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Bud pollination and other techniques are ineffective in breaking late-acting self-incompatibility in *Ceiba chodatii* (Malvaceae – Bombacoideae)

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ABSTRACT

There are two homomorphic self-incompatibility (SI) mechanisms among flowering plants — gametophytic self-incompatibility (GSI) and sporophytic self-incompatibility (SSI). Whilst SI has the advantage of promoting outbreeding in natural populations, it can also be a problem for horticulturists and agronomists. Thus, a number of techniques have been successfully employed to break both GSI and SSI, including bud pollination, saline pre-treatment of the stigmas and simply selfing ageing flowers. We applied these three techniques to determine whether they would be effective at breaking SI in *Ceiba chodatii*, a species with late-acting self-incompatibility (LSI). We self- or cross-pollinated buds ranging from 1–4 days prior to anthesis and checked ageing by selfing or crossing flowers at 1–4 days after the onset of anthesis, with those at four days showing petal wilt. None of the selfed buds or ageing flowers set fruit, unlike the majority of crossed flowers. Likewise, all flowers to which 1 % saline was applied to the stigmas prior to selfing failed to set fruit. We conclude that unlike GSI and SSI, which have pre-zygotic control of self-pollen function, LSI is likely to be immune to these techniques since the focus of rejection occurs after self pollen tubes have reached the ovary.

Keywords: bud self-pollination, *Ceiba chodatii*, late-acting self-incompatibility, selfing ageing flowers, selfing saline-treated stigmas

Introduction

Most flowering plants have bisexual flowers and so are susceptible to self-pollination and consequent inbreeding. Many species avoid self-fertilisation if self-pollination occurs because they possess a self-incompatibility (SI) mechanism that prevents self-pollen functioning effectively. Three such SI mechanisms have been established: homomorphic gametophytic (GSI), homomorphic sporophytic (SSI) and heteromorphic (HetSI). At a Mendelian level, both homomorphic mechanisms function with (usually) one multiallelic locus (S) and incompatibility occurs when the same s alleles are present in the pollen and stigma (Nettancourt 1977; Hiscock & Kües 1999; Silva & Goring 2001; Takayama & Isogai 2005). In GSI, the S-genotype of the haploid pollen phenotype determines the incompatibility reaction, whereas in SSI the incompatibility reaction of the pollen is determined by both s-alleles present in the pollen parent.

A rather more contentious self-incompatibility mechanism termed ‘late-acting self-incompatibility’ (LSI)

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was proposed by Seavey & Bawa (1986) and has been reviewed by Sage *et al*. (1994) and Gibbs (2014). In taxa showing this phenomenon, self-pollination does not yield fruits despite the fact that self pollen tubes grow to the ovary where, in many cases, ovule penetration and fertilisation occur. The occurrence of such fertilisations has led some critics to argue that LSI is a misnomer and lack of fruit set following selfing is caused by the effects of early acting inbreeding depression (EID) involving deleterious recessive alleles (Klekowski 1988; Nic Lughadha 1998). This controversy has remained unresolved and a form of stalemate has prevailed with most papers that report LSI effects also adding the caveat that EID may occur. However, it is noteworthy that studies on post-pollination events in bombacoid and bignon species (two groups with prevalent LSI) have shown that although initial endosperm divisions proceed normally in fertilised ovules in selfed pistils, no cell divisions occur in the "resting zygotes" that are characteristic of these families before pistil abscission (Gibbs & Bianchi 1993; Bittencourt 2017). Effectively in such taxa, there are no embryonic divisions in which EID effects may manifest.

Diallel crosses with sib family arrays have been used to determine the genetic control in many taxa with homomorphic SI (see Gibbs 2014 for review); the relatively few studies using sib progeny diallels to elucidate the genetic control of LSI have produced some ambiguous results, but studies with three species, *Theobroma cacao* (Knight & Rogers 1955; Cope 1962; Lanaud *et al*. 2017), *Asclepias exaltata* (Lipow & Wyatt 2000), and *Handroanthus heptaphyllus* (Bianchi *et al*. 2021) have indicated a possible major gene control analogous to that in conventional homomorphic SI mechanisms.

