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POSTZYGOTIC CONTROL OF SELFING IN *HYMENAEA STIGONOCARPA* (LEGUMINOSAE-CAESALPINIOIDEAE), A BAT-POLLINATED TREE OF THE BRAZILIAN CERRADOS

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The pollination biology and breeding system of *Hymenaea stigonocarpa*, a common tree of the Brazilian cerrados, were investigated. The flowers have nocturnal anthesis, produce copious nectar, and are visited by at least four species of bats, including the specialized nectivorous *Glossophaga soricina* and the less specialized frugivores *Platyrhinus lineatus* and *Carollia perspicillata*. Controlled hand-pollination results indicate the species is basically allogamous. Almost all selfed flowers abscised within 7–8 d, but rejection was due to a postzygotic mechanism, since self-pollen tubes grew to the ovary and penetrated ovules with equal facility to cross tubes, and selfed ovules at 5 d contained a zygote with endosperm nuclei. The causes of selfed pistil abscission were not established, but selfed ovules were smaller than crossed ones at every stage over the first 5 d postpollination, indicating an early recognition or differential development of selfed ovules.

Keywords: Leguminosae, Hymenaea, Brazilian cerrado, bat pollination, breeding system, reproductive biology.

Introduction

Hymenaea is a genus of tropical and subtropical resin-producing trees reputed to be the main source of fossil amber in the Neotropics (Langenheim 1973). The genus includes 14 species that, with the exception of one remarkable disjunct in East Africa–Madagascar, are distributed in Mesoamerica, the West Indies, and most of South America (Lee and Langenheim 1975). The 13 Neotropical *Hymenaea* species are mainly trees of evergreen forests, but some occur in seasonal woodlands, particularly the caatinga thorn forest of northeastern Brazil and the cerrados of central Brazil. The very widespread species *Hymenaea courbaril* ranges from savanna-type vegetation to humid Amazonian forest, but *H. courbaril* var. *stilbocarpa* only occurs in riverine forests in the cerrado region. In contrast, another species with a wide-ranging distribution, *Hymenaea stigonocarpa*, is largely restricted to seasonal woodland habitats such as the caatingas and, particularly, the cerrados of central and southeastern Brazil.

Lee and Langenheim (1975) proposed—on the basis of inflorescence type, large flower size, and the presence of additional nectary tissue in the rim of the hypanthium—that bat pollination was likely to occur in the 11 species of sect. *Hymenaea*, with bee pollination most likely in the three species (including the African *Hymenaea verrusoa*) of sect. *Trachylobium*. However, field observations on floral biology and even basic data on the timing of anthesis were lacking for all these species except *H. courbaril*, the only species of the genus to have been studied for its reproductive biology. Carvalho (1961) and Vogel (1968) reported bat visits to the flowers of *H. courbaril* in Amazonian Brazil, Heithaus et al. (1975) published a list of bats that visited flowers of this species in Costa Rica, and Crestana et al. (1985) also described bat visitation to this species (as *Hymenaea stilbocarpa*) in southeastern Brazil. Bawa (1974) reported *H. courbaril* to be self-incompatible. However, subsequent studies on a number of Neotropical legume species (Bawa and Webb 1984; Seavey and Bawa 1986; Gibbs and Sassaki 1998; Lewis and Gibbs 1999) have shown that apparent self-incompatibility may be due to postzygotic rejection phenomena. We describe the first detailed study of the pollination biology and breeding system of the cerrado-distributed *H. stigonocarpa*, based on field studies at two sites in central Brazil.

Material and Methods

The main study site was in the Brasília Botanic Garden (BBG) located in the Distrito Federal (15°52′S, 47°51′W) at an altitude varying from 1025 to 1150 m. The BBG has an area of 4500 ha, of which 80% is natural vegetation. In 1989, the flowering period was monitored for 10 trees of *Hymenaea stigonocarpa* located along a track in an area of fairly closed cerrado woodland. We made daily observations to determine floral biology and longevity. Nectar volume and sucrose equivalents were measured using micropipettes and a hand refractometer. Floral visitors were observed and photographed; specimens were captured using mist nets placed beside or under the trees. Hand-pollinations were carried out with flowers that had been isolated previously as mature buds in nylon gauze or paper bags. Further studies on the pollination biology and the breeding system of *H. stigonocarpa* were carried out in 1996 using eight isolated trees in cerrado remnants at the Glo-

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ria Experimental Farm (18°5′S, 48°12′W, alt. ca. 800 m) of the University of Uberlândia (Minas Gerais state), located some 400 km south of Brasília.

