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RESEARCH

What causes self-sterility in *Couroupita guianensis* (Lecythidaceae)?

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Abstract

Couroupita guianensis is a native (but not endemic) tree to the Brazilian Amazon rainforest, well known by the pleasant scent of their showy flowers gathered in cauline inflorescences, and their peculiar spherical, heavy, and woody fruits, which makes the species a common component of urban landscapes in Brazil and around the tropical world. Formerly suggested to be xenogamous, this species has been treated as predominantly allogamous but self-compatible since the first criterious study of its breeding system. In this work we revisit the question of self-sterility of *C. guianensis* in a breeding system study based on controlled pollination experiments and light microscopy analysis of post-pollination events. Only cross-pollinated and open-pollinated flowers produced fruits that reached maturity twelve months later, indicating strong self-sterility. Extended pistil longevity and fruit initiation were observed after selfing, but almost all selfed fruits aborted at the first month after pollination exhibiting significantly greater length and width than unpollinated pistils at the time of abscission. No sign of prezygotic self-incompatibility reactions was verified, and ovule penetration, fertilization, and the nuclear endosperm initiation in selfed pistils/fruits did not differ from the same events in crossed ones up to abscission of the formers. Due to the long resting zygote condition in this species, selfed fruit abscission occurred before normal embryogenesis initiation in crossed seeds, so that there was no embryo formation in selfed seeds. No evidence for early-acting inbreeding depression was found in this study. The data indicate postzygotic late-acting self-incompatibility as the main cause of self-sterility in *C. guianensis*.

Keywords Late-acting self-incompatibility · Breeding system · Reproductive biology · Xenogamy

Introduction

Self-incompatibility (SI) is defined as a genetically determined physiological mechanism that prevent fertilization by self- or self-related pollen in fertile co-sexual plants, which diminishes the risk of inbreeding and optimizes the potential to outbreed, generating or maintaining genetic diversity within a species (Nettancourt 2001; Silva and Goring 2001; Hiscock and McInnis 2003; Rea and Nasrallah 2008; Raduski et al. 2012; Barrett 2013). As a rule, SI constraints fertilization by preventing self-pollen germination on the stigma, or by arresting self-pollen tube growth to the ovary and ovule penetration. Three major types of conventional SI systems were established based on their fundamental mechanisms of genetic control: homomorphic gametophytic (GSI), homomorphic sporophytic (SSI), and heteromorphic (HetSI) (Ganders 1979; Gibbs 1990; Nettancourt 2001; Allen and Hiscock 2008; Barrett 2013). Homomorphic SI systems typically are characterized by Mendelian genetic control of a single multi-allelic *S* locus to determine the incompatibility reaction. In GSI, the single *S* allele expressed by the haploid male gametophyte interacts with both *S* alleles of the diploid genome of the pistil, with pollen tube/pistil allelic identity resulting in inhibition of the pollen germination on the stigma or, more frequently, in the pollen tube growth inhibition or suppression along the pistil transmitting tract (Nettancourt 2001; Allen and Hiscock 2008). In SSI, both *S* alleles of the diploid pollen parent genome trigger the allelic-identity-SI reaction, typically

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by the arresting of the germination of incompatible pollen on the stigma (Nettancourt 2001; Allen and Hiscock 2008). Heterostyly is associated with HetSI, and self-recognition in this system usually is based on a single diallelic or two diallelic *S* loci in distylous and tristylous species, respectively (Ganders 1979; Lewis and Jones 1992). More recently, a fourth SI mechanism, also a dialellic *S*-locus based system but not associated with floral heteromorphy (homomorphic dialellic SI), has been reported for some genera of Oleaceae (Saumitou-Laprade et al. 2010, 2017; Vernet et al. 2016). A common property in all these four SI mechanisms is the presyngamic nature of the incompatibility reaction.

However, in the last more than five decades of breeding system studies, several lines of evidence have been accumulated in favor of the recognition that a fifth mechanism, late-acting SI (LSI), operates in different angiosperm taxonomic groups (Seavey and Bawa 1986; Sage et al. 1994; Gibbs 2014). In plants supposed to present LSI, incompatible pollen tubes germinate on the stigma and grow down to ovary, as well as the compatible one, and typically most ovules in self-pollinated pistils are penetrated, with the incompatibility reaction in some cases expressing post-zygotically (Sparrow and Pearson 1948; Seavey and Bawa 1986; Gibbs and Bianchi 1993; Sage et al. 1994; Gibbs 2014). Especially in the cases where the plant clearly is self-sterile and the effective fertilization of most ovules is followed by a uniform abortion of the selfed-pistils, critics have argued that early-acting inbreeding depression (EID), with deleterious recessives affecting selfed embryo development, should be a more likely explanation for the failure of fruit set in selfed flowers of alleged LSI species (Klekowski 1988; Krebs and Hancock 1991; Nic Lughadha 1998; Brandvain et al. 2024). Diallel crosses with sibling progeny arrays have been the standard procedure used to determine the genetic control of the SI, but due to the long-lived tree or shrub condition typically found in LSI taxa (Gibbs 2014), very few of such studies have been made in an attempt to discover the major gene control involved in the incompatibility mechanism of LSI species (Knight and Rogers 1955; Cope 1962; Brewbaker and Gorrez 1967; Brandham and Owens 1978; Lipow and Wyatt 2000; LaDoux and Friar 2006; Bianchi et al. 2021).

It is also important to point out that, as defined above, SI strictly speaking should be restricted to those taxa for which a genetic control has indeed been established. However, due to the difficulties in performing diallel crosses with sibling progeny arrays in non-herbaceous long-lived plants, the majority of the breeding system studies provide only circumstantial evidence of SI. This is the case for all reports of SI in Lecythidaceae until then, and it is possible that some, if not all species referred as self-incompatible in this family may in fact be cases of 'self-sterility', a neutral term that does not imply underlying mechanisms or causes for reduced or null seed set by selfing (Gibbs 2014).

Lecythidaceae A. Rich. is a Pantropical angiosperm family (Mori 2004; Souza and Lorenzi 2019) often ecologically dominants in Amazonian forests – the family was ranked third in total number of trees in a large-scale ecological study of lowland Amazonian forests (Steege et al. 2006). This family comprises woody plants that may range from small (e.g., *Eschweilera nana* Miers) to very large (up to 55 m tall) trees (*Bertholletia*, *Cariniana*) (Mori 2001, 2004). The flower in these plants may be actinomorphic or zygomorphic, with the floral symmetry being determined mostly by pollinator selective pressures on the androecium (Mori et al. 2007, 2017). Usually numerous, the stamens are variably fused, so that in actinomorphic-flowered taxa the filaments are fused at the base to form a staminal ring (androphore, in the sense of Prance 1976); on the other hand, in zygomorphic-flowered taxa the staminal ring is unilaterally expanded to form an androecial hood (ligule), a floral feature exclusively found in this family (Mori 2001; Mori et al. 2007, 2017). Except for a few cases of bat and perhaps beetle pollination, the flowers in Lecythidaceae are pollinated mainly by bees (Knudsen and Mori 1996; Mori 1988; Mori et al. 2007, 2017; Tisou and Mori 2007). Fruits and seeds in Lecythidaceae may present many adaptations for animal, wind, or water dispersion (Prance and Mori 1978).

Although breeding system studies in this family are scarce, Neotropical Lecythidaceae in general have been referred as predominantly or totally "self-incompatible" (Mori and Kallunki 1976; Mori and Prance 1990; Maués 2001; Cavalcante et al. 2012; Potascheff et al. 2014), or they normally present high within-population outcrossing rates (O'Malley et al. 1988; Buckley et al. 1988; Lepsch-Cunha and Mori 1999; Wadt et al. 2015; Tambarussi et al. 2016; Guidugli et al. 2016; Kubota et al. 2024), being completely dependable on animal pollinators to reproduce by seeds. In one of the first studies on pollination biology in Lecythidaceae, Prance (1976) verified that in bagged flowers of 10 inflorescences of *Couroupita subsessilis* Pilg., none of them set fruits, whereas unbagged neighboring inflorescence did, which indicated the allogamy in this species. Still in those early years of studies on reproductive systems in Lecythidaceae, similar simple flower-bagging procedures were also used to evidence allogamy in *B. excelsa* (Prance 1976) and *Lecythis minor* Jacq. (syn. *Lecythis elliptica* Kunth) (Jackson and Salas 1965). Based in these studies, Prance (1976) commented that *C. guianensis* should also be allogamous, since a single tree grown at the Fairchild Tropical Garden in Florida (USA) does not set fruit, whereas groups of trees grown in other places set fruit abundantly. In a study that included controlled experimental pollinations, Mori and Kallunki (1976) demonstrated that *Gustavia superba*

(Kunth) O. Berg is not self-compatible and must be outcrossed to set fruits. These authors also mention a personal communication of G. T. Prance, according to which the same abovementioned single *C. guianensis* tree, cultivated at the Fairchild Tropical Garden in Florida, did not set fruit until it was pollinated with pollen brought from another tree cultivated at Fort Myers, which points to the necessity of between-genets crossing (xenogamy) for fruit set in this species. Mori and Kallunki (1976) concluded that if SI occurs in two distantly related genera, such as *Couroupita* and *Gustavia*, this condition would be of general occurrence in the Lecythidaceae.

However, evidence against the presence of SI has been found in some species of Lecythidaceae, such as in *Eschweilera ovata* (Cambess.) Miers (Gusson et al. 2006; Tambarussi et al. 2016). In a breeding system study in 13 trees of *C. guianensis* cultivated at the Horto Botânico do Museu Nacional, Rio de Janeiro (Brazil), Ormond et al. (1981) recognized the species as allogamous but self-compatible. From 100 self-pollinated flowers, only two (2%) set fruits (vs. 12% and 4% of cross and natural pollinated flowers, respectively), and independent of the kind of pollination treatment young fruits aborted in large numbers. Microscopic analysis of fresh sections indicated no difference in the rate of pollen germination and pollen tube growth between selfed, crossed and natural pollinated pistils. Moreover, the authors reported that, in one of the years of study (1980), flowering was observed in a single local tree that subsequently set fruit, which was viewed as a reinforcement of their own results of controlled experimental pollinations in favor of self-compatibility in *C. guianensis*. Nonetheless, it must be pointed that no mention of a criterion of fruit development monitoring is cited in the study of Ormond et al. (1981), and it has been reported that lone trees of this species, cultivated in isolation, may abort their "fruits" (supposedly derived from unpollinated flowers) when they reach 3 cm in diameter (G. T. Prance, cited by Mori and Kallunki 1976).

As referred above, the available data on the reproductive system of *C. guianensis* appear to be conflicting, and this subject deserves a reinvestigation. If the species proved to be self-sterile, the apparently normal pollen germination on the stigma and pollen tube growth throughout the transmitting tract with the ultimate penetration of ovules in self-pollinated pistils reported by Ormond et al. (1981) may also be congruent with LSI or EID. In this study, we sought to deepen knowledge about the breeding system of *C. guianensis* using controlled pollination experiments and light microscope analysis of post-pollination events. The following questions were addressed in our study: (1) Do our experimental pollinations support the occurrence of self-compatiblility in *C. guianensis*, as indicated by the study of Ormond et al. (1981)? (2) Once confirmed, could the self-sterility in *C. guianensis* be explained as a case of LSI or EID? (3) What does our analysis on post-pollination events indicate regarding the most likely cause of self-sterility in *C. guianensis*? We also aimed to contribute for the embryology in Lecythidaceae.

Material and Methods

Species and Study Site

Couroupita guianensis is found on floodplain riverbanks throughout the Amazonian Region. Due to their showy, perfumed flowers and large cannonball like fruits, it is cultivated as an ornamental worldwide in tropical regions (Mori et al. 2007). In Brazil, it is popularly known as "abricó-de-macaco", and their spherical, woody, and heavy fruits earned the species the name "cannonball tree". The flowers of *C. guianensis* are displayed in long cauliflorous inflorescences (racemes) borne on the trunk and mature branches, and mature fruits fall to the ground and crack open to expose a flashy pulp containing numerous seeds. These seeds are dispersed by animals that feed on the pulp.

