Effects of Self-, Chase and Mixed Self/Cross-pollinations on Pistil Longevity and Fruit Set in Ceiba Species (Bombacaceae) with Late-acting Self-incompatibility

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INTRODUCTION

Late-acting self-incompatibility (LSI) has proved to be a contentious topic since it was introduced by Seavey and Bawa (1986). In general terms, the phenomenon applies to species which do not possess conventional self-incompatibility (SI) mechanisms, but in which self-pollinations do not yield fruits, despite the fact that selfed flowers fail to form fruits despite apparently successful growth of the pollen tubes to the ovules, is a contentious and still poorly understood phenomenon. Some studies have indicated pollen tube–pistil interactions, and major gene control. Others favour an early acting inbreeding depression explanation.

Methods Experimental pollinations, including selfs (in a subsample of which the style was cut before pollen tubes reached the ovary), chase self/cross-pollinations, crosses, and mixed self/cross-pollinations were used to study floral/pistil longevity and effect on fruit set and seed yield in two Ceiba species known to have LSI. Possible explanations for these discordant results are discussed.

Key words: Self-pollination, chase pollination, cross-pollination, pistil longevity, fruit set, Ceiba, self-incompatibility.

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Background and aims Late-acting self-incompatibility (LSI), in which selfed flowers fail to form fruits despite apparently successful growth of the pollen tubes to the ovules, is a contentious and still poorly understood phenomenon. Some studies have indicated pollen tube–pistil interactions, and major gene control. Others favour an early acting inbreeding depression explanation.

Methods Experimental pollinations, including selfs (in a subsample of which the style was cut before pollen tubes reached the ovary), chase self/cross-pollinations, crosses, and mixed self/cross-pollinations were used to study floral/pistil longevity and effect on fruit set and seed yield in two Ceiba species known to have LSI. Possible explanations for these discordant results are discussed.

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Extended floral longevity initiated with self-pollen tubes growing in the style indicates some kind of pollen tube–pistil interaction. Fruit set only in chase pollinations up to 3 h implies that self-pollen tubes either grow more slowly in the style or penetrate ovules more slowly on arrival at the ovary compared with cross-tubes. This agrees with previous observations indicating that the incidence of penetrated ovules is initially lower in selfed compared with crossed pistils. However, the low seed yield from mixed pollinations indicates that self- and cross-pollen tubes arrive at the ovary and penetrate ovules more or less simultaneously. Possible explanations for these discordant results are discussed.
presence of compatible cross-pollen on the stigma does not usually assist any self-pollen also present to break the SI rejection. Indeed, if this were to occur, the value of the SI mechanism would be severely limited. However, it appears that in LSI species, in which self-pollen is normally able to grow to the ovules before rejection occurs, mixed pollinations may have different consequences.

In this study, the two species (Ceiba chodatii and C. speciosa) which have LSI (Gibbs and Bianchi, 1993) were used to look at the effects of self-pollinations on the timing of pistil abscission, and the effects of chase (self-pollen followed at varying intervals by cross) and mixed (self/cross) pollinations on fruit and seed set were investigated.

MATERIALS AND METHODS

The trees studied

Hand pollinations were undertaken with three trees of Ceiba chodatii (Hassl.) Ravenna in Rosario, Argentina, and one tree of C. speciosa (St Hil.) Ravenna, in São José do Rio Preto, Brazil. Both species have been traditionally classified in the genus Chorisia, but following Gibbs and Semir (2003), species of Chorisia have been included in the genus Ceiba. These species have large, showy flowers which are produced prolifically over a period of several weeks. The five staminal filaments are united to form an 8–10 cm tube around the ovary and style, with a collar of five anthers located just below the exerted style and globose stigma. The ovary has axile intruded placenta with around 250–350 ovules.