Self-pollinations were made with pollen from the same flower, and cross-pollinations involved donor trees some 30 m distant. A series of tagged flowers was used to estimate natural fruit set at the BGG site. Most hand-pollinated flowers at both sites were left to census for fruit set, but subsamples of selfed and crossed pistils were fixed in FAA (70% ethanol with 5% glacial acetic acid/formaldehyde) at daily intervals up to 10 d after pollination to study pollen-tube growth using leucoaniline blue staining and fluorescence microscopy (Martin 1959). For this purpose, a portion of the thick carpel wall was carefully trimmed away in the field to facilitate penetration of the fixative. Subsequently, in the laboratory, the remaining ovary wall was trimmed away to leave a row of ovules mounted on the placenta. Most of these placenta/ovule portions were then softened in 8 M NaOH solution in an oven at 60°C for ca. 20 min and quickly rinsed in water, and then ovules and placenta were further dissected on a microscope slide in a droplet of the stain before applying a cover slip. Complementary observations on ovule size and the presence of a pollen "tail" at the micropyle were made using unsoftened ovules mounted in aniline blue. Detached ovules from 5-d-old selfed versus crossed pistils were embedded in resin (LKB Historesin kit) and sectioned at 4 μm using a Reichert microtome and stained in toluidine blue (O'Brien and McCully 1981).

Results

Pollination Biology

Hymenaea stigonocarpa flowers between January and March, in the middle of the rainy season when the trees are in leaf. Trees of *H. stigonocarpa* produced ca. 10–20 flowers per night during the flowering peak and several hundreds of flowers over the total flowering period of 2 or 3 wk.

The flowers, the largest in the genus, occur in short panicles. The thick sepals, which reflect back during anthesis, and the white, ca. 22 × 12–mm petals, sit on the rim of a broad, subsessile hypanthium. The 10 exserted, ca. 40-mm, stamens have large, ca. 9-mm versatile anthers. The single, fleshy carpel, which is borne on a ca. 6–8-mm stipe, has a mean ovule number of 12.9, range 8–18 ($n = 25$). The carpel terminates in an elongate style with a capitate stigma (wet-papillate; Owens 1989) that is located at the same level as the anthers. Some flowers with reduced, malformed pistils were observed at both study sites, rendering functionally staminate flowers. These flowers were rare (less than 1%), and it was not possible to say if the reduced pistils were the result of a failure in development or the action of pathogens.

Flowers started to open at dusk from 1730 hours on, but it varied somewhat from plant to plant. Flowers were usually completely opened, with pollen available, the style erect, stigma receptive, and nectar available ca. 2 h later (ca. 2000 hours both in Brasília and Uberlândia). Usually, before the stamens and petals were completely unfolded, the style was projected from the flower and the stigma seemed receptive. Nectar was secreted from the onset of anthesis from a nectary located below a fleshy disk at the base of the pistil stipe and accumulated within the hypanthium. At 1930 hours, previously bagged flowers yielded 20–110 μL of nectar, with 6%–15% of sucrose equivalents (*sensu* Inouye et al. 1980). By 2100 hours, 0.5–1.5 mL, with a sucrose content of 12%–19% ($n = 6$ in Brasília and $n = 8$ in Uberlândia) could be withdrawn from the same flowers. By this time, in bagged flowers held in an upward position, the amount of nectar was sufficient to fill the hypanthium and was held by the bases of the filaments and petals. Nectar was produced copiously and continuously during the night, although at a decreasing rate. Flowers lasted 1 night; petals and stamens dropped the following day. The residual hypanthium and pistil of unpollinated flowers fell by the fourth or fifth day after the anthesis.

Visits by bats and hawkmoths began as soon as the flowers were fully open (ca. 2000 hours) and continued intermittently throughout the night. The most common visitor captured and photographed at the flowers was *Glossophaga soricina*. These bats visited the tree canopy either alone or in small groups, foraging at many flowers. This bat hovered in front of the flowers and inserted its head to lap the nectar in very characteristic fleeting visits of less than a second or so. They were also observed to lick the anthers for pollen in recently opened flowers (fig. 1). Other larger bat species were also observed visiting the flowers of *H. stigonocarpa*. *Platyrhinus lineatus* is a frugivorous species that is a less specialized flower visitor. These bats repeatedly approached a flower without touching it, perhaps in a reconnaissance flight, before subsequently landing on the flower, clutching the petals, sepals, or pedicel while inserting its head inside the flower (fig. 2). In contrast to the fleeting visits of *Glossophaga*, those of *Platyrhinus* lasted several seconds. These bats were usually alone and visited few flowers. Another frugivorous species, *Carollia perspicillata*, was also captured under the canopy of *Hymenaea* trees and was observed visiting flowers by clasping the petals like *P. lineatus*. A specimen of the large bat *Phyllostomus hastatus* was also netted below an isolated tree of *H. stigonocarpa* at the Gloria Farm but was not observed visiting the flowers.