The fieldwork was conducted from 2015 to 2017 (controlled pollination experiments), and from 2021 to 2023 (field procedures for histological analysis of post pollination events) with planted trees at the Universidade Estadual Paulista "Júlio de Mesquita Filho" (lat. 20°47′05″S, long. 49°21′36″W, municipality of São José do Rio Preto, São Paulo, Brazil). The zygomorphic flower of *C. guianensis* is perigynous, 5–6 cm in diameter; the perianth presents 6–8 triangular sepals and 6–8 oblong petals; the staminal ring shows 600–700 small stamens that liberate fertile pollen grains in monads (Mori and Prance 1990); the flattened androecial hood emerges laterally from the staminal ring and folds over the top of the gynoecium; at its distal portion, the androecial hood contains 180–220 staminodes that liberate only sterile pollen grains in tetrads (Mori and Prance 1990); both the androecium and corolla abscise at the afternoon end of the single-day anthesis (Schoemberg 1983a), the semi-inferior, syncarpous, 6–8-carpellate and multiovulate ovary displays a complex morphology – it is multilocular with axile placentation at the base, unilocular with parietal placentation at the middle portion, and again multilocular at the apex (Schoemberg 1983a) –, with the middle portion of each carpel opening in a short ventral slit (Tisou 1994), and the external upper part of the ovary exposed outside of the hypanthium; the terminal style is very short and displays a star-shaped (in transverse section) central channel; the stigma is also star-like lobed, the number of lobes corresponding to the number of carpels; the edges of the stigmatic lobes are covered with secretory hairs involved in

pollen capturing (Ormond 1981). The anatropous, bitegmic, and tenuinucellate ovule presents a *Pollygonum* type megagametophyte with ephemerous antipodal cells and two polar nuclei that fuse to form a secondary nucleus at the embryo sac maturity (Tisou 1994). According to Schoemberg (1983a, b), the nuclear type endosperm of *C. guianansis* is completely consumed before the seed maturity, and the embryo development initiates around the seventh month after fertilization.

Pollination Experiments and Fruit Set

Controlled manual pollinations were performed in six individuals. After removing open flowers, whole inflorescences were enclosed in organza bags to exclude all the potential pollinators. Subsequently, flowers at an early-anthesis stage were either self-pollinated – i.e., using pollen from fertile stamens (staminal ring) of the same flower (test for autogamy) or from another flower of the same plant (test for geitonogamy) – or cross-pollinated – i.e., using pollen from fertile stamens of another individual (test for xenogamy) – and rebagged. A number of bagged flowers were left not pollinated to check for spontaneous (automatic) self-pollination, or had the stigma beheaded to check for agamospermy. Non-legitimate pollination – i.e., with pollen from the androecial hood of a flower of different individual – was also tested in some of the begged flowers. Pollen deposition was carried out by frictioning anthers of the staminal ring (legitimate pollination) or those of the androecial hood (non-legitimate pollination) over the stigma. Hand pollinations were carefully performed to cover all the receptive surface of the stigma with pollen in order to prevent failure of ovule fertilization due to limited pollination. For the flower treatment tagging, we used color lines attached to the flower pedicel, and the calix marking with color waterproof plastic glues (each color indicating a particular treatment). A self-adhesive plastic label attached to the pedicel, with the anthesis date printed on it, was used for each flower/fruit monitoring in the following days. Each inflorescence was unbagged daily to check for abscised pistils/initiating fruits and rebagged, while persistent fruits were monitored up to maturity. Natural fruit-set was monitored by tagging unbagged flowers.

Ovule-Seed Conversion Efficiency and Verification of Embryos

To estimate how many ovules per ovary develop into seeds in mature fruits derived from natural pollination, ovule and seed counts from the same trees used in the experimental pollinations were performed. Two fresh pistils from each tree were dissected to remove the ovary wall, and the ovules were counted under a stereomicroscope. 2–5 mature fresh fruits from each tree were dissected and had their well-developed seeds counted to estimate seed-set per fruit. Additional fruits formed spontaneously (i.e., by natural pollination) in seven intermediate stages – fruits around 4, 6, 8, 10, 12, 14, 16 cm wide; one sample from each tree – had their pulp dissected to extract fresh developing seeds that were examined in stereomicroscope to check for the presence of embryos, or were subjected to fixation for obtaining histological serial sections (see below).

Pistil and Fruit Longevity

As a necessary complement of the breeding system study, pistil longevity was analyzed after the following three different treatments: unpollination, self-, and cross-pollination. To this end, such treatments and respective tagging procedures were carried out as described above in additional flowers of the same individuals. The pistils were monitored daily up to each single pistil/fruit abscission or presentation of the first signs of dehydration (assumed as a clear sign of abortion), when its longevity was registered. The abscised/aborted pistil/fruit was then collected and submitted to measurements and fixation for posterior histological analysis (see below). Maximum pistil/fruit length and width were measured with a digital caliper. Persistent fruits (i.e., those that did not abort) were monitored in the trees until maturity. Non-abscised pistils/fruits that started to exhibit clear signs of dehydration and darkening were disregarded from the sampling for fixation and measurements, not only due to the doubt about when they really started to abort, but also due to the possible interference of the dehydration in the precision of the pistil growth measurement, as pistil dehydration may results in its shrinkage (Fig. 3a, white arrow).

In Situ *Pollen Germination and Pollen Tube Growth Analysis*

Cross- and self-pollinations were also performed in the same trees for the analysis of pollen germination and pollen tube growth using epi-fluorescence microscopy (Martin 1959). Pistils collected 6, 12, 24 and 48 h after hand pollination were dissected to remove the ovary wall and were fixed in a 5:5:90% solution of formaldehyde 4%, acetic acid and 50% ethanol (FAA50; Johansen 1940). The pieces were cleared and softened with 9 N NaOH at 65 °C for 20–40 min, and stained with leuco-aniline blue. In each ovary, some ovules were gently scraped out into the droplet of aniline blue before the cover-slip application in order to facilitate viewing penetrated ovules showing a visible pollen tube 'tail' at the micropyle (Gibbs and Bianchi 1993). These preparations were examined using an Olympus BX51 photomicroscope

equipped with a reflected fluorescence system (mercury burner USH-103OL, cube model U-MWU2, excitation filter BP330-385, dichronic mirror DM400, and barrier filter BA420). 2–3 samples of each pollination treatment/time interval were examined.

Histological Analysis

Unpollinated, selfed and crossed pistils or young fruits collected as cited above (section Pistil longevity) were dissected to remove the ovary wall/pericarp and fixed in 4% paraformaldehyde/1% glutaraldehyde in 0.1 M sodium phosphate buffer at pH 7.2 (Mcdowell and Trump 1976). Large pieces (young fruits) were divided into smaller parts before their immersion in the fixative solution. Additionally, unpollinated pistils (a day before anthesis or at the anthesis initiation stage), and non-abscessed selfed and crossed pistils/fruits (from the 1st to the 4th day after pollination and in several subsequent developmental stages) were also collected and subjected to the same fixation procedure for the histological verification of the early events in mature ovules, as well as fertilization and post-fertilization events. The pieces were dehydrated in an ethanol series, infiltrated with and embedded in glycol methacrylate, and serially sectioned into 4 µm slices using a Leica RM 2255 rotary microtome. The sections were stained in toluidine blue O (O'Brien et al. 1964) and, after permanent mounting with Permount®, examined and photographed using an Olympus BX51 photomicroscope equipped with an Olympus Q-Colour 5 digital camera.

Statistical Analysis

Simple linear regression analysis (Zar 1999) was used to verify the dependence of pistil/fruit longevity on pistil/fruit size (length and width) before abscission in unpollinated, self-pollinated, and cross-pollinated flowers. Pistil/fruit longevity up to abscission was statistically compared between the different treatments using the Mann–Whitney test (Zar 1999). To compare the pistil/fruit size at the time of abscission, One-way ANOVA (Zar 1999) was used considering parametric data of the three independent groups. In this analysis, to reduce the negative effect of early pistil abscission on the estimation of the pistil/fruit size at the time of abscission, data from samples that aborted below the 6th day were discarded in all treatments. The χ^2 statistic (Zar 1999) was used to compare the number of seeds presenting a developing endosperm on the 2nd, 3rd, and 4th day after self-pollination vs. cross pollination. Three fruits from each day were randomly selected, and the presence or absence of endosperm in all seeds was verified in the histological serial sections of each sample. The χ^2 test was also applied to compare unpollinated and pollinated (self + cross) pistils with respect to the degeneration of ovules and seeds. For this purpose, three samples of unpollinated, self-pollinated, and cross-pollinated pistil/fruits, aging 7, 14 and 20 days after anthesis, were randomly selected for microscopy analysis of serial sections, and all healthy and degenerating/degenerated ovules/seeds were counted. Ovules/seeds that did not show signs of degeneration (see below) were considered healthy. Statistical analysis was done using BioEstat 5.3 software (Ayres et al. 2007).

Table 1 Experimental pollinations and fruit set in *Couroupita guianensis*

Pollination treatment		Flowers	Fruits	Fruits (%)
Hand cross-pollination	(xenogamy)	185	37[a]	20
Hand self-pollination	(autogamy)	93	0	0
	(geitonogamy)	92	0	0
Spontaneous self-pollination		232	0	0
Beheaded stigma (agamospermy)		35	0	0
Illegitimate pollination		44	0	0
Natural (open) pollination		548	11	2

[a]Fruits that presented significant growth exceeding two months of development. Due to the attack of parasites and predators, only 15 of these fruits (8.2% of crossed flowers) reached maturity

Results

In the study site, *C. guianensis* flourished almost all year round, with peaks occurring from March to June and September to December. The anthesis of each flower began at approximately 7:00 a.m. Anther dehiscence in ligule staminodes occurred shortly afterwards, but dehiscence in fertile stamens of the staminal ring was not complete before 09:00 a.m. At around 05:00 p.m., the corolla and the entire androecium (i.e. all the androecial hood + staminal ring) fell off, leaving only the pistil and the persistent calyx in the inflorescence. "Fruit" initiation (i.e., the post-anthesis pistil swelling) was verified in both unpollinated and manually (self and cross) pollinated monitored flowers, but most of these fruits aborted in the following weeks after anthesis (see below). Viable fruits took around 12 months to reach maturity.

Experimental pollinations and seed set – Only cross pollinated and natural pollinated flowers set fruits that reached maturity (Table 1). Pistils in mature flowers presented 1128.5 ± 175.3 ovules per ovary (mean ± standard deviation, ranging from 850 to 1488, n = 12). Mature fruits resulting from natural pollination presented 274.5 ± 81.6 well developed seeds (mean ± standard deviation, ranging from 136 to 461, n = 15). The percentage of ovules per ovary that developed into mature seeds per fruit (ovule-seed conversion efficiency) was 24.3%.

Pistil/Fruit Longevity and Post-Anthesis Growth

Pistils kept intact (unpollinated) never developed into fruits, although there was some growth of them before their abscission. The linear regression analysis revealed a positive correlation between pistil/fruit growth prior to abscission and pistil/fruit longevity for all the three flower treatments (Fig. 1). However, the coefficients of determination (R^2) for unpollinated pistils were low, indicating that only 16% and 38% of the variability in the pistil length and width (respectively) was explained by the pistil longevity. The abscission of unpollinated pistils occurred in a continuum between the third and 22nd day, and of the 100 flowers tagged, 75 pistils underwent abscission from the seventh to the 15th day after anthesis (Fig. 2). Some aborted pistils remained in the inflorescence without undergoing abscission (Fig. 3a), especially those with a longevity of more than 15 days. The complete dehydration of these decomposed but not abscised pistils was observed around the 30th day.

