the style, is equivalent to that of ‘self’ pollen, indicating that they might be potentially controlled (at least in part) by the same mechanism(s). Interspecific barriers to successful fertilisation that are controlled by the self-incompatibility mechanism may be manifest as unilateral incongruity where the pollen of a self-compatible (SC) species is inhibited in the style of a self-incompatible (SI) species, but not in reciprocal crosses (SI × SC rule) (Lewis and Crowe 1958; de Nettancourt 1977, 1997). Further investigations of reciprocal crosses by pollinating K. ericoides with K. pomifera pollen may help to determine the existence of any unilateral incompatibility between these species, however, given that K. ericoides has been recorded to be self-compatible (SC) (Burrell 1965; de Lange et al. 2005), any unilateral incongruity may not conform to the SI × SC rule. The implication of the S-locus in the rejection of interspecific pollen-tubes has been demonstrated for a number of genera such as Nicotiana (Pandey 1981; Murfett et al. 1996; McClure et al. 2000; Hancock et al. 2003) and Lycopersicon (Bernacchi and Tanksley 1997), but it is likely to be modified by other mechanisms. In the present study, the largely self-incompatible K. pomifera successfully hybridised with K. ambiguа but not K. ericoides; this variation in response may be evidence that the interspecific barrier between K. ericoides and K. pomifera is likely to be functionally distinct from that of self-incompatibility in K. pomifera. Exceptions to the SI × SC rule are not uncommon (Lewis and Crowe 1958; de Nettancourt 2001), however, and Mutschler and Liedl (1994) proposed that self- and interspecific-incompatibility were functionally distinct. In Brassicaceae, for instance, morphological similarities between the SI and UI reaction were found among several...
species (Hiscock and Dickinson 1993; Takada et al. 2005), but segregation analysis indicated that the UI was independent of the S-locus (Takada et al. 2005).

In the present study, the growth of *K. ericoides* pollen-tubes terminated in the style of *K. pomifera* with distinct swelling of their tips, which was not observed in any of the ‘self’ pollen-tubes. Such swollen pollen-tube tips have been recorded in interspecific crosses between species of *Solanum* (Fritz and Hanneman 1989) and *Psychotria* (Fairev 2002). The disparity in the morphology of ‘self’ and interspecific pollen-tubes in *Lilium* (Ascher and Peloquin 1968), *Lycopersicon* (Liedl et al. 1996) and *Capsicum* (Onus and Pickersgill 2004) led these authors to propose that the mechanism(s) of interspecific rejection may be distinct from those of self-incompatibility. In this study, self-pollen-tubes of *K. pomifera* were observed to enter the ovary in some cases, but this was not the case for any of the samples examined for *K. pomifera × K. ericoides* artificial crosses. Therefore, given the difference in the morphology of self and interspecific pollen-tubes and the more comprehensive interspecific incompatibility barrier in the style, it is possible that self and interspecific incompatibility mechanisms in *K. pomifera* are distinct. Hogenboom (1973, 1975, 1984) described interspecific incongruity as being independent of the self-incompatibility mechanism and arising as a consequence of evolutionary divergence between two species. Incongruity described by Shivanna (1997) is more of a passive process and not the result of active pollen recognition and rejection, but from the absence of specific genetic information in one partner (i.e. pollen) for some relevant character of the other (i.e. pistil). While genetic divergence between the three species used in this study is yet to be determined, differing degrees of such divergence may explain why successful hybrids of *K. pomifera* were formed with *K. ambiguia*, but not with *K. ericoides*. Liedl et al. (1996) reported that the phenotypic manifestation of self-incompatibility was distinct from interspecific incompatibility between *Lycopersicon esculentum* and *L. pennellii* and therefore independent of each other. In a QTL analysis of unilateral incongruity between *L. esculentum* and *L. hirsutum*, it was demonstrated that the mechanism was associated with the S-locus indicating the likelihood that SI and UI were not necessarily independent (Bernacchi and Tanksley 1997). Kuboyama et al. (1994) proposed that in *Nicotiana tabacum*, the differences observed in pollen-tube morphology following interspecific pollination with *N. rustica*, *N. repanda* and *N. trigonophylla* were indicative of the differences in physiological causes of incongruity between the three crosses. Murfett et al. (1996), however, reported that the morphology and site of pollen-tube rejection among *Nicotiana* species were not reliable indicators of their mechanism despite the involvement of the S-locus in both self- and interspecific-incompatibility reactions. Molecular analysis of the S-locus has revealed that SI represents not just a single system, but a ‘collection of divergent mechanisms’ (Takayama and Isogai 2005), which is likely to explain the variability in both self- and SI-mediated interspecific incompatibility. Therefore, while the apparent phenotypic differences between self- and interspecific-incompatibility in *K. pomifera* indicate they may be independent, further investigations are required to determine the genetic control of each.

**Conclusions**

*Kunzea 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 unrelated genotypes are required within a plantation in a design that optimises the level of cross-pollination between them to maximise fruit size and yield.

No barriers to hybridisation were found between *K. pomifera* and *K. ambiguia*, 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*, since potentially useful variation in *K. ambiguia* is readily accessible for breeding of improved cultivars of *K. pomifera*.

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 pistillate parent), with *K. ericoides*. Such barriers 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*. Further investigations, however, are required to determine the specific mechanism(s) involved in this barrier.

With the notable exception of one accession (Ba), successful outcross pollination is a prerequisite for fruit development in *Kunzea pomifera*. The parthenocarpic fruit of Ba was found to be substantially smaller than that resulting from intraspecific pollination. 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 the likelihood for a good potential to exploit
this variation in breeding to maximise yields of commercial cultivars.

Acknowledgments T. P. was the recipient of an Australian Postgraduate Award (APA), and the project received funding support from the Rural Industries Research and Development Corporation (RIRDC) and RWS Nicholas Agricultural Science Scholarship. Neville Bonney kindly donated the ‘Ba’ accession for use in this study.

References


Rohitha BH, Klinac DJ (1990) Relationship between seed set and fruit weight and shape of nashi (Pyrus serotina Rehder var. culta Rehder). NZ J Crop Hort Sci 18:133–136