Ceiba chodatii. Tree A, which was used as a pollen source, was a cultivated street tree located some 1.5 km from the other two trees. Trees B and C were cultivated trees located in the forecourt of the Colegio Gregoria Matorras de San Martín, Av. Córdoba, Rosario. These are mature trees with canopies of some 20 m, and separated on either side of the school façade by some 18 m. Access to lower branches was by means of tall stepladders, and access was also possible to flowers in the uppermost canopy of tree B by leaning over the parapet of the flat roof of the college.

Ceiba speciosa. This species was a cultivated tree, or possibly a survivor of natural woodland in the area, on the campus of the University at São José do Rio Preto, São Paulo state, in south-east Brazil. Again access to flowers on lower branches was by stepladders.

Self-pollinations with C. chodatii and C. speciosa to study effects on pistil longevity

Mature flower buds were enclosed in light muslin bags during the afternoon prior to opening. The following morning, the bags were removed and a 3 cm length of plastic tube (cut from commercial drinking straws) was inserted around the exposed stigma and pushed into the neck of the staminal tube. The plastic tube, secure in this position, prevented any contact with the stigma by potential pollinators so that further bagging before or after hand pollinations was unnecessary.

With both species, flowers were self-pollinated by first removing the plastic tube, and then dabbing the stigma with pollen from a dehisced anther of a separately collected flower. The tube was then replaced and the flower labelled with a lightweight aluminium foil tag giving day of pollination. The thread securing the tag was tied around the stout pedicel of the flower, and further secured there with a dab of glue. In both species, a control group consisted of flowers with the plastic tube inserted which were left unpollinated.

With flowers of C. speciosa self-pollinations were made at around 1500–1700 h each day. Self-pollinated flowers were divided into two groups: (1) left without further treatment, or (2) the day following pollination, at 0900–1000 h (i.e. some 18 h after self-pollination), a small window was cut near the base of the staminal tube with a scalpel, and using this access, the style was severed. Previous studies with C. speciosa (Gibbs and Bianchi, 1993) had shown that self- and cross-pollen tubes begin to reach the ovary and penetrate ovules after 24 h. By 18 h post-pollination, therefore, pollen tubes were unlikely to have reached the ovary. As a control, pistils from five cut-style treated flowers and five uncut flowers were fixed in 70 % formalin-aceto-alcohol and subsequently examined for pollen tube growth using aniline blue staining and fluorescence microscopy (Martin, 1959). Cumulatively over several days, each of these treatments was applied to 50 flowers of C. chodatii, and 80 flowers of C. speciosa. Monitoring for abscission consisted of a daily search for fallen flowers at the base of the tree, distinguished by their aluminium tag, and noting the date of pollination and the date of collection.

Cross- and mixed pollinations with C. chodatii

Mature buds were bagged as before and the following morning their stigmas were either cross-pollinated, using pollen from one of the other two trees, or a mixture of 1 : 1 or 2 : 1 self-pollen : cross-pollen was applied to the stigma. This was achieved by applying self-pollen using an anther to half or two-thirds of the globose stigma, and then applying cross-pollen to the remainder of the stigma. In all cases, a large charge of pollen was applied to the stigma. A plastic tube was inserted around the stigma as before, and the flowers monitored for fruit set. A total of 23 flowers were crossed, and 33 flowers were submitted to mixed pollinations. Fruits were harvested just prior to dehiscence of the capsule valves, and seed set, as judged visually by their well-developed, rounded appearance, assessed. Independently, samples of five flowers from each of trees B and C were collected, and the number of ovules in their pistils counted. Trees B and C formed a few fruits by natural pollination each year. Access to most of these fruits was usually impossible, but 13 were collected and the number of seeds per capsule counted.

Since distribution around the mean values for longevity of unpollinated and selfed flowers was not normal, we used the Mann–Whitney test with Tukey type test to compare mean values (Zar, 1996). Mean seed set from cross- and mixed pollinations were compared using Student’s t-test.
Chase pollinations with C. chodatii

Mature buds on trees B and C were enclosed in bags. The following morning, with flowers open, they were self-pollinated and a short plastic tube inserted to protect the stigma as before. Such flowers were then divided into five groups, and cross-pollen, obtained from one of the other two trees, was subsequently applied to the stigma at one of 1, 2, 3, 4 or 8 h intervals after selfing. The plastic tube was replaced to prevent any contaminant pollination, the flowers were tagged, and monitored for abscission or fruit set. Fruits were harvested and the number of seeds counted was counted in subsamples of four and two fruits obtained from self + cross at 2 h and at 3 h, respectively.