Self-pollinated pistils showed a more evident growth (compared with unpollinated pistils) in the period between flower anthesis and pistil/fruit abscission (Fig. 1), with R^2 indicating that 53% and 71% of the variability in the selfed pistil/fruit length and width (respectively) was explained by the longevity. All the initiating selfed fruits aborted at an early stage of development, never reaching maturity. The abscission of such pistils/fruits occurred between the ninth and 34th day after pollination, with 69 fruits (out of 100 treated flowers) undergoing abscission between the 19th and 27th day (Fig. 2). Eventually, aborted fruits did not undergo abscission exceeding 35 days of longevity. The abortion of these fruits was confirmed by the stagnation of their growth, as well as by their dissection revealing dried seeds surrounded by decomposed tissues. Periderm formation was observed on the outer surface of the pericarp of the longest-lived fruits (around 25 days or more) derived from self-pollination (Fig. 3a), a feature that was not observed in unpollinated pistils.

Only fruits derived from cross-pollination reached maturity around the 12th month after anthesis. However, many flowers subjected to cross-pollination did not generate viable fruits, although these fruits presented notable growth before aborting (Fig. 1), with R^2 indicating that 75% and 70% of the variability in the crossed pistil/fruit length and width (respectively) was explained by the longevity. Of the 100 flowers treated, the abscission of 60 pistils/fruits occurred from the 4th to the 57th day after pollination, with most abscissions (50 pistils/fruits) occurring before the 30th day (Fig. 2; only aborted pistils/fruits are plotted in this graphic). Periderm formation on the pericarp was also

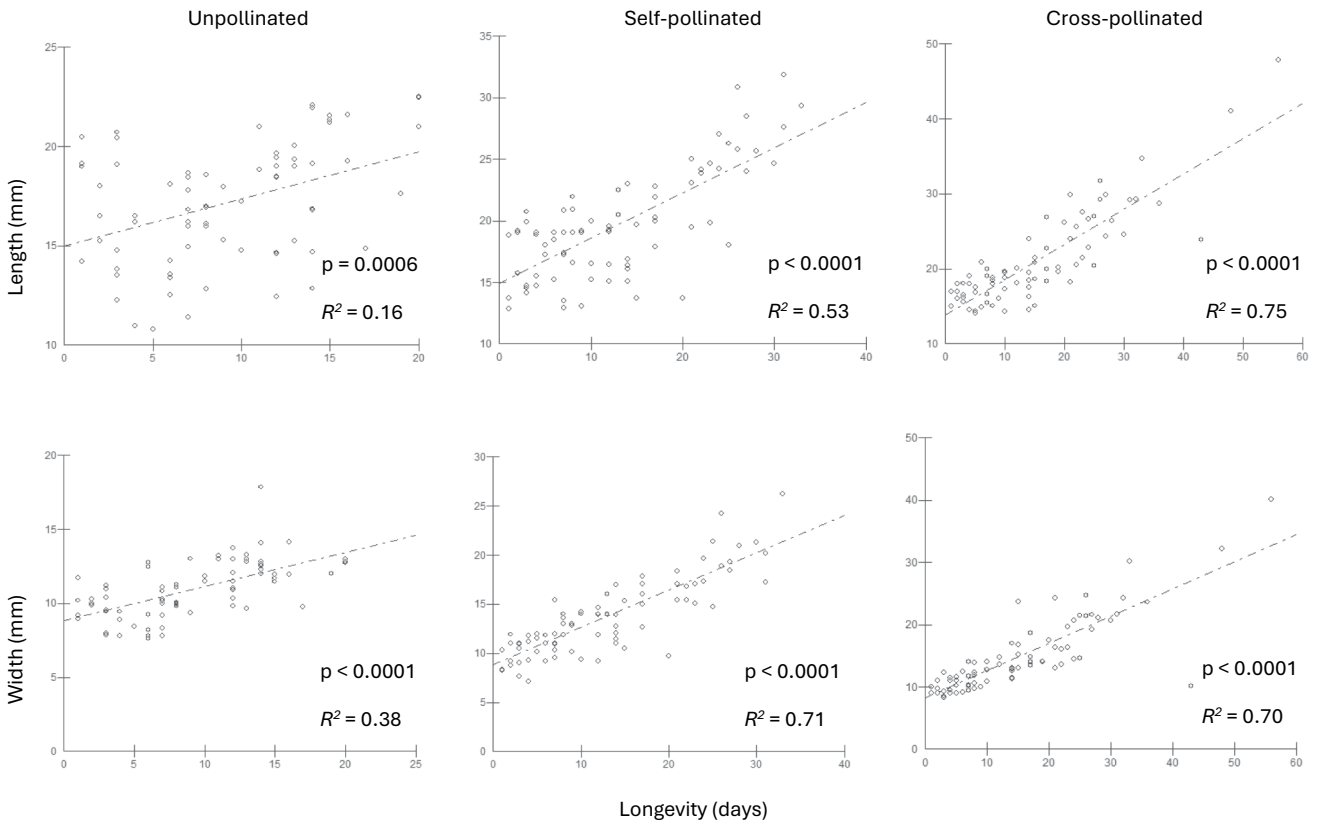


Fig. 1 Simple linear regression analysis tasting the dependence of pistil/fruit growth (length and width) before abscission on pistil/fruit longevity in unpollinated, self-pollinated, and cross-pollinated flowers of *Couroupita guianensis*

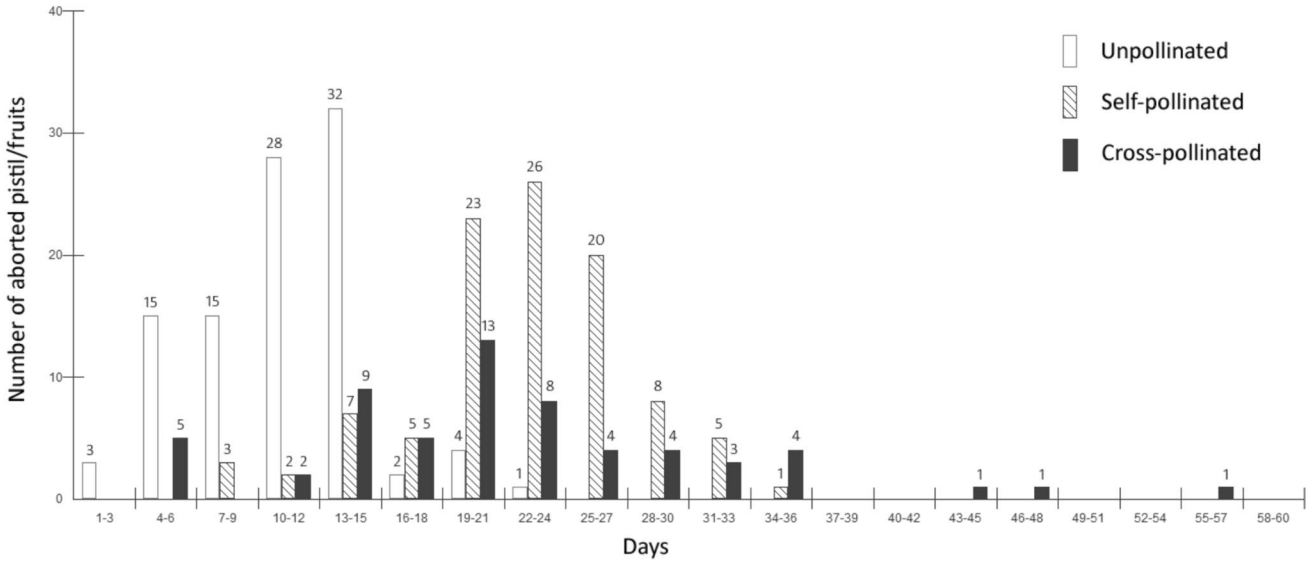


Fig. 2 Progress of pistil/young-fruit abscission after anthesis in unpollinated, self-pollinated, and cross-pollinated flowers of *Couroupita guianensis*. The number above each vertical bar indicates the number of pistils/fruits that underwent abscission/abortion at each time interval

observed in crossed fruits roughly from the 25th day of development. Except for those intensely attacked by parasites and predators, crossed fruits that exceeded two months of development reached maturity (15 fruits).

Longevity in self-pollinated pistils was greater than in unpollinated ones, although none of these treatments generated viable fruits (Fig. 4). Regarding the abortion of self- vs. cross-pollinated pistils, the statistical test did not indicate a significant difference for the interval from anthesis to abscission, even though the fruits initiated by cross-pollination (but not viable) aborted until the second month after pollination (Fig. 2). When aborting, unpollinated pistils presented length and width values significantly lower than those of pollinated pistils, while abscised selfed vs. crossed fruits did not show significant differences in length and width (Fig. 5).

Pollen Germination and Pollen Tube Growth

Dozens of germinated pollen grains were observed in both selfed and crossed pistils six hours after pollination (Fig. 3b), with many pollen tubes penetrating the transmitting tract below the stigma and some of them reaching the ovary. Profuse ovule penetration by pollen tubes was observed 12–24 h after pollination also in pistils of both treatments (Figs. 3c–e). Due to the sub-sessile condition of the terminal stigma, the distance that pollen tubes had to growth down into the ovary to penetrate ovules never surpass 10 mm long, so that it was not possible to measure pollen tube growth speeds throughout the transmitting tract in self- vs. cross-pollinated pistils with the adopted procedure. Moreover, due to the compact and massive nature of the ovary, together with its great number of ovules, counts of penetrated ovules were not feasible in this study. However, at the 24- and 48-h stages, most of the ovules in selfed and crossed pistils in which the visualization of the micropyle was possible showed a pollen tube into it (Figs. 3d and e). No morphological sign of pollen tube/transmitting tissue incompatibility reactions – such as pollen tube tip swelling and burst or irregular deposition of callose plugs along the pollen tube – was observed in the selfed pistils. Effectively, no difference was observed in this study between self- vs. cross-pollinated pistils relative to pollen germination, pollen tube development, and ovule penetration.

Ovule Anatomy

The anatropous, bitegmic ovule attaches to the placenta by an elongated funicle (Fig. 6a). The number of cell layers in the outer integument varies from 6 to 10, depending on the observed region. Both the outer and inner epidermis of the outer integument exhibit pavement cells, while parenchymatic polygonal cells fill the internal layers (Fig. 6b). The cells of the outer epidermis in this integument are rich in cytoplasmic phenolic compounds (evidenced by its greenish color in toluidine-blue-O-stained sections), while the cells in all the layers below (without considering the procambium), including those of the inner epidermis, show vacuolated cytoplasm and eccentric nucleus. The inner integument is formed by 4–6 layers of cells. The pavement cells of the outer epidermis, as well as the polygonal cells of the parenchyma in the layers below are vacuolated, but the cells of the inner epidermis at the region that surrounds the embryo sac are anticlinally elongated and show dense cytoplasm and prominent central nucleus (Fig. 6b); therefore,