Self-incompatibility can present problems in agricultural and horticultural research, *e.g.*, by preventing the establishment of inbred lines, and various techniques have been established to circumvent homomorphic SI mechanisms. The most common involves bud pollination (Nettancourt 1977; Shivanna 1982; Shivanna & Johri 1985), which has been employed to obtain selfed progeny in GSI species, *e.g.*, *Nicotiana* and *Petunia* (Pandey 1963; Rangaswamy & Shivanna 1969) and SSI species, *e.g.*, *Brassica* (Attia 1950; Cabin *et al*. 1996). Self-incompatibility has also been shown to break down naturally in old flowers in diverse taxa, *e.g.*, *Brassica* (Kakizaki 1930), *Lilium* (Ascher & Peloquin 1968), *Campanula* (Stephenson *et al*. 1992; Vogler *et al*. 1998) and *Leptosiphon* (Goodwillie *et al*. 2004). Other techniques that have been used successfully to break SI are the application of saline solution to the stigmas (Monteiro *et al*. 1988; Tlngdong *et al*. 1992; Carafa & Carratu 1997; Hiscock 2000) and high ambient CO_2 concentration (Nakanishi & Hinata 1973; Dhaliwal *et al*. 1979; O'Neill *et al*. 1984).

None of these techniques has been applied to any species with LSI. Here we report the use of bud and old flower selfings, and also application of saline solution to the stigma prior to selfing, to determine whether these methods permit selfing in *Ceiba chodatii*, a species that, as in other bombacoid taxa, has been shown to present the LSI syndrome (Gibbs & Bianchi 1993; Gibbs *et al*. 2004).

Materials and methods

Ceiba chodatii (Hassl.) Ravenna (Malvaceae – Bombacoideae) is a ventricose tree of up to 20 m or so with a distribution in SW Paraguay, S Bolivia and NW Argentina (Gibbs & Semir 2003). It is widely cultivated in urban settings in the latter country, together with the congeneric and inter-fertile *C. speciosa*. It flowers prolifically with a wide latitudinal range of blooming season from August-September in Paraguay, Bolivia and NW Argentina to January-April in Santa Fe and Buenos Aires provinces (Fig. 1A). Flowers are large (around 12 cm), with ivory to pale yellow petals. The five stamens are united by their filaments to form a tube surrounding the multi-ovulate ovary and from which the apex of the style and stigma are exserted (Fig. 1B). Our studies were undertaken with two trees of *C. chodatii* in a school patio in the city of Rosario, Argentina. These trees, situated some 18 m from each other, have flowers produced at a height that is accessible with a step-ladder, and are conveniently protected from public curiosity. They have been used previously by us in studies on aspects of LSI in this species (Gibbs *et al*. 2004).

Bud and old flower pollinations

We made self- and cross-pollinations using flower buds at 4.0-4.5 cm, which were around four days prior to anthesis (4 DPA), 5 cm (3 DPA), 6-7 cm (2 DPA) and 9 cm (1 DPA). Flower buds at 2 DPA, with which most pollinations were made, were around 7 cm long, with a pistil 5.9 ± 0.17 cm (n= 5), comprising an ovary 0.57 ± 0.12 cm, and style/stigma 5.3 ± 0.29 cm; the staminal tube of filaments and anthers was 5.1 ± 0.12 cm. This compared with flowers at anthesis which had an ovary of 1.02 ± 0.13 cm and style/stigma of 10.4 ± 0.97 cm, with a pistil 11.4 ± 0.96 cm (n= 5).

Pollinations with flower buds at different stages were affected by cutting a small hole in the petals to expose stigma at the mouth of the tube of the unopened anthers. Geitonogamous selfs were made using pollen from newly opened flowers on the same tree, and crosses with pollen from flowers of the other tree. To prevent any further contaminant pollination, we isolated the stigma by inserting a small length of plastic drinking straw into the staminal tube (Fig. 1C).