RESULTS

Floral longevity

The percentages of surviving flowers/pistils per day after selfing, selfing with cut-style, and unpollinated controls for C. chodatii and C. speciosa are given in Fig. 1A and B. The mean number of days for which flowers/pistils survived in each species following each treatment is given in Fig. 2A and B. Pistil longevities in self- and unpollinated flowers in C. chodatii, and the three treatments with C. speciosa: unpollinated, selfed and style cut, and selfed, were significantly different (P < 0.001). Fluorescence microscopy observations of pollen tube growth in pistils from selfed and cut-style, or selfed and uncut style pollinations in C. speciosa, collected at 18 h post-pollination, all showed no pollen tubes in the ovary.

Hand cross- and mixed pollinations with C. chodatii

Hand cross-pollinations (n = 23) between C. chodatii trees A, B and C (Table 1) yielded an overall mean seed set of 232 (s.d. = 55-2) seeds/fruit. Since ovaries of these trees had a mean of 307 (s.d. = 60-4) ovules/ovary (n = 10), ovule conversion to seeds (O/S) is high in this species at around 73%. Mixed pollinations (Fig. 3), both 1 : 1 self/cross (n = 28), and 2 : 1 self/cross (n = 5), all produced fruits, but with a lower yield of seeds/fruit (mean of 93, s.d. = 47-2, and mean of 63, s.d. = 20-7, respectively), representing an O/S of around 30% and 20%. Seed yield from crossed pistils was significantly different from that of 1 : 1 and 2 : 1 mixed pollinations (P > 0.05). The sample of 13 pods formed from natural pollinations in these trees (Table 1) gave a mean seed set of 56 (11–150) seeds/fruit (O/S around 18%).

Chase pollinations with C. chodatii

Results of chase pollinations are given in Table 2. All pistils with cross-pollen added to the stigma up to 3 h after selfing yielded fruits, but with diminished seed set, whereas
**TABLE 1.** Mean seed yield in fruits Ceiba chodatii from hand-cross-, hand-mixed self/cross-1 : 1, and 2 : 1, and from natural pollinations.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean seed yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cross-pollinations (n)</td>
<td></td>
</tr>
<tr>
<td>B × A (8)</td>
<td>234.4 (s.d. = 42.1)</td>
</tr>
<tr>
<td>B × C (9)</td>
<td>200 (s.d. = 30.2)</td>
</tr>
<tr>
<td>C × B (6)</td>
<td>276 (s.d. = 72.8)</td>
</tr>
<tr>
<td>Mixed pollinations</td>
<td></td>
</tr>
<tr>
<td>B₂ × C₁ (12)</td>
<td>67.7 (s.d. = 24.9)</td>
</tr>
<tr>
<td>B₁ × A₁ (7)</td>
<td>113.9 (s.d. = 44.5)</td>
</tr>
<tr>
<td>C₁ × A₁ (6)</td>
<td>79.7 (s.d. = 23.2)</td>
</tr>
<tr>
<td>C₁ × B₁ (3)</td>
<td>170 (s.d. = 67.7)</td>
</tr>
<tr>
<td>B₂ × C₁ (3)</td>
<td>56.7 (s.d. = 18.6)</td>
</tr>
<tr>
<td>C₂ × B₁ (2)</td>
<td>37.5 (s.d. = 26.2)</td>
</tr>
<tr>
<td>Natural pollinations</td>
<td></td>
</tr>
<tr>
<td>B (11)</td>
<td>64 (s.d. = 43.2)</td>
</tr>
<tr>
<td>C (2)</td>
<td>15.3 (s.d. = 6.4)</td>
</tr>
</tbody>
</table>

**Fig. 3.** Mean seed yield in fruits from flowers of *Ceiba chodatii* with cross-, mixed 1 : 1 and mixed 2 : 1 self/cross-pollinations. Different letters above columns indicate significant difference at *P* > 0.05.