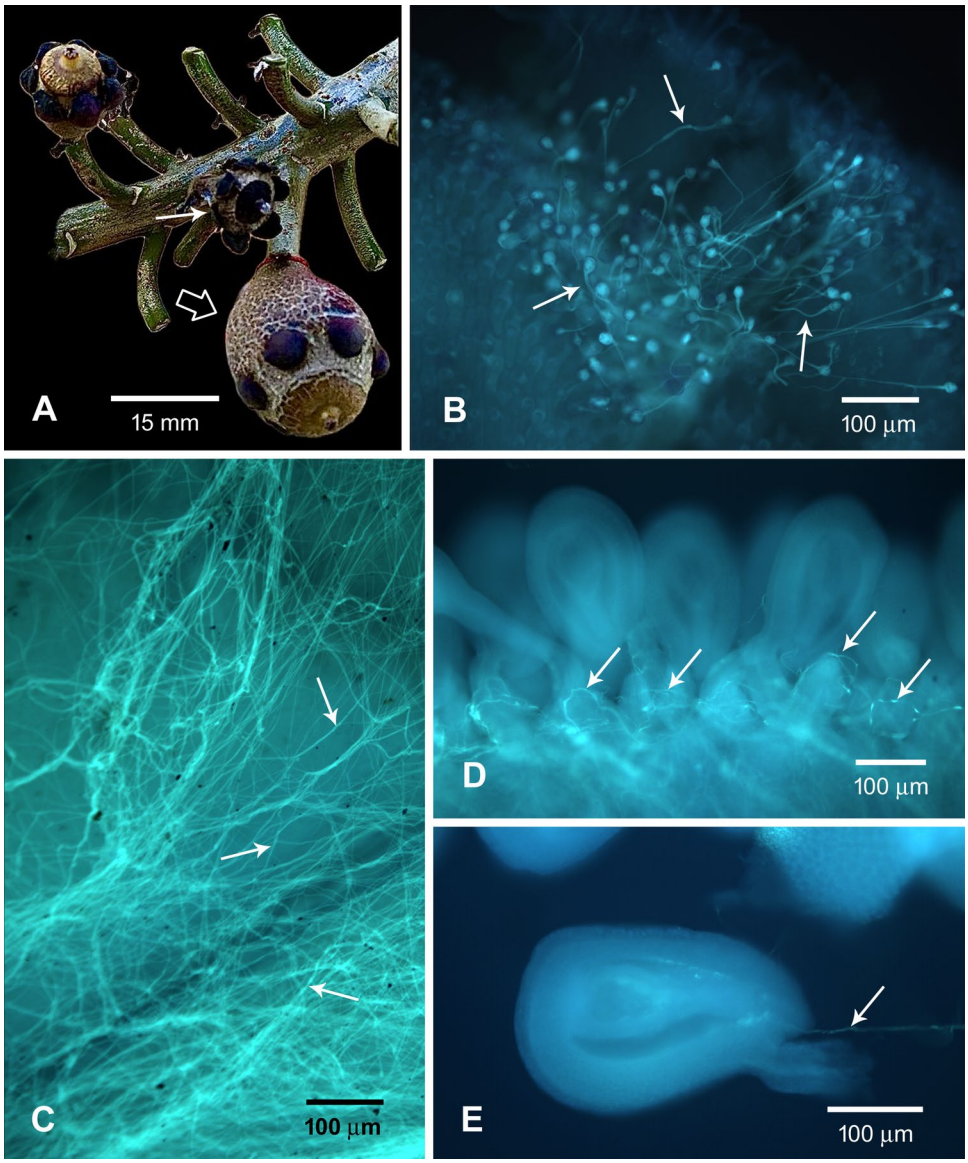


Fig. 3 *Couroupita guianensis*. **a** Part of an inflorescence showing pistils after corolla + androecium shedding and a 34-day selfed fruit (black arrow). The white arrow indicates a dehydrate persistent unpollinated pistil. **b–e** Epifluorescence photomicrographs of self-pollinated pistils. Germinating pollen grains on the stigma, 6 h after pollination (**b**). Pollen tubes at the upper part of the ovary, 24 h after pollination (**c**). Ovules penetrated by pollen tubes, 24 h after pollination (**d**, **e**). White arrows indicate pollen tubes

the inner epidermis at this region of the inner integument is sharply differentiated in an endothelium. Binucleated cells were often observed in this endothelium. The micropyle is endostomic – i.e., with the inner integument projecting beyond the margin of the outer integument –, and cells of the inner integument in most part of the micropyle tend to be periclinally stretched (Figs. 6a and b). An agglomerate of hypostase cells with dense cytoplasm was commonly observed next to the chalazal end of the embryo sac (Fig. 6b). The procambial strand of a future post-chalazal non-ramifying vascular bundle extends from the funicle throughout the outer integument, up to the anti-rapheal side of the ovule (Figs. 6a and b).

In a median longitudinal section of the ovule, the *Polygonum* type megagametophyte (embryo sac) exhibits an elliptic shape with an acuminate micropylar end (Figs. 6a and b). The egg cell apparatus exhibits two side by side piriform and elongated synergids, with their narrowed ends (bearing the filiform apparatus) toward the internal opening of the micropylar channel (Figs. 6c and f). Each synergid usually presents a large vacuole at the chalazal pole of the protoplast, and the nucleus and most of the cytosol at the micropylar pole (Fig. 6c). The egg cell has a less regular shape (compared to that of synergids), and usually exhibits a cytoplasm largely vacuolated and the nucleus positioned at the chalazal pole of the cell (Figs. 6d and f). At the pre-anthesis stage, the polar nuclei often are seen into a cytoplasmic strand of the largely vacuolated central cell, roughly positioned at the center of the embryo sac (Fig. 7a). However, in most ovules observed just before the flower opening, the fusion of the polar nuclei had already occurred. A central cell secondary nucleus was observed in all the open flower ovules

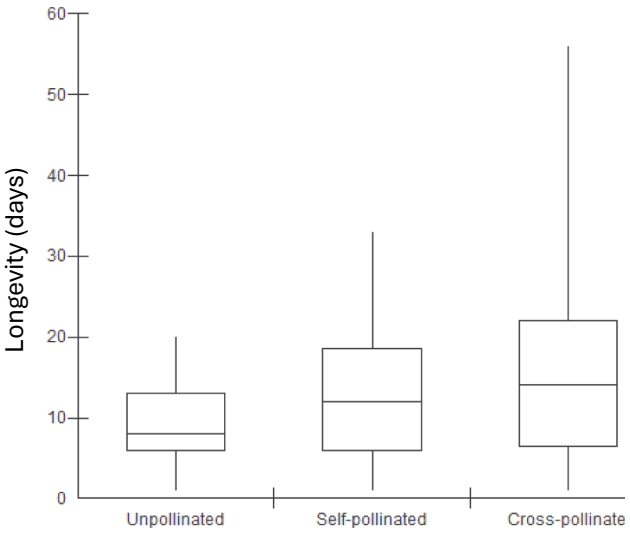


Fig. 4 Pistil/young-fruit longevity up to abscission in *Couroupita guianensis*. Boxes represent the lower quartile, median, and upper quartile. Error bars represent the variation between minimum and maximum values. Mann–Whitney test indicated significant difference between longevity of unpollinated and self-pollinated pistils ($p=0.0173$), but not between self- and cross-pollinated ($p=0.3180$)

examined in this study. The secondary nucleus is larger than any other cell nucleus of the embryo sac, positioning itself close to the egg cell (Figs. 6e and 8d). Antipodals were seen only in ovules of flower buds (pre-anthesis stage; Figs. 7a and b) since they degenerate before the megagametophyte maturity.

Histological Events in Unpollinated Pistils

In unpollinated pistils, from the sixth day on after the flower opening, a progressive anticlinal elongation of the endothelial cells was observed to the point of narrowing the cavity where the embryo sac is housed (Fig. 7c and d). At the same time, parenchymatic cells of the subjacent layers of the inner integument expanded showing a higher cytoplasmic density than before, and their walls underwent slight thickening staining more intensely purple by the toluidine blue O (Figs. 7c and d). No significant changes were observed in the outer integument until the pistil abscission.

From the seventh day on, we observed the degeneration of several ovules, originally evidenced by lysis in endothelial cells that exhibited increasing cytoplasm staining followed by cell collapse (Figs. 7c–f), although in some ovules the degeneration of the outer integument preceded that of the endothelium (not shown). Progressively, cells of both the integuments, chalaza and funicle, undergo lysis and collapse (Figs. 7e–h). The degeneration proceeds up to the point where only traces of the ovules persist – i.e., collapsed tissues involving some remnant cells of the outer and inner integuments (Fig. 7g). Carpel degeneration begins in the placentae (Figs. 7e–g), occurring mainly in the oldest pistils. In non-abscessed unpollinated pistils with longevity of more than 14 days, most ovules exhibited the abovementioned signs of degeneration. In abscised pistils, some of the ovules were still "healthy" (i.e., with normal structuring of their components), while others were in process of degeneration or completely degenerated, depending on the longevity achieved by the pistil. No difference was noted in

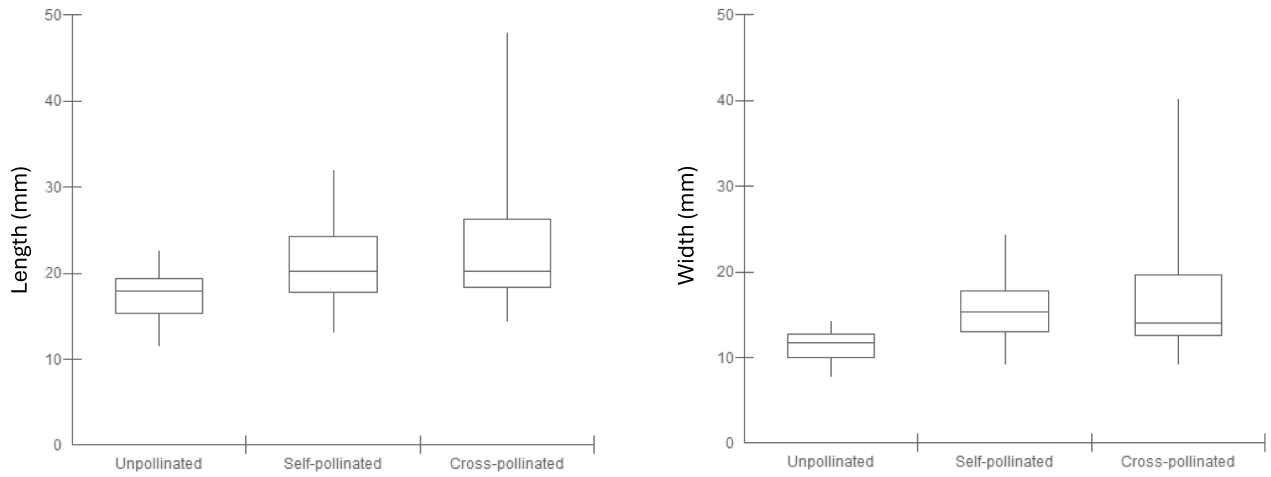


Fig. 5 Pistil/young-fruit size at the time of abscission in *Couroupita guianensis*. Boxes represent the lower quartile, median, and upper quartile. Error bars represent the variation between minimum and maximum values. Measurements of pistils with longevity of less than six days were not included. One-way ANOVA revealed significant differences in length ($F(2, 164)=14.22$, $p<0.0001$) and width ($F(2, 164)=22.88$, $p<0.0001$), with post-hoc comparisons using Tukey test indicating that the significant differences for both dimensions were between unpollinated vs. self-pollinated ($p<0.01$), and unpollinated vs. cross-pollinated ($p<0.01$) pistil/fruits