A total of 87 buds were either self- or cross-pollinated with buds at 4 DPA (n= 14), 3 DPA (n= 4), 2 DPA (n= 42), 1 DPA (n= 11), and at start of anthesis (n= 6). A subsample of 10 in total were fixed in FAA (Lersten & Curtis 1988), comprising five pistils of each treatment that suffered abscission at 6 days in selfs and five pistils harvested whilst

Figure 1. *Ceiba chodatii*. **A.** Tree branches. **B.** Flower. **C.** Hand-treated flower buds with an aperture cut in the petals and a straw-protected stigma. Scale bar in B = 22 mm, in C = 16 mm.

still developing at 8 or 10 days in crosses. The ovaries of these pistils were subsequently dissected to remove the pericarp to expose the ovules/young seeds.

We also made cross- and self-pollinations with old flowers at 24 h, 48 h, 72 h and 96 h post-anthesis, the latter about to enter senescence (petal wilt). These flowers were initially protected from pollinators by enclosing them whilst in bud in paper bags and subsequently with the use of a portion of drinking straw inserted into the staminal tube. Pollinations, both self from flowers on the same tree, and cross from the other tree, were with pollen of flowers in their first day of anthesis.

Saline treatment

With flowers on the first day of anthesis, that had been bagged previously, we applied a 1 % saline solution (1 % NaCl in 0.1 % Tween 20 detergent diluted in deionized distilled water) as reported by Hiscock (2000) [modified from Carafa & Carratu (1997)] and by Sun *et al*. (2005). We applied self-pollen to the treated stigmas after a 5 min interval (n= 11), and to others after 60 min (n= 8). Stigmas of treated flowers were again protected from contaminant pollen by inserting a plastic straw into the staminal tube as before.

Results

Flower bud pollinations

All self-pollinated buds (total n= 49), ranging in size from 4 cm (4 DPA) to 9 cm (1 DPA), abscised between 4-12 days after pollination (DAP) (Fig. 2); the majority (71.4 %) of flower buds in these size ranges that were cross-pollinated (n= 28) formed normal fruits that were scored at one month (Tab. 1). However, a small proportion of these developing fruits began to show distal necrosis of the exocarp (close to the style) due, apparently, to bacterial-fungal attack. Such infected capsules did not mature and remained mummified on the trees.

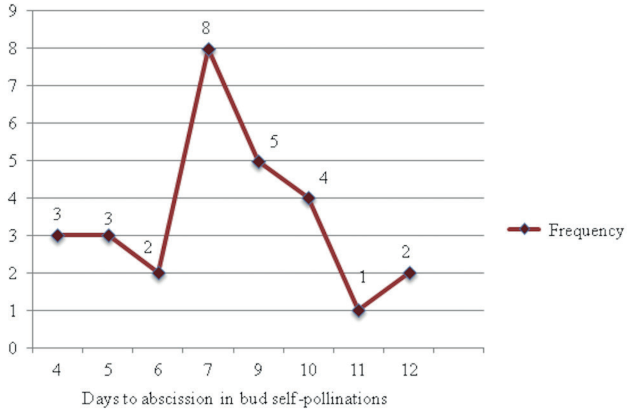

Figure 2. *Ceiba chodatii* self-pollinated buds abscission. Frequency of days after pollination (DAP) to abscission for buds from four to one day prior to anthesis.