The flowers were also visited by hawkmoths (Sphingidae), which had either a hovering or a settling behavior similar to the bats, though they usually remained below the level of the anthers or stigma and were not observed to make effective contact with either. *Eumorpha labruscae*, *Cocytius antaeus*, and *Cocytius duponchel* are large hawkmoths captured in mist nets located near an individual of *H. stigonocarpa* to catch bats. Other nonidentified sphingid moth species were observed and photographed visiting *H. stigonocarpa* flowers.

Breeding System

Hand-pollinations yielded 45% fruit set from crosses and 2% from selfs (table 1). Most selfed pistils abscised by the eighth day after anthesis, but the two pistils that persisted developed into normal fruits over 6–7 mo later. Seed viability in these selfed fruits was not tested but appeared to be normal. Fluorescence microscopy observations indicated that in both treatments some pollen tubes had reached the ovary by 24 h, with some ovule penetrations. Based on pooled observations from several pistils for each interval, the incidence of ovule penetrations did not differ significantly between selfs and crosses at 24, 48, and 72 h, but at 96 h, significantly more

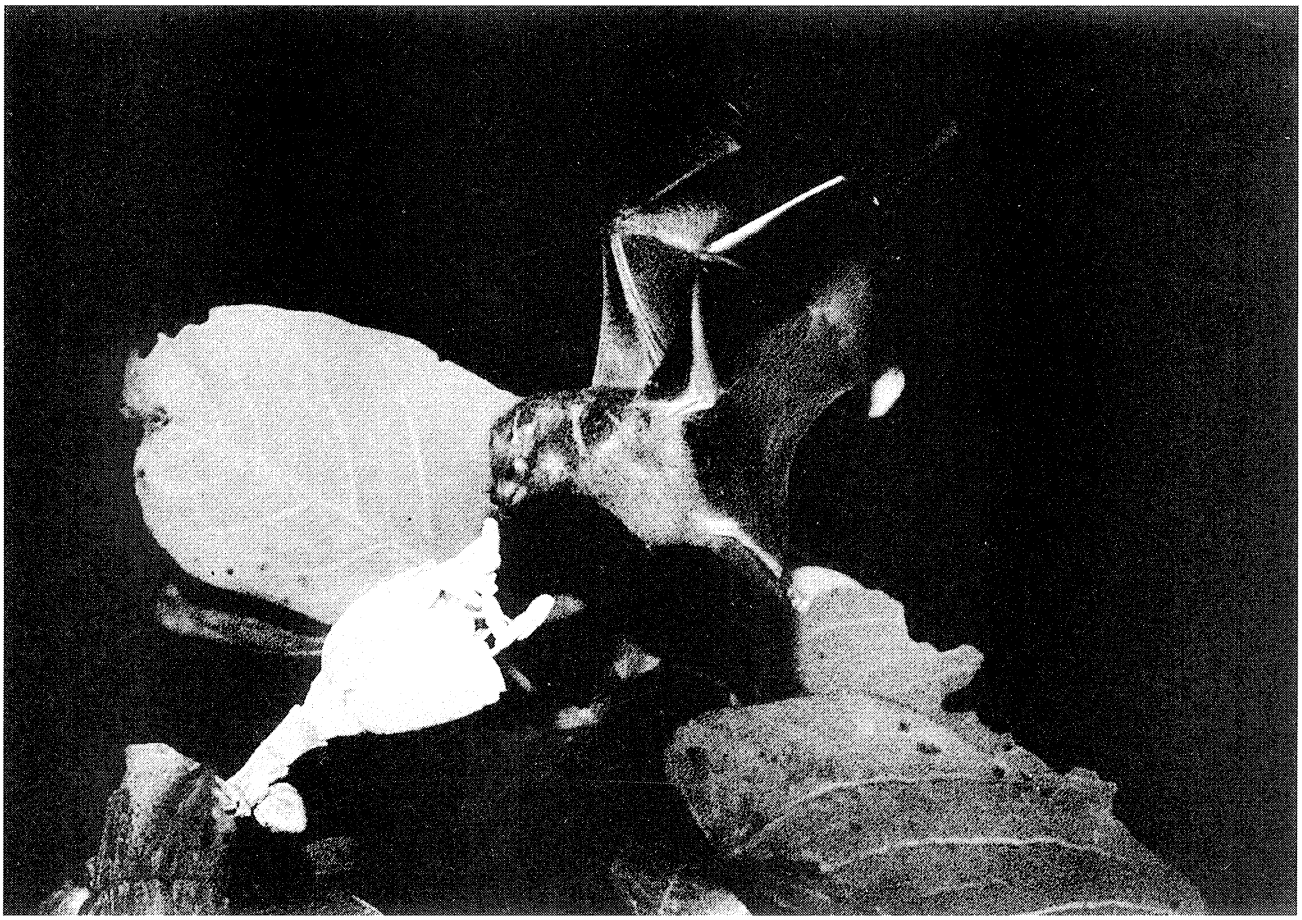

Fig. 1 *Glossophaga soricina* visiting *Hymenaea stigonocarpa* flower. In this case, the bat hovered in front of the flower to lap pollen from the anthers. The same hovering behavior was observed when this species of bat was visiting the flowers for nectar.

ovules were penetrated in crossed pistils than in selfed (table 2).

In both types of pollination, some ovules remained unpenetrated despite the obvious excess of pollen on the stigma. The position of such nonpenetrated ovules varied from pistil to pistil in both selfs and crosses, but ovule positions 1–5 (from the base of the style) were almost invariably penetrated and positions 10–12 were more likely to be unpenetrated. Ovule size differed significantly between crossed and selfed pistils at each interval from 24 to 96 h (fig. 3). Sectioned ovules from selfed pistils fixed at 5 d indicated that ovule penetration was followed by fertilization, with the presence of an undivided zygote with endosperm nuclei at this stage as in crossed ovules (fig. 4). One-week-old selfed fruits, i.e., at about the time they were abscised, contained rows of uniformly sized ovules comparable to, although smaller than, those in crossed fruits (fig. 5).

Discussion

Pollination Biology