**TABLE 2.** Fruit set and seed yield following chase (self/cross) pollinations in *C. chodatii*.

<table>
<thead>
<tr>
<th>Stigma selfed, and cross pollen applied after</th>
<th>Fruit set (%)</th>
<th>Seed yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 h (n = 20)</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>2 h (n = 30)</td>
<td>100</td>
<td>64, 69, 87, 129 (n = 4)</td>
</tr>
<tr>
<td>3 h (n = 27)</td>
<td>100</td>
<td>122, 144 (n = 2)</td>
</tr>
<tr>
<td>4 h (n = 15)</td>
<td>0*</td>
<td></td>
</tr>
<tr>
<td>8 h (n = 14)</td>
<td>0*</td>
<td></td>
</tr>
</tbody>
</table>

*Pistils abscised at 5–8 d following pollination.

no pistils with cross-pollen added at 4 or 8 h following selfing yielded fruits. Mean overall seed set in the subsample of fruits counted (n = 6) was 102 seeds/fruit.

**DISCUSSION**

**Pistil longevity**

In *C. speciosa*, the self-pollinations in which the style was cut around 18 h post-pollination, effectively denied self-pollen tubes access to the ovary. Nevertheless, merely the presence of self-pollen tubes in the style was sufficient to cause a slight but significant delay in pistil abscission from a mean of 4.9 d in unpollinated flowers to 5.5 d in selfed and cut-style flowers. Moreover, only 7% of unpollinated flowers had pistils which survived for 5 d, whereas 19% of selfed and cut-style flowers had pistils which survived more than 5 d. Such delay is presumably due to some kind of interaction or ‘cross-talk’ between pollen tubes growing in the style and the ovules in the ovary. Various studies have shown that the presence of pollen tubes growing in the style may, for example, trigger sink movements of metabolites from bracts to ovary (Linskens, 1975), or that contact between the pollen tube and style is essential in some way to condition the pollen tube to be able to receive signals from the ovule to direct its growth to the micropyle (Higashiyama et al., 1998; Lord and Russell, 2002). Most such studies have been with compatible pollinations in species with or without conventional SI mechanisms, but Sage et al. (1999) reported for *Narcissus triandrus*, a species with LSI, that ovule maturation and ovule degeneration within the ovary may differ, depending upon whether self- or cross-pollen tubes are growing in the style.

A more marked delay in pistil abscission occurred in flowers which were selfed and with the style left intact, which had a mean longevity of 8.1 d (s.d. = 1.6) and 7.94 d (s.d. = 1.0), compared with 5.3 d (s.d. = 1.2) and 4.9 d (s.d. = 1.6) for unpollinated flowers, in *C. chodatii* and *C. speciosa*, respectively. The major influence here was not pollen tube growth but the fact that self-pollen tubes penetrate the ovules. This ability of successful self-pollen tube growth to the ovules to delay pistil abscission in LSI species also occurs in other Bombacaceae, such as *Ceiba pentandra* (Gribel et al., 1999), and *Pseudobombax munguba* (Gribel and Gibbs, 2002), and in the Bignoniaceae, in *Tabebuia ochracea* and *T. caraiba* (Gibbs and Bianchi, 1993). However, in another bignonaceous species, *Spathodea campanulata* (Bittencourt et al., 2003), selfed pistils fall at 3–4 d. In this latter species, despite self-pollen tube growth to the ovule where ovule penetration, fertilization, and initiation of the endosperm occur, selfed pistils did not survive for any longer than unpollinated flowers.