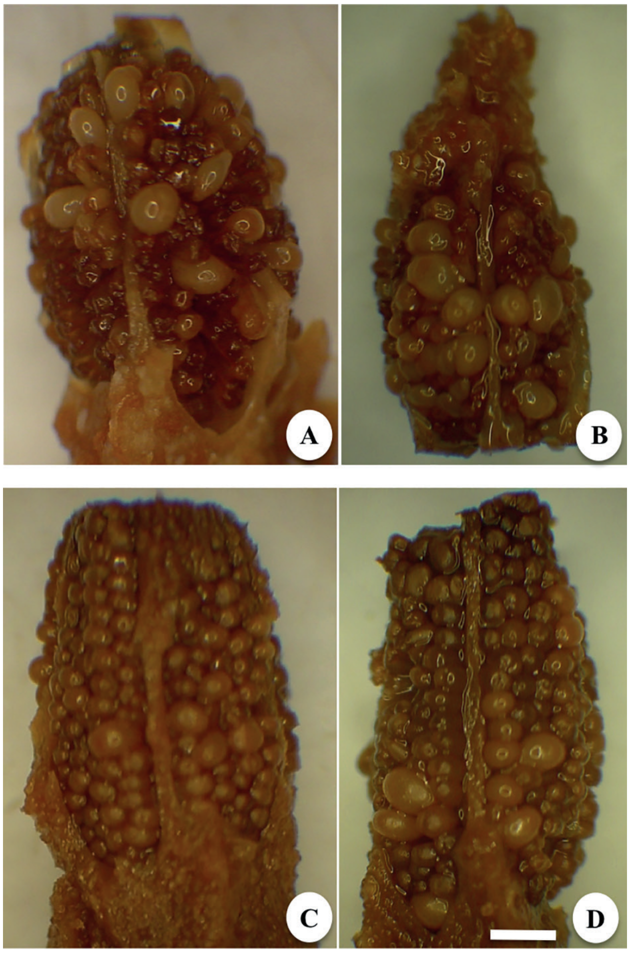

Figure 3. *Ceiba chodatii*. Dissected ovaries with the pericarp removed in flower bud pollinations. **A-B** selfed pistils abscised after six days. **C-D** cross-pollinated pistils fixed after eight and ten days (respectively). All show notable differences in ovule/young seed size. Scale bar = 2.5 mm.

The exposed ovules/young seeds after removal of the pericarp of bud pollinated pistils that abscised at six days after self, and harvested at eight, 10 days after cross, revealed a surprising feature. Although almost all ovules had increased in size compared with their pre-pollination state, presumably reflecting pollen tube penetration, all dissected pistils showed some ovules/young seeds that were disparately enlarged (Fig. 3A-D). Such ovule/seed size differences were not observed in the pistils of flowers self- or cross-pollinated on day 1 of anthesis (Fig. 4A, B).

Old flower pollinations

All old flower self-pollinations (24, 48, 72, 96 h post-anthesis) abscised at 6-7 DAP (n= 29). In contrast, all cross-pollinated old flowers (some with petal wilt) formed fruits (n= 14), (Tab. 2). Again, some of these developing fruits suffered bacterial-fungal infection.

Table 1. *Ceiba chodatii*. Results for bud self- and cross-pollinations practised in five different phenology stages in trees B and C during two flowering seasons (2005 and 2011). DPA = days prior anthesis; DA = day one of anthesis. Letters '$_B$' and '$_C$' indicate the tree pollination origin. Percentages of fruit set after cross-pollinations appear between brackets.

Flower Buds		Self-pollination		Cross-pollination		
DPA	Size (cm)	No. Pollinations	Abscised Flowers	No. Pollinations	Abscised Flowers	Fruit set (%)
4	4-4.5	3_B	3	5_B 6_C	2_B 1_C	8 (72.7)
3	5	–	–	4_C	1_C	3 (75)
2	6-7	9_B 21_C	30	6_B 6_C	2_B 2_C	8 (66.6)
1	9	10_C	10	1_B	0	1 (100)
DA	12.5	6_C	6	–	–	–
Total		**49**	**49**	**28**	**8**	**20 (71.4)**

Table 2. *Ceiba chodatii*. Old flowers hand self- and cross-pollinations at intervals of 24, 48, 72 and 96 h post anthesis in trees B and C. Letters '$_B$' and '$_C$' indicate the tree pollination origin. Percentages of fruit set after self- and cross-pollinations appear between brackets.