Details of floral anthesis for *Hymenaea courbaril* given by Lee and Langenheim (1975), based on unpublished observations by G. W. Frankie, are generally very similar to our observations with *Hymenaea stigonocarpa*, except that the protandry reported for *H. courbaril*, with the style initially remaining coiled within the flower, was not found in *H. stigonocarpa*. Moreover, Crestana et al. (1985) did not observe such protandry in *H. courbaril* var. *stilbocarpa* in São Paulo State. On the contrary, as in *H. stigonocarpa*, these authors reported that the style, with the stigma apparently receptive, was projected from the bud at preanthesis, indicating protogyny.

Hymenaea stigonocarpa is one of a group of at least five bat-pollinated trees found in the central Brazilian cerrados, the others being *Caryocar brasiliense* (Gribel and Hay 1993), *Lafoensia pacari* (Sazima and Sazima 1975), *Bauhinia rufa*, and *Pseudobombax longiflorum* (R. Gribel, unpublished studies). Another species, *Pseudobombax tomentosum* is also visited by bats, but Gribel (1988) has argued that this species is better adapted to pollination by marsupials.

Hymenaea stigonocarpa, as *C. brasiliense*, presents an exposed nectary that characterizes more primitive chiropterophilous flowers that can be explored both by short-tongued Phyllostomidae and more specialized Glossophaginae (Vogel 1968). Bat visitors to *H. stigonocarpa* are reported also to

Table 1

Results of Hand-Pollination Treatments to Determine the Breeding System of *Hymenaea stigonocarpa*

	Flowers	Mature fruits	
		n	%
Cross-pollination	47	21	45
Self-pollination	50	2	4
Open pollination	110	5	5

Table 2

Incidence of Ovule Penetration in Selfed and Crossed Pistils

Time	Selfed	Crossed	χ^2	df	*P*
24 h (2/3)[a]	17 (22)[b]	18 (23)	0.0063	1	>0.95
48 h (3/3)	22 (34)	23 (37)	0.049	1	>0.90
72 h (3/3)	21 (38)	29 (38)	3.74	1	>0.05
96 h (4/3)	29 (64)	34 (42)	13.37	1	<0.01

[a] Number of pistils self-pollinated/cross-pollinated.
[b] Total number of ovules.

pollinate *L. pacari* (Sazima and Sazima 1975) and *C. brasiliense* (Gribel and Hay 1993), namely, *Glossophaga soricina* and *Platyrhinus lineatus*. *Carollia perspicillata* was another possible pollinator of *C. brasiliense*, but these bats were not observed directly at the flowers. In their survey of bat foraging habits in a Costa Rican seasonal forest (by capture and pollen analysis), Heithaus et al. (1975) reported pollen of *H. courbaril* present on individuals of *G. soricina*, *C. perspicillata*, *Phyllostomus discolor*, *Artibeus jamaicensis*, *Artibeus literatus*, and *Artibeus phaeotis*.