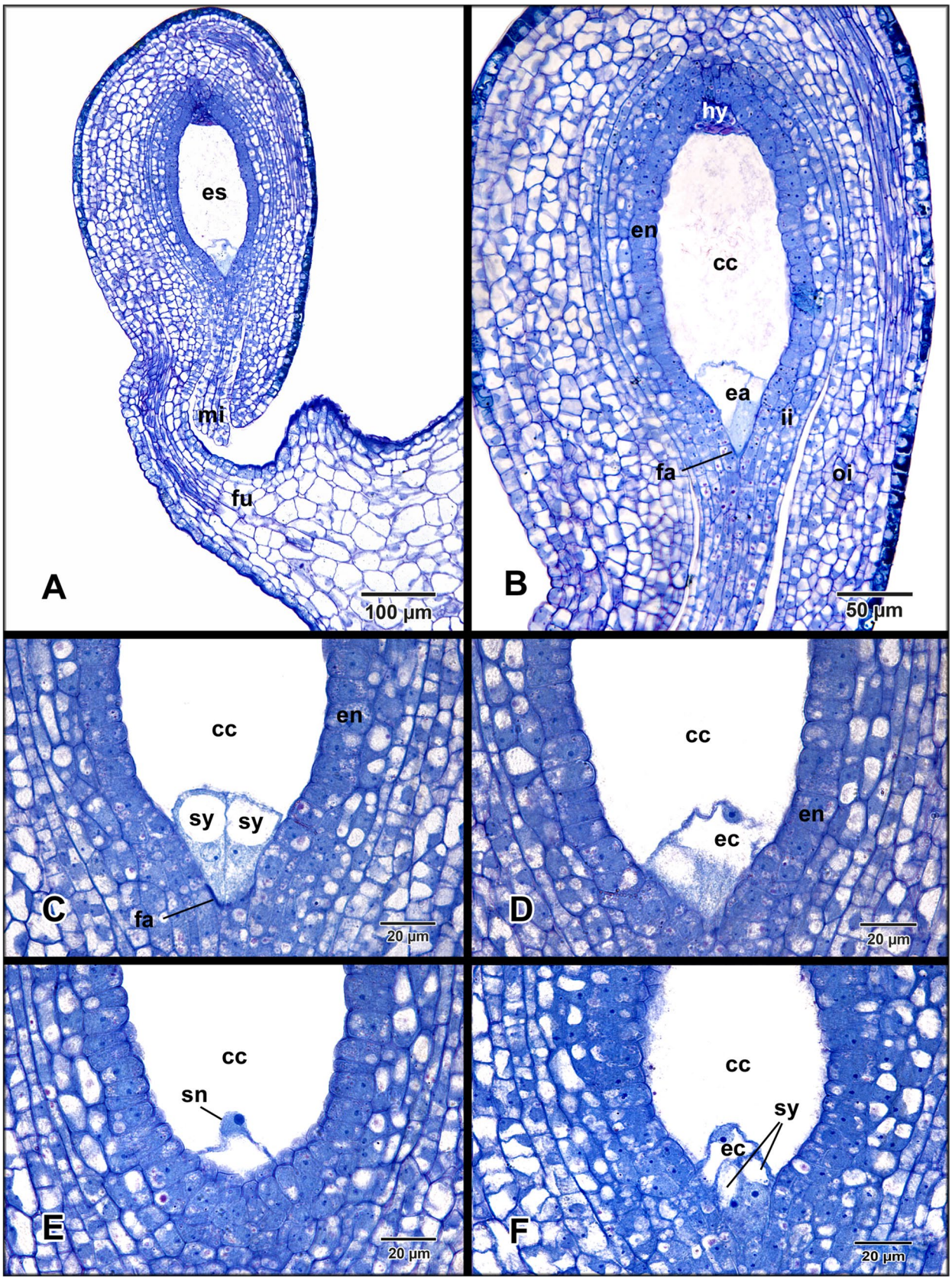

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Fig. 6 Light photomicrographs of longitudinal sections of ovules in *Couroupita guianensis*. General aspect of the ovule at the anthesis stage (**a**). The same in a high magnification exhibiting the embryo sac (**b**). Serial sections of the micropylar portion of a same ovule showing the two synergids (**c**), the egg cell (**d**), and the secondary nucleus (**e**). Single section of the micropylar portion of another ovule exhibiting all the cell components of the egg apparatus (**f**). *cc* central cell, *ea* egg apparatus, *ec* egg cell, *en* endothelium, *es* embryo sac, *fa* filiform apparatus, *fu* funicle, *hy* hypostase, *ii* inner integument, *mi* micropyle, *oi* outer integument, *sn* secondary nucleus, *sy* synergids

the degree of degeneration in ovules and carpels between aborted pistils and pistils of the same age but collected before abortion.

No sign of endosperm and/or a zygote-like cell formation was verified in ovules of unpollinated pistils. Megagametophyte degeneration varied according to the onset of endothelial cell lysis. In 7-day pistils, intact ovules (Fig. 8a) still showing a well-preserved endothelium presented a structurally normal egg apparatus (Figs. 8b and c) and a recognizable secondary nucleus into the embryo sac (Fig. 8d). Subsequently, the collapse of the embryo sac accompanied the changes described above for the endothelium and underlying cell layers.

Histological Events in Self- and Cross-Pollinated Pistils

Numerous pollen tubes were found into the ovary locule of both selfed and crossed pistils at the following days after pollination (Fig. 9a). From the first day on after anthesis, one of the synergids in pollen tube penetrated ovules appeared strongly stained, and the penetrated synergid exhibited a cytoplasmic loop around the chalazal pole of the egg cell in most of these ovules (Fig. 9b). In subsequent stages, the synergids collapsed and were absorbed.

Endosperm development was observed also in ovules of pistils submitted to both pollination treatments. The first nuclear divisions of the endosperm occurred from the second day after the flower opening (Fig. 9c). Four days after anthesis, each endosperm already presented several nuclei in a widely vacuolated coenocyte, with the nuclei peripherally positioned (Fig. 9d). The large vacuole of the endosperm often exhibited a pinkish granular content (Figs. 10a). Initiating endosperms in fruits originated by self-pollination did not show any structural difference in relation to those formed by cross-pollination (Figs. 10 a–d). The quantitative analysis indicated an increase in the number of initiating seeds bearing a developing endosperm during the four days following pollination, but no significant difference between selfed and crossed fruits (Table 2). Endosperm cellularization was observed only in seeds of fruits derived from cross-pollination, and only in fruits older than 50 days (Figs. 10e and f). In fruits derived from self-pollination, whose longevity never surpassed 34 days, only nuclear phases of the of the endosperm were observed.

Like the observed for the endosperm initiation, the zygote formation was verified in ovules of both self- and cross-pollinated pistils (Figs. 10a and b; Figs. 11a and b). The zygote typically exhibited a rounded shape, with a large vacuole occupying the majority of its internal volume. The nucleus and the bulk of the cytosol were positioned at the chalazal pole of the zygote, toward the developing endosperm. A cell wall enveloping the entire zygote protoplast, together with differentiated cytoplasmic plastids around the nucleus (Fig. 11c) were frequently observed in our histological sections, particularly at the more advanced developmental stages. In fruits resulting from cross-pollination up to two months' post-anthesis, histological analysis revealed no evidence of embryogenesis; only resting zygotes were detected. However, embryos in advanced stages of development, exhibiting cotyledons to the naked eye, were checked in seeds of fruits from the eight cm wide stage onwards, and our histological analysis revealed proembryos showing the embryo proper at an early globular stage in seeds of fruits with six cm wide, which corresponded to about four months of development (Fig. 11d). Effectively, in fruits initiated by self-pollination, except for the signs of cell differentiation cited above (i.e., plastid maturation and wall formation), the resting zygote remained unaltered and did not initiate divisions until the fruit abscission.

In both self- and cross-pollinated pistils/young fruits, in ovules bearing no endosperm – i.e., ovules that eventually were not fertilized –, the histological changes of the endothetium and underlying cells in the inner integument where like those observed in ovules of unpollinated pistils. In endosperm-bearing ovules, the post-anthesis changes in the inner integument involved also the anticlinal elongation of the endothelial cells and a volumetric expansion of the parenchyma cells located between the endothelium and the outer epidermis, together with an increase of cytoplasmic density in cells of both tissues (Figs. 12a and b). However, in many of these endosperm-bearing ovules (presumably the viable ones) a shrinkage of the embryo sac/endosperm cavity was not observed (Figs. 12a and b), and no significant integumentary cell lysis or collapse were observed in the first two weeks after pollination. Moreover, the seed enlargement was accompanied by an increase in cell layers in the outer integument, notably by periclinal divisions from the inner layers of cells (including from the inner epidermis), close to the inner integument (Fig. 12b).

Around the 20th day after pollination, collapsed cells begin to appear in the inner integument, around the endothelium (Figs. 12c and d). Progress of cell collapse of the inner integument layers around the endothelium in the subsequent days was followed by degeneration also of the inner layers

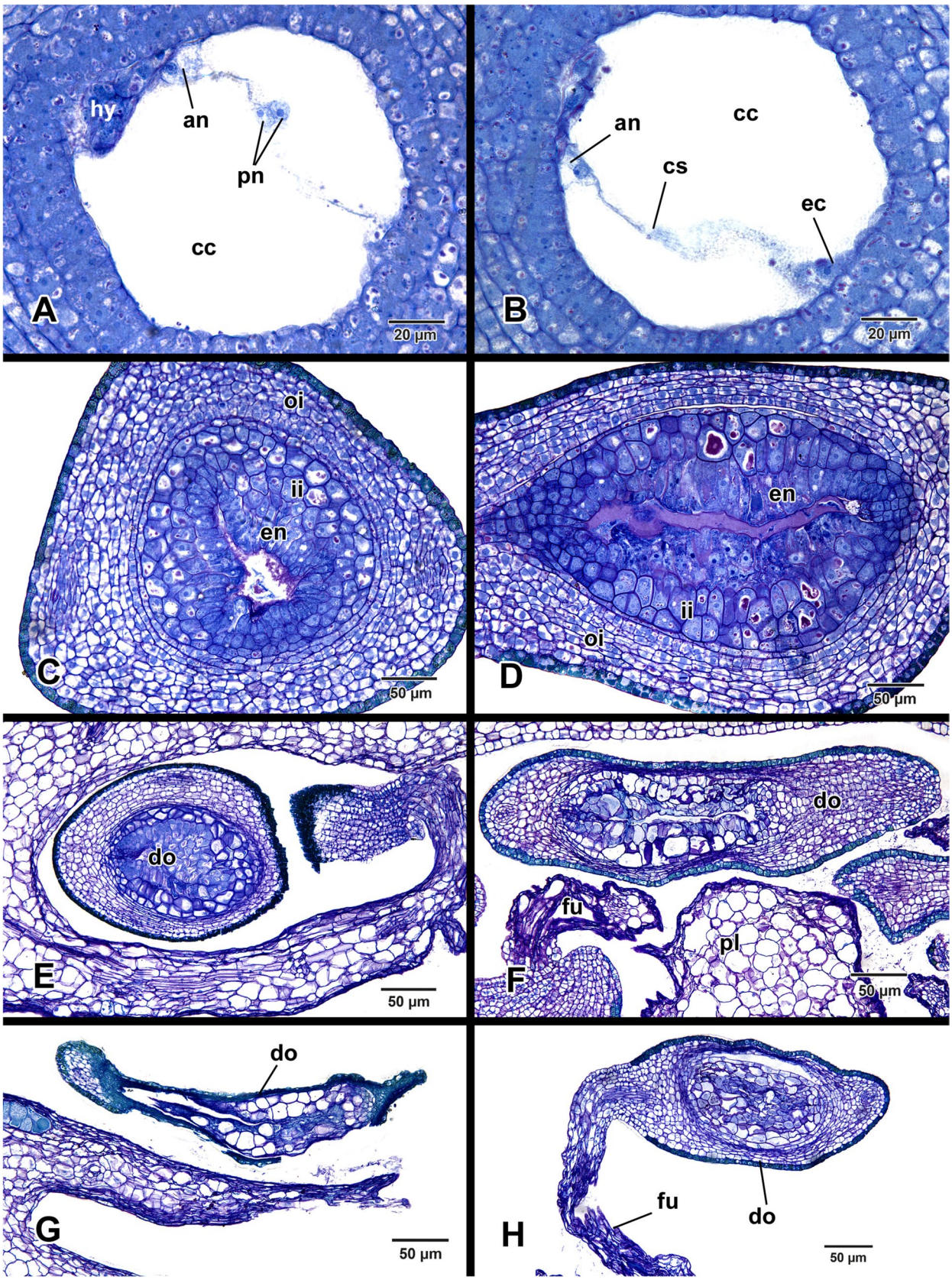

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Fig. 7 Light photomicrographs of ovule sections in *Couroupita guianensis*. Oblique serial sections of a same mature ovule but before anthesis (**a–b**). Transverse (**c–f**) and longitudinal (**g–h**) sections of ovules of unpollinated pistils, 7 (**c**), 9 (**d**), 11 (**e**) 12 (**f**), 15 (**g**), and 16 days (**h**) after anthesis. *an* antipodals, *cc* central cell, *cs* cytoplasmic strand, *do* degenerating ovule, *ec* egg cell, *en* endothelium, *fu* funicle, *hy* hypostase, *ii* inner integument, *oi* outer integument, *pl* placenta, *pn* polar nuclei

of cells in the outer integument, starting from the 32nd day (Figs. 12c–f). Changes occurred also in the endothelium because their cells, that initially became columnar, later tended to become rounded in shape (Figs. 12c and d). In seeds of crossed fruits, the initial degeneration of the endothelium was observed coincidently with the first stages of the endosperm cellularization (Figs. 12e and f).