Flower Age	Self-pollination		Cross-pollination		
Hours post anthesis	No. Pollinations	Abscised Flowers	No. Pollinations	Abscised Flowers	Fruit set
24	16_C	16	3_C	0	3
48	3_B 2_C	5	2_B 2_C	0	4
72	3_B 2_C	5	2_B 2_C	0	4
96	3_C	3	3_C	0	3
Total	**29**	**29 (0)**	**14**	**0**	**14 (100)**

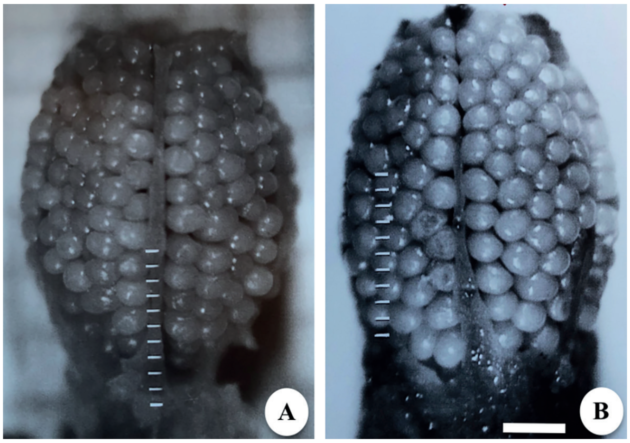

Figure 4. *Ceiba chodatii*. Dissected ovaries with the pericarp removed in flowers pollinated on the first day of anthesis and fixed six days after pollination. **A.** self-pollinated pistil. **B.** cross-pollinated pistil. In both cases ovule/young seeds are similar in size. Scale bar = 2.5 mm.

Saline treatment

All selfed flowers pollinated after pre-treatment of the stigma with saline solution failed to set fruit. With those pollinated 5 min after treatment, pistils abscised at 12 DAP, and those selfed after a 60 min interval abscised at 8 DAP (Tab. 3).

Table 3. *Ceiba chodatii*. Hand self-pollinations in flowers with saline treated stigmas of trees B and C. Days to abscission are indicated between brackets.

Trees	1 % NaCl solution selfed after 5 min	1 % NaCl solution selfed after 60 min
	Nº Fruit set / Nº self-pollinations	Nº Fruit set / Nº self-pollinations
B	0/ 5 (12 d)	0/ 2 (8 d)
C	0/ 6 (12 d)	0/ 6 (8 d)

Discussion

That bud pollinations have proven successful to break SI with various Brassicaceae species, *e.g.*, *Brassica rapa*, *Lesquerella fendleri* and *Raphanus sativus* (Cabin *et al*. 1996) might be expected given that the incompatibility reaction in this SSI family is at the stigma surface. It is likely that the female SI agent in these species - a receptor kinase (SRK) that spans the plasma membrane of the stigmatic papilla cells - and which potentially interacts with the male determinant protein moieties born on and in the wall of the pollen (Sherman-Broyles & Nasrallah 2008), is not *in situ* until shortly before anthesis; as a consequence, normally incompatible self pollen would not be challenged at the stigma surface and pollen tubes can grow to the ovary. Given that the final site of rejection with LSI species such *Ceiba chodatii* is at or in the ovule it is likely that there is no marked stigmatic barrier to self pollen at anthesis or earlier phases.

However, that bud pollination can overcome SI in species such as *Nicotiana* and *Petunia* (Pandey 1963) is more enigmatic. Both species have an RNase based GSI mechanism in which self rejection occurs when pollen tubes are growing in the style (Franklin-Tong VE & Franklin-Tong FC 2003; Kao & Tsukamoto 2004). Suyama *et al*. (2013) also report breaking SI in *Hydrangea* spp. and hybrids (unknown SI mechanism but not SSI) using bud, or warm water immersion of the stigmas, or a combination of both treatments. These results imply that the metabolites in the stylar transmitting tract that inhibit self pollen tube growth in GSI taxa may also only be synthesized immediately prior to anthesis. In contrast, whatever the control of LSI in *Ceiba chodatii* it seems that the SI system is functional well before anthesis.