Hymenaea stigonocarpa is the only species of the cerrado bat–pollinated group to flower in the middle of the rainy season (January–February), while the other bat-pollinated species, including *H. courbaril* var. *stilbocarpa* (Crestana et al. 1985), all flower at the end of the dry season or beginning of the rains (September–October). Heithaus et al. (1975) noted that utilization of flowers versus fruits by bat species varied seasonally and that pollen loads occurred most frequently in the dry season, such that *G. soricina* was an exception in that pollen was absent from most bat individuals captured in the wet season. These authors also noted that primarily frugivorous bats such as *C. perspicillata* and *Artibeus jamaicensis* only utilized nectar when it was abundant in the dry season. However, our study indicated for the central Brazilian cerrados that

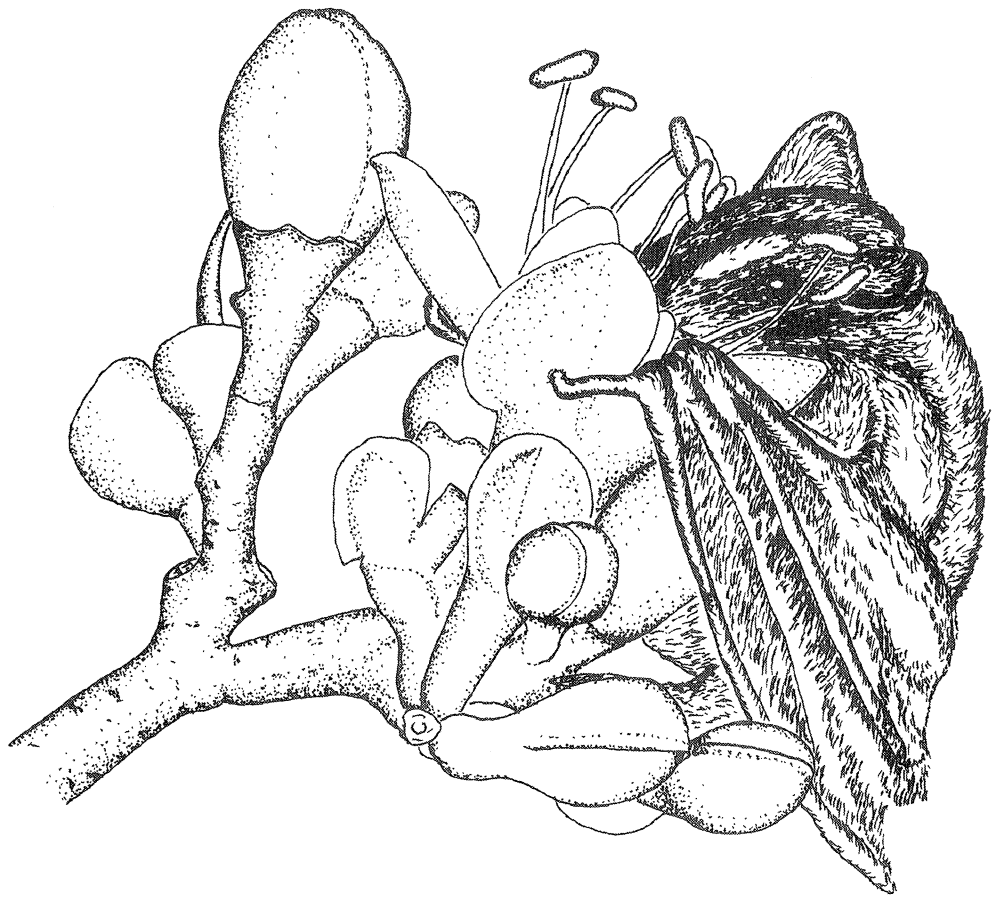

Fig. 2 Settling visit of *Platyrhinus lineatus* to a flower of *Hymenaea stigonocarpa*. The bat landed on the flower, clutched the sepals and receptacle, and inserted its head into the flower to drink the nectar. Drawn from photograph.