The abortion of non-viable ovules/young seeds was marked by the cell degeneration and collapse in the integumentary and chalazal tissues, as observed in ovules of unpollinated pistils. Like the shrinkage and collapse of the embryo sac observed in late stages reached by the ovules in unpollinated pistils, endosperm degeneration and collapse occurred in aborting seeds in fruits derived from both self- and cross-pollination (Fig. 12g). All these signs of degeneration begin to appear in aborting seeds from the seventh day after pollination, but a non-collapsed nuclear endosperm was observed in a few degenerating seeds up to 20 days of fruit development (Fig. 12h). As in unpollinated pistils, in fruits resulting from self- and cross-pollination, no difference was noted in the degree of degeneration in the seeds between aborted fruits and fruits of the same age but collected before abortion.

A significant increase in the number of degenerated ovules/seeds – i.e., ovules/seeds exhibiting advanced stages of collapsed integumentary and chalazal tissues or a collapsed endosperm – was observed up to 20 days after anthesis for all the three pistil treatments (Table 3). No statistical difference was observed between self- and cross-pollination, but the number of degenerated ovules/seeds was significantly greater in unpollinated pistils compared with initiating fruits (pollinated pistils) at least up to 20 days after anthesis (Table 3). It is worth highlighting that there was a delay in ovule/seed degeneration in fruits derived from pollinated pistils, relative to unpollinated ones.

Discussion

Only cross-pollinated flowers set fruits that developed to maturity in our controlled experimental pollinations, and the fruiting rate through natural pollination was very low (only 2% of almost 550 tagged flowers). The absence of fruiting in the tests for agamospermy and non-legitimate pollination indicated the inability to set seeds by autonomous apomixis and confirmed the sterility of the pollen produced by the androecial hood, respectively. Although some self-pollinated pistils started to form fruits, almost all these fruits aborted during the first month after pollination, whereas persistent viable fruits resulting from cross- and natural pollination reached maturity. These results indicate the occurrence of strong self-sterility which was corroborated by the procedure we used to verify pistil/fruit longevity in unpollinated, selfed end crossed flowers (all the mature fruits obtained were from cross-pollination). Our results differ from those of Ormond et al. 1981, confirming the xenogamous nature of fertilization originally alleged for *C. guianensis* (Prance 1976; Mori and Kallunki 1976).

Epi-fluorescence microscopy analyses in this study indicated that the efficiencies of pollen germination on the stigma and pollen tube growth to reach and penetrate ovules into the ovary seem not to differ between self- vs. cross-pollination treatments. Although we could not count and compare the number of penetrated ovules in selfed vs. crossed pistils, the abundance and uniformity of pollen tube growth throughout the transmitting track, together with the observation of many penetrated ovules in both treatments are at variance with the self-pollen germination inhibition on the stigma, or the arresting of self-pollen tube growth down to the ovary typically observed in taxa that exhibit conventional homomorphic SSI or GSI (Seavey and Bawa 1986; Sage et al. 1994; Gibbs 2014). In fact, the lack of a style in the gynoecium of *C. guianensis* seems to favor some kind of delayed incompatibility reaction, which contrasts with the mechanism typically observed in plants with conventional homomorphic GSI, since it is usually within the transmitting tract of the style (or before entering the ovary) that rejection of self-pollen tubes occurs in this kind of SI. The histology of post-pollination events in this study also revealed numerous pollen tubes crowding the ovary locule, as well as many penetrated ovules in both selfed and crossed pistils at the first days following pollination.

Despite the complete self-sterility verified in *C. guianensis* in this study, self-pollinated pistils clearly exhibited an extended longevity relative to unpollinated ones. The highest longevity achieved by fruits initiated by self-pollination (34 days) corresponds to a small fraction (9.3%) of the time spent by viable fruits to complete their development. Comparatively, the highest longevity displayed by an unpollinated pistil (22 days from anthesis to abscission) corresponded to only 6% of the whole time a viable fruit need to reach maturity. Most unpollinated pistils aborted into two weeks after anthesis, and the degeneration rate of their ovules was significantly higher than those of pollinated (selfed and crossed) pistil/fruits. Contrarily, most self-pollinated pistils exceeded the 14-day landmark for abscission of the unpollinated pistils, and continued to grow undergoing

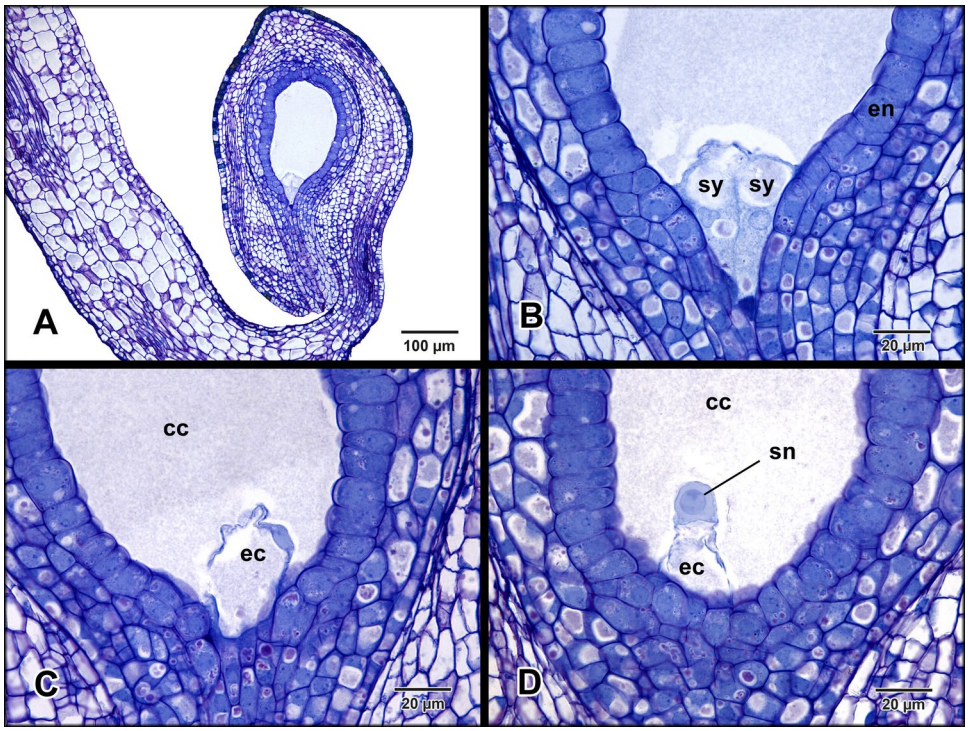


Fig. 8 *Couroupita guianensis*. Light photomicrographs of longitudinal sections of an ovule of unpollinated pistil, seven days after anthesis. General aspect of the ovule (**a**). Serial sections of the micropylar portion of the ovule showing the two synergids (**b**), the egg cell (**c**), and the secondary nucleus (**d**). *cc* central cell, *ec* egg cell, *en* endothelium, *sn* secondary nucleus, *sy* synergids

abscission only around the 4th week after self-pollination, but exhibiting endosperm in their normally developing seeds which clearly attest fruit initiation.

A similar extended longevity of self-pollinated pistils has been reported as an outcome of the delayed nature of the self-rejection process implicated in the LSI mechanism (Gibbs et al. 2004; Primo and Machado 2009; Gandolphi and Bittencourt 2010; Bittencourt et al. 2011; Gibbs 2014; Bittencourt 2017, 2019). Moreover, a small but statistically significant growth of the ovary in self-pollinated pistils (relative to unpollinated ones) before their uniform abortion only a few days after pollination has also been reported for some bignoniaceous species that present LSI (Gandolphi and Bittencourt 2010; Bittencourt et al. 2011; Bittencourt 2017, 2019). Although the short time frame before abscission does not appear to differ between self- vs. unpollinated pistils in some LSI species (Bittencourt et al. 2003; Gibbs 2014), it is not known how much a delayed abortion of selfed pistils could be generalized for taxa that present LSI. However, the occurrence of such a phenomenon in self-incompatible species of phylogenetically unrelated families, such as Bignoniaceae (Gandolphi and Bittencourt 2010; Bittencourt et al. 2011; Bittencourt 2017, 2019), Malvaceae (Gibbs et al. 2004), Capparaceae (Primo and Machado 2009), and Lecythidaceae (this study) may indicate that it is a common feature among taxa that present LSI.

The discharge of the pollen tube content into one of the synergids after penetration of the megagametophyte was evidenced by the strong purple coloration (due the action of the toluidine-blue O staining) acquired by the cytoplasm of the penetrated synergid, which facilitated to visualize the penetrated ovules. The observation of ovules showing a penetrated synergid just one day after both self- and cross-pollination is congruent with the studies by Ormond et. al. (1981), who also observed great part of the ovules penetrated by pollen tubes 24 h after pollination. The observation of many ovules with the penetrated synergid exhibiting a cytoplasmic loop around the chalazal pole of the egg cell, signaling the transfer of male gametes to the egg and central cell (Ye et al. 2002; Bittencourt 2017), evidenced the operation of double fertilization in ovules of both selfed and crossed pistils.

Another evidence for effective fertilization of ovules in self-pollinated pistils was the observation of the zygote and the initial development of the endosperm, and their absence from ovules of unpollinated pistils. It is important to point out that the reconstitution of the cell wall around the chalazal end of the fertilized egg cell, as observed in this study, is an indication that this cell became a zygote (Schultz and Jensen 1968; Olson and Cass 1981). Our observations demonstrate that the resting zygote period in *C. guianensis* is very long. Embryogenesis initiation in fruits derived from hand pollination was not observed because the resting zygote remained undivided until the latest stages of seed development analyzed, even in seeds of crossed fruits. In a study of fruit anatomy in *C. guianensis*, Schoenberg (1983b) reported the beginning of zygote divisions around the 7th month after cross-pollination. However, in our own