The dissected pistils from both abscised self- (at six days) and harvested cross- (at eight and 10 days) pollinations revealed that the mass of fertilised ovules with a zygote and dividing endosperm had enlarged, but they also showed the curious feature of some very much larger ovules (Fig. 3A-D). We assume this was a consequence of the fact that prior to anthesis, the ovules may show asynchronous megagametophyte development, as has been reported in various taxa (Sage *et al*. 1999; Lentini *et al*. 2020). As a consequence, the precocious arrival of pollen tubes in the ovary following our bud pollinations would encounter only some ovules with mature 7-celled gametophytes and so capable of penetration and fertilisation, but others still undergoing megagametophyte mitoses and so with a delay in accepting pollen tubes. A further consequence would be that the former, early penetrated ovules would have a head start of endosperm nuclei division post-fertilization, hence their relatively enlarged size in our abscised (self-) and harvested (cross-) pistils. We have no observations on megagametophyte development in *Ceiba* ovaries but this hypothesis receives some support from the fact that the disparate ovule sizes observed following precocious (bud) pollinations have not been seen in other studies of pistils with self- or cross-pollinations made at day one of anthesis - either with dissected removal of the ovary wall, or in previous studies with this species employing fluorescence microscopy preparations (MB Bianchi, unpubl. obs.).

All species for which application of saline solution to the stigma has successfully overcome SI have SSI, *e.g.*, *Brassica campestris*, *Brassica napus*, *Brassica oleracea*, *Senecio squalidus*, *Eruca sativa* (Monteiro *et al*. 1988; Tlngdong *et al*. 1992; Carafa & Carratu 1997; Hiscock 2000; Sun *et al*. 2005) and so have the stigmatic surface as the locality for the SI reaction. Species with LSI such as *Ceiba chodatii* may possibly have interactions between the stigma or transmitting tract of the style and self pollen (see below) but this mechanism only becomes evident with events in the ovary, *i.e.*, delayed pollen tube penetration of ovules, or post-fertilisation failure, and of course, self-pistil abscission. Again, it is unsurprising that saline on the stigma did not affect LSI.

Loss of self-incompatibility in ageing flowers has been reported for SSI species (*Brassica*, *Leptosiphon*) and GSI species (*Lilium*, *Campanula*) (Kakizaki 1930; Goodwillie *et al*. 2004; Ascher & Peloquin 1968; Good-Avila *et al*. 2001). Some authors have viewed such loss as an adaptive feature, since if cross pollination has failed, selfing may ensure reproductive success. However, our aged flowers of *Ceiba chodatii* maintained their LSI, although it was notable that many developing fruits from cross-pollinated controls suffered bacterial-fungal attacks - the infestation presumably being initiated with pollination of the ageing stigma/style. All selfed ageing flowers abscised at 6-7 days, in comparison with a mean of 8 days (4-11 days) for selfed flowers of *C. chodatii* pollinated on day 1 of anthesis (Gibbs *et al*. 2004).

Another technique employed to break SI in species is the mentor effect. The Russian plant breeder Michurin pioneered a technique in the 1950s - 60s to mix small quantities of a compatible pollen with heterospecific normally incompatible pollen to obtain success with incompatible interspecific hybridizations. Stettler (1968) modified Michurin's technique by exposing the compatible mentor pollen to gamma radiation, then mixing this dead pollen with live normally incompatible pollen to successfully obtain hybrids between *Populus* species that were inter-incompatible. This technique to mix dead compatible pollen with live incompatible pollen has been used successfully to obtain inter-specific hybrids and also, strikingly, to break self-incompatibility (Howlett *et al*. 1975). Moreover, various studies have shown that a mixture of live, normally incompatible self pollen with compatible heterospecific (usually congeneric) pollen can induce some selfing. This phenomenon occurs in species with GSI and SSI (Pandey 1977; Desrochers & Rieseberg 1998; Mráz 2003).