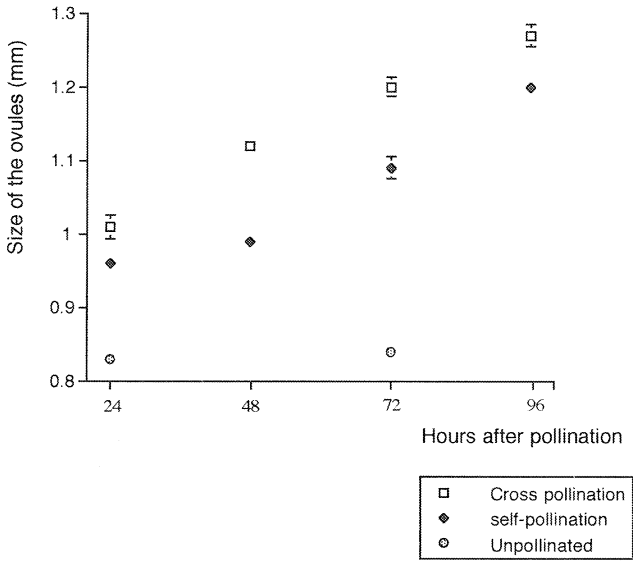

Fig. 3 Ovule size of *Hymenaea stigonocarpa* after self- and cross-pollination. Pooled results from different pistils ($n = 3$) at 24, 48, 72, and 96 h after pollination. Bar = SEM.

primarily frugivorous bats such as *P. lineatus* and *C. perspicillata* will continue to utilize a nectar-rich source such as flowers of *H. stigonocarpa* during the wet season, despite the relative abundance of fruits in the cerrado and adjacent gallery forest at this time. The diversity of bat pollinators to *H. stigonocarpa* flowers and the overlap of such visitors with other bat-pollinated species in the area, despite the different flowering season, strongly support the view that the Neotropical chiropteran fauna associated with pollination are relatively generalist and widespread (Koopman 1981).

Breeding System

Our study indicates that *H. stigonocarpa* is an outbreeding species in which selfing is controlled by a postzygotic rejection mechanism. In an early study, based simply on success of cross- versus self-pollinations, Bawa (1974) reported *H. courbaril* to be self-incompatible. However, in a subsequent review paper, Seavey and Bawa (1986) drew attention to some neglected cases in the literature of species in which selfed flowers are rejected despite successful growth of self-pollen tubes to the ovary, a phenomenon that they termed "late-acting self-incompatibility" (LSI), also termed "ovarian self-incompatibility" by some authors (Sage et al 1994). Seavey and Bawa (1986) listed five legume species, members of the subfamily Papilionoideae,