**Chase and mixed pollinations**

All chase pollinations in which cross-pollen was applied up to 3 h after selfing formed fruits, albeit with reduced seed set, whereas those with cross-pollen applied at 4 and 8 h failed to set fruit. It is likely that the latter treatments were effectively self-pollinations, in which all ovules were penetrated by self-pollen, and which, as usual, fail to set fruit. When cross-pollen was applied up to 3 h following selfing, despite the head start by the self-pollen, it can be assumed that a proportion of cross-pollen tubes reached the ovary within a time which permitted them to penetrate a sufficient number of ovules to prevent abscission, and allow fruit development.

The results from the mixed pollinations are in striking contrast to this scenario. All flowers submitted to mixed pollinations, with self- and cross-pollen applied simultaneously to the stigma, formed fruits with a low seed set compared with fruits obtained from hand cross-pollinations.
(Table 1). If self-pollen tubes either grow more slowly in the style and so arrive ‘late’ at the ovary, as the chase pollinations imply, and/or they penetrate ovules more slowly than cross-tubes, as previous studies indicate (Gibbs and Bianchi, 1993), it would be expected that, following mixed pollinations, the relatively faster cross-pollen tubes would arrive first at the ovary, and penetrate the majority of the ovules before the arrival of self-tubes. The low seed set in fruits from mixed pollinations indicate that, on the contrary, a large proportion of ovules were penetrated by self-pollen tubes, and subsequently aborted, although some may survive to give viable seeds, as in other LSI species (Bertin and Sullivan, 1988; Gribel et al., 1999; Gribel and Gibbs, 2002). The results reported here imply that usually no more than half of the ovules received cross-pollen tubes, since one would expect most crossed ovules to develop normally to seeds.

Three explanations are possible for these discordant results following chase vs. mixed pollinations: (1) effects due to localized pollen placement on the stigma, (2) pollen load size, or (3) interactions between self- and cross-pollen tubes affect their growth in the style.

(1) Applying self- and cross-pollen to distinct sectors of the stigma, might, despite syncarpy, have led to pollen tubes preferentially entering some ovary locules. We are inclined to discount this possibility since Gribel et al. (1999), with Ceiba pentandra, and Gribel and Gibbs (2002) with the confamilial Pseudobombax munguba, obtained similar depressed seed set following mixed pollinations after applying premixed self/cross-pollen loads over the whole stigma.

(2) A number of studies have shown a relationship between pollen load size on the stigma and fruit set (for review, see Stephenson et al., 1995). Quesada et al. (2001), working with Pachira quinata, another bombacaceous species with LSI (as Bombacopsis quinata; Sandiford, 1998), reported that only flowers which received a mean pollen load of 422 pollen grains on the stigma (2.6 : 1 pollen grains per ovule), developed fruits. Furthermore, in such fruits, these authors estimated that around 23 pollen grains were needed for each mature seed. By analogy, one could hypothesize that in our mixed pollinations with C. chodatii, the quantity of cross-pollen on the stigma was diluted, and the number of grains was insufficient to fertilize more than a proportion of ovules in the ovary, leaving others available for the later arrival of self-pollen tubes.

There is also an inclination to discount the effect of pollen load in the study reported here. Quesada et al. (2001) were reporting pollen loads following natural pollinations, which therefore probably comprised mixed self/cross-pollinations. Consequently, their estimations of the number of compatible pollen grains needed to produce mature seeds could have been influenced by counts of an unknown proportion of self-pollen tubes growing in the styles. In our study, with crosses and mixed pollinations, we applied pollen to the stigma by hand and, in such cases, a large overcharge of pollen is normally involved. This applies particularly in these Ceiba species, since the globose stigma is relatively large and of easy access, and abundant pollen was available from the anthers.

(3) Another possible explanation is that when cross- and self-pollen tubes are growing together in the style, they interact, in such a way that either the rate of (normally slow) self-pollen tubes is enhanced, or the rate of (normally fast) cross-pollen tubes is impaired. Or, arriving at the ovary together, the normally slow rate of ovule penetration by self-pollen tubes is enhanced, or that of cross-pollen is impaired. To distinguish these possible effects, it will be necessary to: (a) count the number of pollen grains on the stigmas following our hand cross- and half cross-pollination procedures; and (b) carefully monitor at intervals, pollen tube growth in styles, and their arrival at the ovary, following self-, cross- and mixed pollinations.