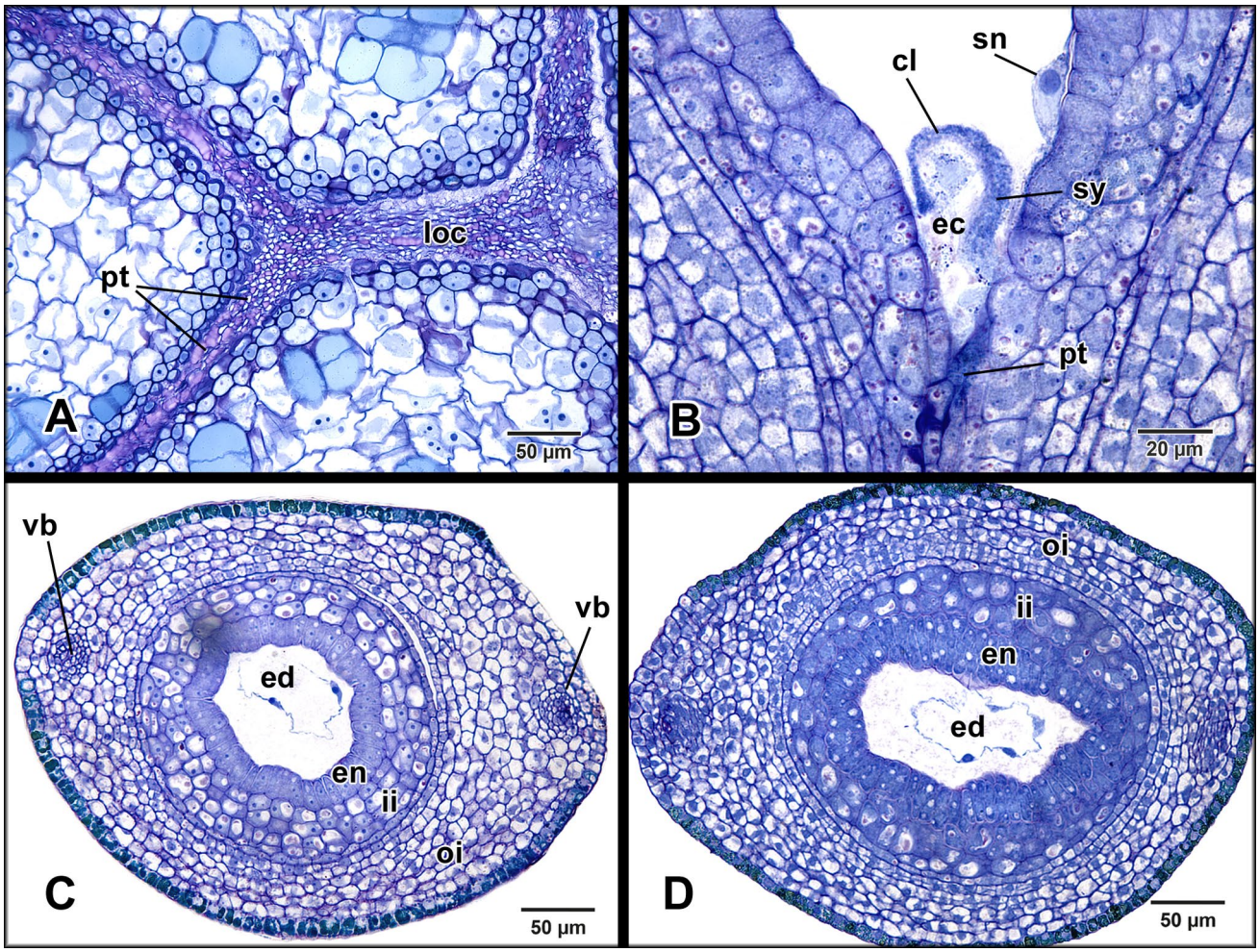


Fig. 9 Light photomicrographs of the pistil in *Couroupita guianensis*. Transverse section of the ovary at the middle portion of the locular cavity, three days after self-pollination (**a**). Longitudinal section of the micropylar portion of an ovule at the 2nd day after self-pollination showing a penetrated syergid (**b**). Transverse sections of ovules/seeds at the 2nd (**c**) and 4th days (**d**) after self-pollination, containing a 2-nucleate and a 10-nucleate endosperm, respectively (only the four nuclei positioned at the same plane of section are seen in **d**). *cl* synergid cytoplasmic loop, *ec* egg cell, *ed* endosperm, *en* endothelium, *ii* inner integument, *loc* locule, *oi* outer integument, *pt* pollen tubes, *sn* secondary nucleus, *sy* penetrated synergid, *vb* vascular bundle

analysis, seeds from spontaneously formed fruits (i.e., derived from natural pollination) with only four months of development already exhibited embryos at an early globular stage.

Several studies in self-sterile species of Bignoniaceae, one of the main clusters of LSI species in angiosperms (Gibbs and Bianchi 1999; Gibbs 1914), have revealed discrepancies in the post-pollination events between self- and cross-pollinated pistils that favors of LSI. A delay in ovule penetration and/or fertilization in selfed pistils was verified in the studies carried out by Gibbs and Bianchi (1993, 1999), Bittencourt (2019), Bittencourt et.al. (2003, 2011), Bittencourt and Semir (2004, 2005, 2006), and Gandolphi and Bittencourt (2010). Similarly, Bittencourt (2019), Bittencourt et.al. (2003, 2011), Bittencourt and Semir (2005, 2006), and Gandolphi and Bittencourt (2010) reported a delay in the progress of initial phases of endosperm development in selfed pistils, compared to crossed ones. In these cases, a recognition mechanism of self-pollen tube by the pistil seems to operate, despite pistil rejection invariably occurring after fertilization of most ovules, which implies the contentious assumption of a SI mechanism operating post-zygotically (Gibbs 2014; Bittencourt 2017).

As with the quantification of ovule penetration, it was not possible to compare the progress of initial stages of the endosperm development in self- vs. cross-pollinated pistils in this study, as performed in the studies of bignoniaceous LSI species cited above. The ab initio cellular type of endosperm development in Bignoniaceae (Johri et al. 1992) was essential for the step by step following of the initial phases of endospermogenesis by histological sections in these studies. The same procedure was not feasible with the ab

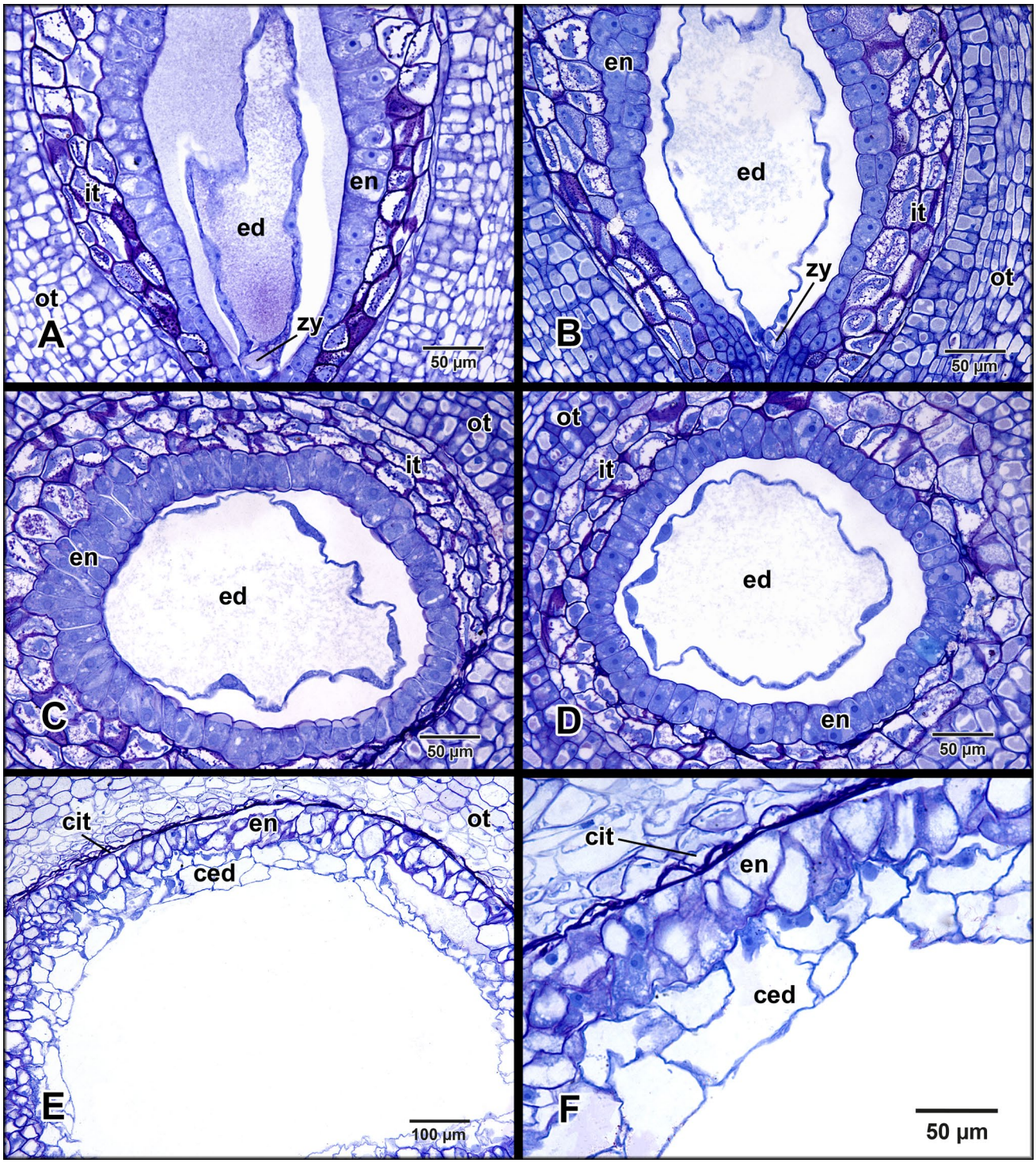


Fig. 10 Light photomicrographs of sections of young seeds in *Couroupita guianensis*. Longitudinal sections of a crossed (**a**) and a selfed seed (**b**), 17 days after pollination. Transverse sections of a crossed (**c**) and a selfed seed (**d**), 21 days after pollination. Cellularized endosperm in a transverse section of a crossed seed, 56 days after pollination (**e**). The same showing endothelium and endosperm cells in higher magnification (**f**). *ced* cellularized endosperm, *cii* collapsed inner integument, *ii* inner integument, *oi* outer integument, *ed* endosperm, *en* endothelium, *zy* zygote

Table 2 Endosperm in early developing seeds derived from self- vs. cross-pollination in *Couroupita guianensis*

	Time after pollination					
	48 h		72 h		96 h	
	Self (%)	Cross (%)	Self (%)	Cross (%)	Self (%)	Cross (%)
Endosperm present	128 (5,78)	91 (4,52)	196 (9,30)	237 (10,20)	429 (20,27)	387 (18,86)
Endosperm absent	2087 (94,22)	1923 (95,48)	1911 (90,70)	2086 (89,8)	1687 (79,73)	1665 (81,14)
Total	2215 (100)	2014 (100)	2107 (100)	2323 (100)	2116 (100)	2052 (100)

Each column pools data from three fruits collected at random. The differences between self vs. cross at 48, 72 and 96 h after pollination were not statistically significant ($\chi^2 = 3.413$, df = 1, $P = 0.0754$; 72 h $\chi^2 = 1.015$, df = 1, $P = 0.3387$; $\chi^2 = 1.324$, df = 1, $P = 0.2664$, respectively)