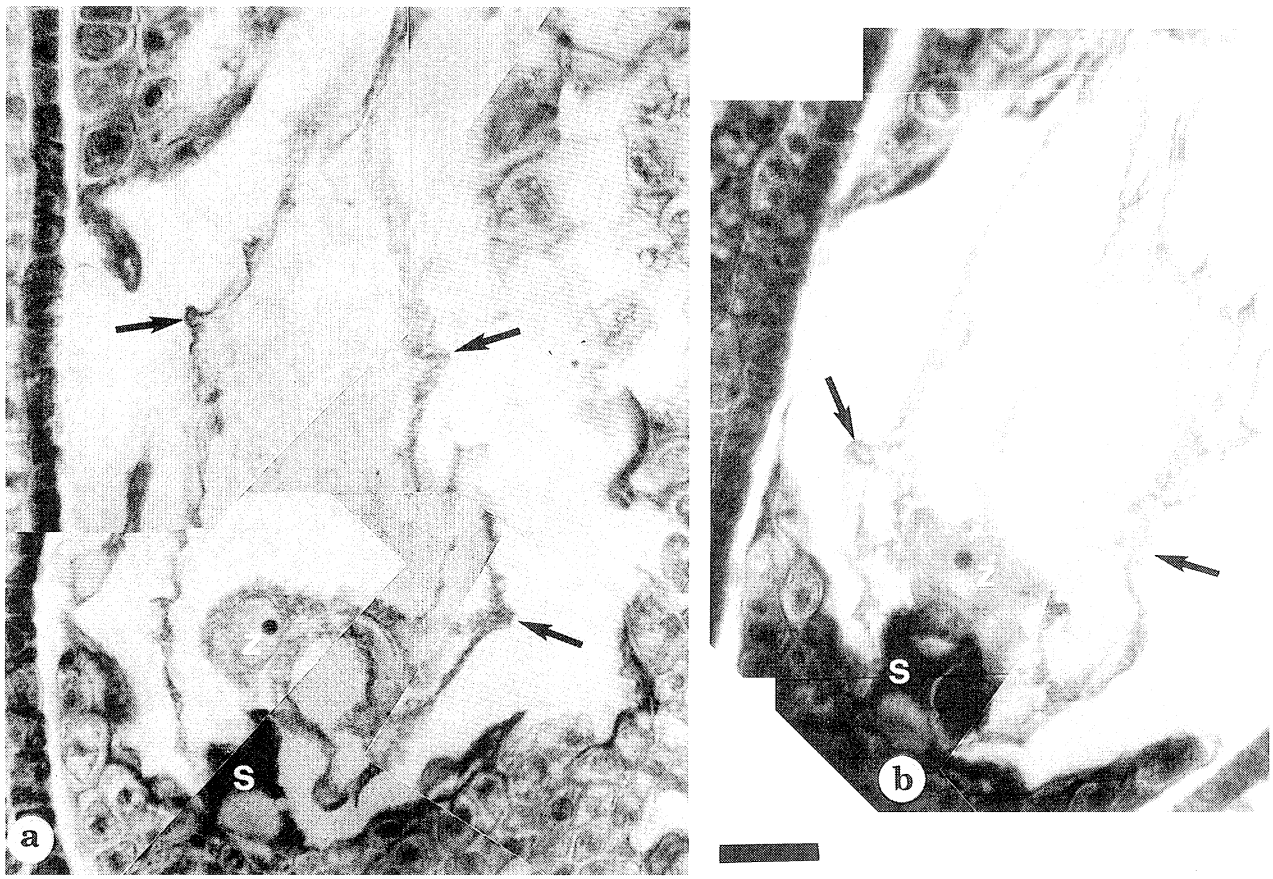

Fig. 4 Partial longitudinal sections of the embryo sac in ovules of *Hymenaea stigonocarpa* at (*a*) 5 d post-self-pollination and (*b*) 5 d post-cross-pollination. Composite views from video prints taken at different focal planes. *S* = synergid, *Z* = zygote, endosperm nuclei arrowed. Resin-embedded sectioned at 4 μm, toluidine blue–stained. Scale bar = 35 μm.

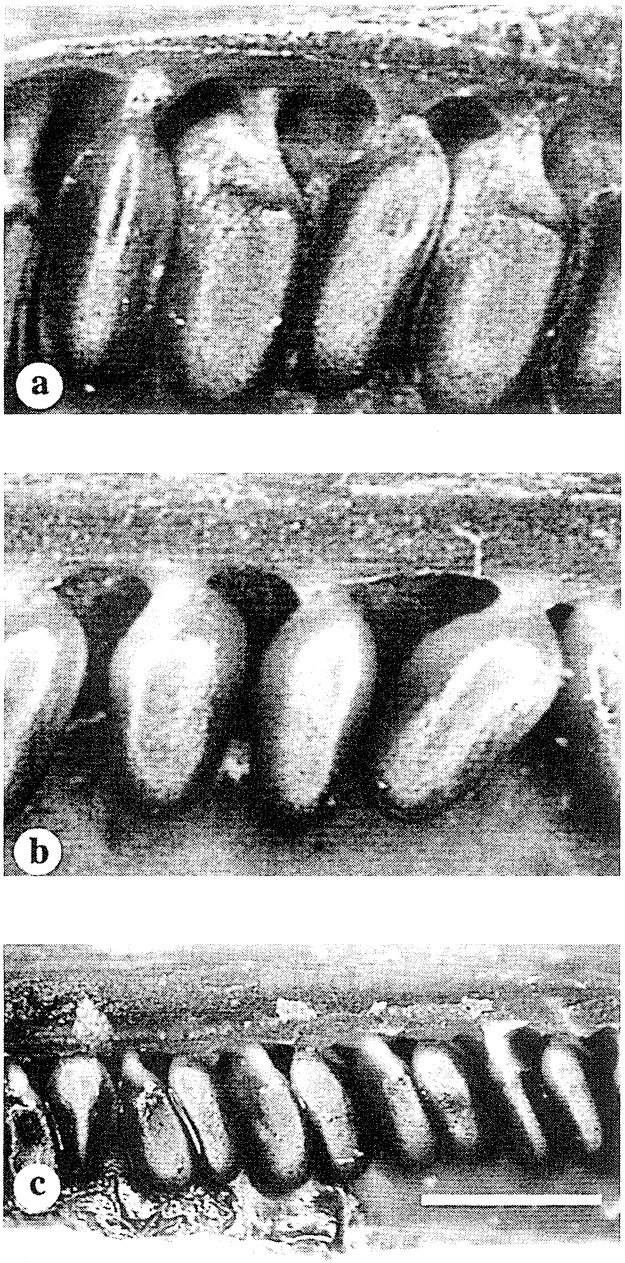

Fig. 5 Views of ovules/young seeds in pistils of *Hymenaea stigonocarpa*: (*a*) cross- and (*b*) self-pollinated after 7 d; (*c*) unpollinated at 24 h. Scale bar = 1 mm.

with LSI; Lewis and Gibbs (1999) also have reported ovarian self-incompatibility in *Caesalpinia calycina*. Control of selfing by postzygotic, ovarian self-sterility–type phenomena in these taxa contrasted with the suggestion of Arroyo (1981), based on circumstantial evidence, that self-incompatibility in the legume family, including Caesalpinioideae, was most likely to be of the classic gametophytic type.