These results highlight an unresolved enigma as to why mixed pollinations in LSI species produce fruits with low seed set. We are confident that our chase and mixed pollinations, albeit with just two trees, give results which are generally applicable to C. chodatii, and probably other LSI species. The relatively slow self-pollen tube growth in the style and/or ovule penetration implied by our chase pollinations are in accord with previous observations of delayed penetration of ovules by self-pollen tubes in this species. Gibbs and Bianchi (1993), working with trees of C. chodatii cultivated in Brazil, reported a lower incidence of ovule penetration in selfed vs. crossed pistils at 24 and 48 h, although by 96 h no significant differences in penetrated ovules were evident. Similar results were also observed in LSI species in the Bignoniaceae (Gribel and Bianchi, 1999; Bittencourt et al., 2003). Likewise, the fruits with low seed set from mixed pollinations reported here agree with similar results found in other Bombacaceae (Gribel et al., 1999; Gribel and Gibbs, 2002), and also in the Bignoniaceae (Bertin and Sullivan, 1988).

Although it is not possible to explain this disparity between the slow functioning of self-pollen tubes in C. chodatii when they are present in pure self- or chase-pollinated pistils, in contrast to their efficacy in mixed pollinated pistils, the fact that in the latter situation self-pollen tubes are able to compete with cross-pollen tubes for ovules may have some striking consequences for the mating system of such LSI species. Using genetic markers to check paternity, mixed pollinations were shown to produce fruits with some selfed seeds in the bombacaceous species Ceiba pentandra and Pseudomobax munguba (Gribel et al., 1999; Gribel and Gibbs, 2002), as found previously in the bignoniaceous Campsis radicans by Bertin and Sullivan (1988). Moreover, Souza et al. (2003) used such isozyme genetic markers to study the mating system in a population of 53 trees of Ceiba speciosa, and reported predominant allogamy but with some mixed mating in this species. These authors considered the latter finding to be discordant with our indication of LSI in C. speciosa (Gibbs and Bianchi, 1993). However, it could be argued that natural pollinations can result in a mixture of self- and cross-pollen arriving on the stigma, and in LSI species such mixed pollinations can yield fruits with a proportion of selfed seed. We would predict, therefore, that species with LSI are likely to exhibit some mixed mating, as found for Ceiba pentandra by Murawski and Hamrick (1992) and C. speciosa by Souza et al. (2003).

As a postscript in this context, it is of interest to comment on the fruits formed on trees B and C from natural pollinations. Due to LSI, cross-pollination between trees B and C,
or another conspecific, is needed to form such fruits. All of the natural fruits which were accessible for collection \((n = 13)\) had low seed numbers (mean of 56 seeds). This contrasts with the mean seed yield of 235 seeds/fruit from hand cross-pollinations, but is similar to that (63) obtained from mixed 2 : 1 self/cross-pollinations.

The low fruit set presumably reflects a lack of the natural sphingid pollinators to these trees in this city centre site. *Ceiba chodatii*, is naturally distributed in southern Paraguay and western Argentina. However, the low seed yield in these fruits implies that the occasional cross-pollinations which do occur involved a large measure of geitonogamous pollinations, and that mixtures of largely self- and some cross-pollen, were transferred to stigmas. This phenomenon was also observed in trees of bat-pollinated *Pseudobombax munguba* growing in their Amazonian habitat (Gribel and Gibbs, 2002), in which naturally produced fruits had a seed content similar to that obtained from experimental mixed pollinations rather than hand cross-pollinations. Evidently, as reported by Waser and Price (1991) in *Ipomopsis aggregata*, late-acting self-incompatibility carries a cost in diminished fecundity due to ovule usurpation by self-pollen tubes.

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**LITERATURE CITED**