Several studies have used the mentor effect with *Theobroma cacao*, a species with LSI. Adu-Ampomah *et al*. (1990) obtained some selfed seed in *T. cacao* trees following application of 1:1 self: mentor pollen, the latter cross compatible pollen that had been irradiated at 60 Gy. With increased proportions of mentor pollen, they obtained better yields of selfed seed. However, mixes with mentor pollen irradiated at 80 Gy did not produce fruits, indicating that for the effect to occur the mentor had to produce pollen tubes. Lanaud *et al*. (2017) report using self pollen mixed with live pollen of the species *Herrania mariae* as the mentor to obtain selfed seeds with *T. cacao*. *Theobroma* and *Herrania* are phylogenetically close genera in the Theobromateae. These classic mentor techniques using irradiated compatible conspecific or live heterospecific pollen produced self-seeds alone in the surviving fruits.

A rather different phenomenon has been demonstrated in studies with diverse LSI species. Application of a mix of live self and live cross pollen on the stigma, or chase self and cross pollinations, may yield fruits with a mix of cross and selfed seeds as in *Campsis radicans* (Bertin & Sullivan 1988). In bombacoids, using genetic markers to determine paternity, although mixed 1:1 self: cross pollinations with one tree of congeneric *Ceiba pentandra* yielded very few selfed seeds (Gribel *et al*. 1999), similar studies with *Pseudobombax munguba* resulted in up to 22 % selfed seeds (Gribel & Gibbs 2002). Although not confirmed with genetic markers, the reduced fruit/seed set following mixed pollinations undertaken with these two *C. chodatii* trees reported by Gibbs *et al*. (2004) was most likely caused by some selfing, whilst a study using Random Amplified Polymorphic DNA (RAPD) markers did indicate the presence of selfed seeds following mixed pollinations (Gibbs *et al*. 1995). Self-seeds were also obtained following mixed pollinations with LSI species *T. cacao* (Glendinning 1960; Opeke & Jacob 1969; Lanaud *et al*. 1987), *Cola nitida* (Jacob 1973), *Asclepias exaltata* (Broyles & Wyatt 1993), *Akebia quinata* (Kawagoe & Suzuki 2005). The assumption is that with the mixed pollen load on the stigma, self- and cross-pollen tubes are equally successful in growing down the style to the ovary, where some ovules are penetrated by self pollen, but sufficient ovules receive cross pollen to prevent pistil abscission (as occurs following self only pollinations) and allows fruit set, albeit with reduced fecundity and a mix of cross and some selfed seeds. Moreover, some studies indicate that when mixed self/cross pollinations occur naturally they result in low seed sets that resemble those from hand mixed pollinations due to ovule discounting by self pollen (Waser & Price 1991; Gribel & Gibbs 2002; Gibbs *et al*. 2004; Vaughton *et al*. 2010). In these cases it seems that we are dealing with a feature of the LSI phenomenon rather than classic mentor effect pollinations.

A number of studies have indicated that the ability of compatible pollen tubes to grow successfully to the ovules is conditioned during their traverse of the style by secretions from the ovular micropyle (Higashiyami *et al*. 1998; Dresselhaus & Franklin-Tong 2013), and a similar phenomenon of 'long distance' recognition of self pollen growing in the style has been reported for various LSI species (Willemse 1996; Sage *et al*. 1999, 2006; Gibbs & Bianchi 1999). Whether at least in some species (or families) LSI is controlled by major gene action, as in conventional SI, remains uncertain. And to what extent a mismatch between signals from self pollen and ovular secretions triggers pedicel abscission such that self-pistils are rejected despite ovule penetration and, frequently, fertilisation, is not known.

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