Seavey and Bawa (1986) emphasized that it may be difficult in some cases of LSI to distinguish between early-acting inbreeding depression effects and any novel form of late-acting cause of rejection of selfed pistils. Klekowski (1988) has criticized the term "LSI," arguing that recessive lethals are a more likely explanation than any novel form of self-incompatibility.

An increasing number of apparently self-incompatible species (Bertin and Sullivan 1988; Oliveira et al. 1991; Waser and Price 1991; Oliveira et al. 1992; Gibbs and Bianchi 1993; Seavey and Carter 1996; Nic Lughadha 1998) have been characterized as having self-sterility associated with successful self-pollen tube growth to the ovary and therefore some form of ovarian sterility. Rejection of selfed flowers or fruits in such cases occurred either within a fairly well defined postpollination interval, ranging from 72 h in *Theobroma cacao* (Cope 1962) to some 7–10 d, as in *Chorisia* and *Tabebuia* species (Gibbs and Bianchi 1993), or over extended periods of weeks or months, as in *Dalbergia miscolobium* (Gibbs and Sassaki 1998) and *Gomidesia* species (Nic Lughadha 1998). In the latter situation, inbreeding depression effects seem the most likely explanation. However, when there is a fairly short period of time in which selfed flowers are uniformly rejected, then the action of lethal recessive alleles is more difficult to explain. In *Theobroma*, *Chorisia*, and *Tabebuia* species, e.g., rejection occurs while there is a resting zygote stage before embryonic divisions occur.

Hymenaea stigonocarpa, whose selfed flowers/pistils fall after a week or so, falls into this latter category of species with ovarian sterility but a well-defined abscission period. The causes of selfed pistil abscission were not established in this investigation, although it may be significant that selfed ovules were smaller than crossed ones at every stage over the first 5 d postpollination, indicating an early recognition or differential development of selfed ovules. Moreover, there was no indication of striking variation in ovule/young seed size within pistils over the first week, as might be expected with deleterious recessives acting at various stages in early embryo development (Seavey and Carter 1996). As Sage et al. (1994) emphasized, fruit abortion may occur long after primary causes of failure are active. It is possible, therefore, that abscission of selfed pistils in *H. stigonocarpa* is a consequence of early recognition events, e.g., at the time of ovule penetration. However, the size difference between selfed versus crossed ovules could also reflect the early action of deleterious alleles, and cytological studies on large numbers of ovules would be necessary to establish whether the rejection of selfed pistils in *H. stigonocarpa* is due to the abnormal development of some ovules.

The very low fruit set from natural pollinations observed in this species (5%) is similar to that reported for other legume species, e.g., 8% in *Dalbergia retusa*, 3.4% in *Myrospermum frutescens* (Bawa and Webb 1984), and 6.6% in *D. miscolobium* (Gibbs and Sassaki 1998), all of which are bee-pollinated, and also in bat-pollinated species of other families, e.g., 3.6% in mass-flowered *C. brasiliense* (Gribel and Hay 1993). In all these species, most flowers fall relatively rapidly rather than initiate fruiting, which implies that either low pollination success or the effects of geitonogamous pollinations may be responsible.

Given the tendency for ovarian sterility phenomena to occur in related taxa (e.g., *Chorisia chodatii*, *Chorisia speciosa*, and *Eriotheca pubescens* in the Bombacaceae; *Tabebuia ochracea*, *Tabebuia caraiba*, and *Campsis radicans* in the Bignoniaceae), it is most likely that the self-incompatibility reported in *H.*

courbaril (Bawa 1974) is also of the ovarian self-sterility type. As noted by Gibbs and Sassaki (1998), there are more cases of LSI or ovarian sterility reported for legume species than for conventional self-incompatibility. Moreover, given the increasing number of taxa reported to have this kind of mechanism, it is possible that phenomena involving postzygotic rejection, whether due to inbreeding depression effects or other causes, may prove to be as widespread in controlling selfing in flowering plant taxa as conventional self-incompatibility mechanisms of the homomorphic type.

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