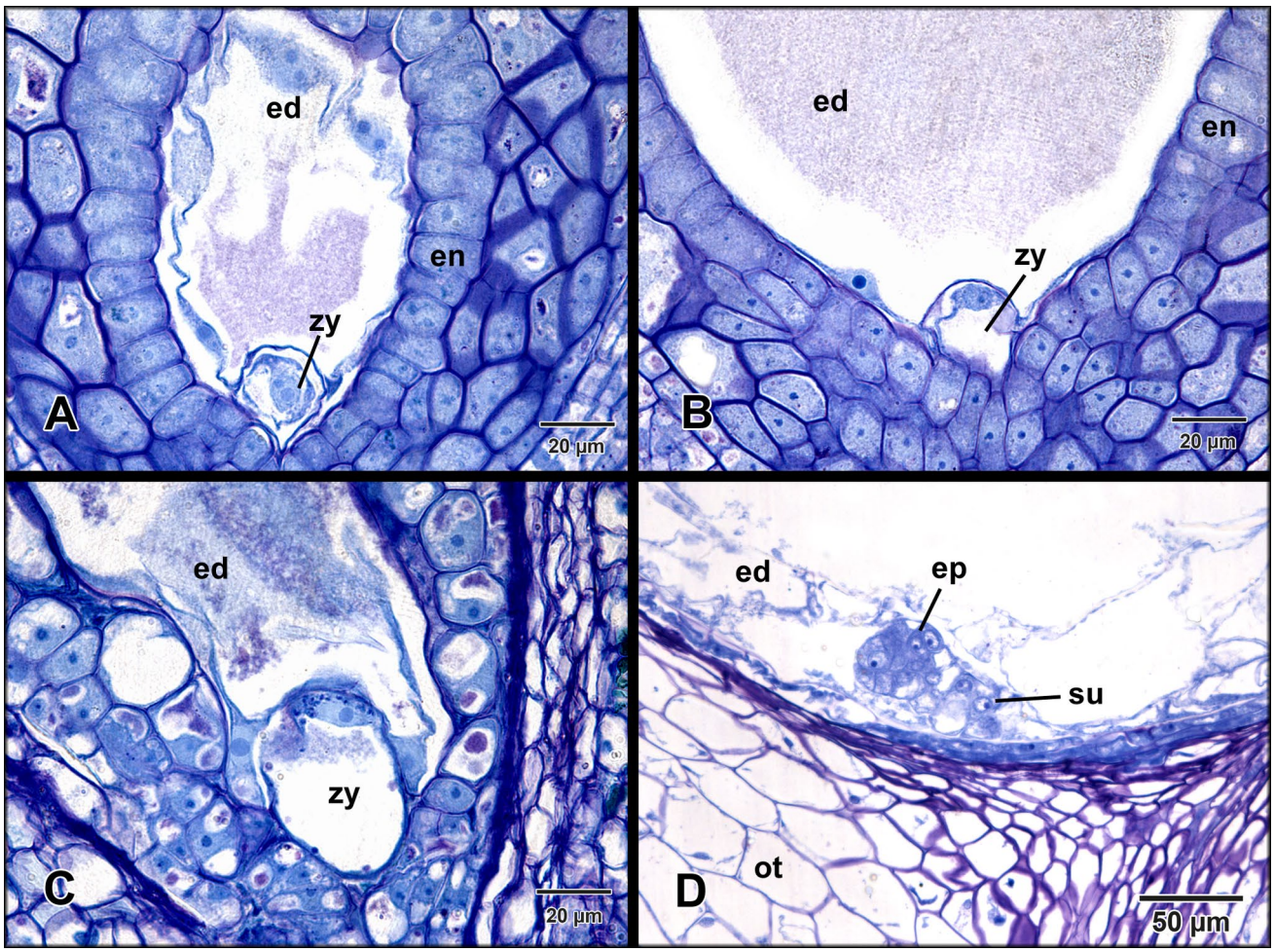


Fig. 11 Light photomicrographs of longitudinal sections of young seeds in *Couroupita guianensis*. Zygote in a crossed (**a**) and in a selfed (**b**) seed, seven and eight days after pollination, respectively. (**c**) Zygote in a sefled seed, 22 days after pollination. (**d**) Proembryo with the embryo proper at an early globular stage in seed of a 6 cm wide naturally pollinated fruit. *ed* endosperm, *en* endothelium, *ep* embryo proper, *oi* outer integument, *su* suspensor, *zy* zygote

initio nuclear type endosperm development that occurs in *C. guianensis*. An estimation of the endosperm development by means of counts of their nuclei during the free nuclear phase as performed in studies with species bearing a nuclear type of endosperm development (Valtueña et al. 2010) was also not possible to perform in this study. However, we were able to count the numbers of young seeds that exhibited or not a developing endosperm, and the comparison of selfed vs. crossed initiating fruits revealed that the proportions of seeds containing endosperm were similar in both treatments from the second to the fourth day after pollination. This indicates that self-pollen tubes in *C. guianensis* are

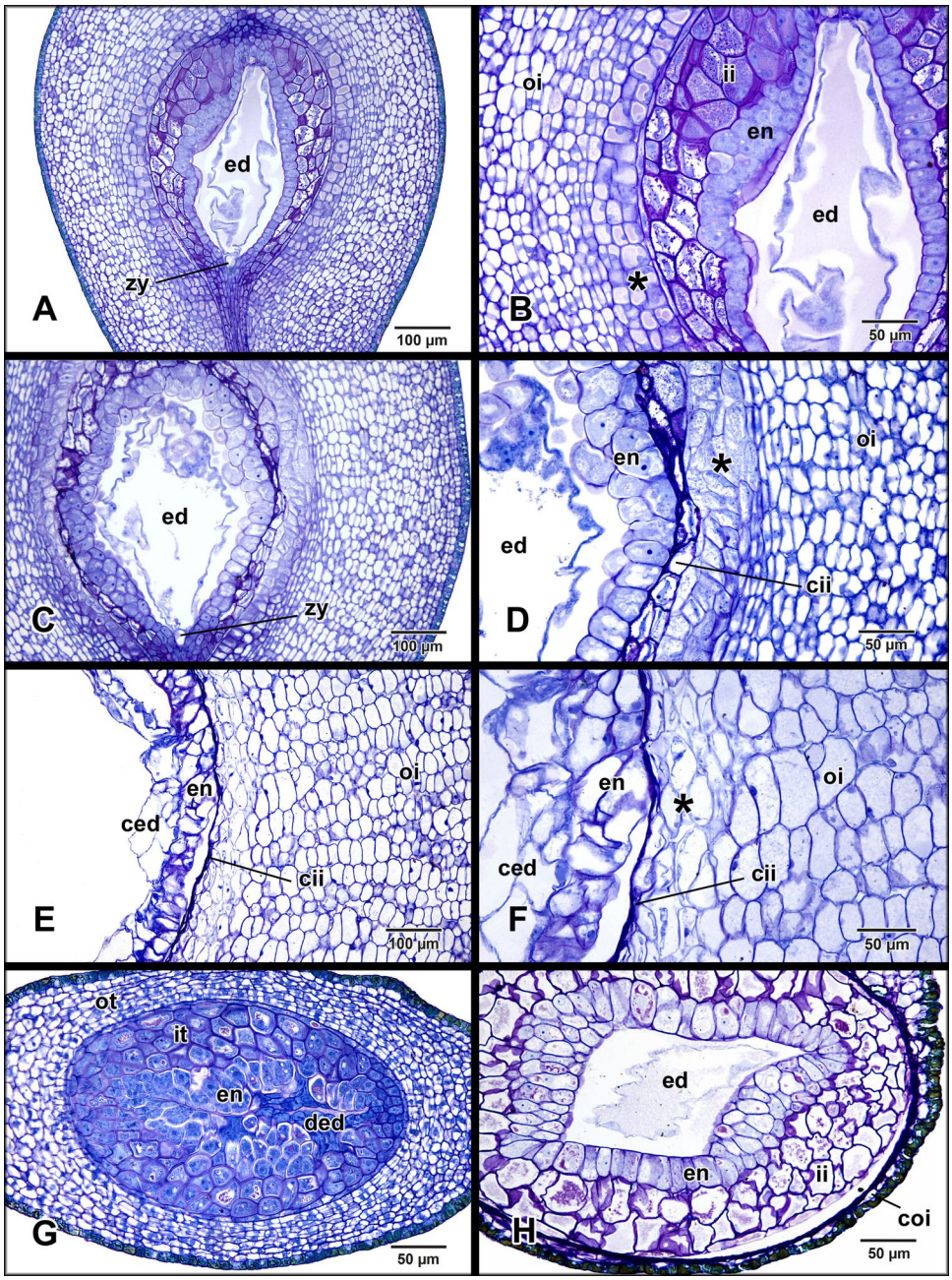


Fig. 12 Light photomicrographs of sections of young seeds in *Couroupita guianensis*. Longitudinal sections showing degeneration of the inner integument and the inner layers of the outer integument (**a–f**) in seeds derived from cross pollination. General aspect of the seed (**a**) and tissues in a higher magnification (**b**), 17 days after pollination. The asterisk in **b** indicates cell proliferation by periclinal divisions in the inner layers of the outer integument. General aspect of the seed (**c**) and tissues in a higher magnification (**d**), 32 days after pollination. General aspect (**e**) and high magnification (**f**) of the interface outer integument–peripheral region of the endosperm, 56 days after pollination. The asterisks in **d** and **f** indicate degeneration of the inner cell layers of the outer integument. Transverse section of a non-viable seed with a degenerated endosperm, seven days after self-pollination (**g**). Transverse section of a non-viable seed containing an apparently normal free-nucleate endosperm but a collapsed outer in tegument (**h**). *ced* cellularized endosperm, *cii* collapsed inner integument, coi collapsed outer integument, *ded* degenerated endosperm, *ed* endosperm, *en* endothelium, *ii* inner integument, *oi* outer integument, *zy* zygote

Table 3 Healthy and degenerated ovule/seeds in unpollinated, self-pollinated, and cross-pollinated pistil/early-fruits of *Couroupita guianensis*

	Time after anthesis								
	7 days			14 days			20 days		
	Unpol (%)	Self (%)	Cross (%)	Unpol (%)	Self (%)	Cross (%)	Unpol (%)	Self (%)	Cross (%)
Healthy	559 (69,7)	637 (88,97)	732 (91,84)	278 (40,35)	502 (83,13)	532 (74,09)	63 (9,78)	555 (73,12)	478 (75,16)
Degenerated	248 (30,3)	79 (11,03)	65 (8.16)	411 (59,65)	138 (21,56)	186 (25,91)	581 (90,22)	204 (36,76)	158 (33,05)
Total	807 (100)	716 (100)	797 (100)	689 (100)	640 (100)	718 (100)	644 (100)	759 (100)	636 (100)

Each column pools data from three pistil/fruits collected at random. The differences between self vs. cross at 7, 14 and 20 days were not statistically significant ($\chi^2=2.2247$, df=1, $P=0.1358$; $\chi^2=2.7730$, df=1, $P=0.0959$; $\chi^2=0.5763$, df=2, $P=0.4478$, respectively). However, these differences were significant between unpollinated pollinated pistil/fruits ($\chi^2=168.6691$, df=1, $P<0.0001$; $\chi^2=254.4563$, df=1, $P<0.0001$;$\chi^2=732.0468$, df=1, $P<0.0001$, respectively)

as efficient in inducting seed initiation as the cross- ones, although selfed-seed development does not surpass a little more than 30 days.

What is the Ultimate Cause of Self-Sterility in *Couroupita guianensis*?

Two main factors may induce strong self-sterility in seed plants: SI and EID. No sign of pre-syngamic pollen tube/pistil incompatibility reactions was observed in self-pollinated flowers, such as pollen tube tip swelling and burst or irregular deposition of callose plugs along the pollen tube (Lush and Clarke 1997). No evidence of self-pollen tube growth/ovule penetration inhibition was found in this study, although this may have resulted from limitations in the comparative in situ pollen tube growth/ovule penetration analyzes, together with an unfavorable pistil morphology (i.e., almost style-less, short, and massive gynoecium, bearing hundreds of ovules to be penetrated by pollen tubes). These results point to the absence of prezygotic self-incompatibility in *C. guianensis*. On the other hand, the precocious abortion of all selfed fruits with many initiating seeds bearing endosperm at early stages of development, as observed in this study, clearly points to some kind of postzygotic self-sterility. The absence of embryo development in seeds of fruits derived from self-pollination discredit the hypothesis that self-sterility in *C. guianensis* would be explained by fruit developmental failure due to embryo expression of deleterious recessive alleles, an expected condition in an EID context (Seavey and Bawa 1986; Sage et al. 1994; Hokanson and Hancock 2000; Wiens et al. 1987; Mahy and Jacquemart 1999; Hao et al. 2012; Gibbs 2014). Although there is some evidence that EID may also arise from endosperm gene expression, selfed fruit developmental failure in these cases results from disharmony in embryo/endosperm development in their seeds (Valtueña et al. 2010), which cannot be applied to the self-sterility verified in *C. guianensis* because selfed fruits in this species, as demonstrated in our study, abort before embryogenesis initiation. Moreover, no evidence for endosperm malfunction was found in *C. guianensis* up to the time of selfed pistil abscission. Both traits – i.e., no endosperm malfunction and no developing embryo to undergo malfunctioning in selfed pistils/fruits – have been pointed as common in postzygotic LSI species (Ferrer and Good 2012; Gibbs 2014).

As pointed out by Gibbs and Bianchi (1999), although LSI type phenomena have been reported for species in diverse families, there is a tendency for LSI to occur in confamilial genera, or congeneric species, that is, to show phyletic clustering. Our study is the first report of LSI in Lecythidaceae, and with *Camellia sinensis* (Theaceae; Chen et al. 2012), *Ipomopsis aggregate* and *I. tenuifolia* (Polemoniaceae; Waser and Price 1991; LaDoux and Friar 2006; Sage et al. 2006), *C. guianensis* correspond to the fourth LSI taxon recognized for Ericales, an order belonging to the Lamiid clade, one of the main clusters of LSI taxa in angiosperms (Gibbs 2014). Considering the scarcity of breeding system studies in Lecythidaceae, the carrying out of new studies of this kind in other species and genera from this and related families would be imperative to expand our knowledge about the LSI phenomenon.

Conclusion

This study indicates strong self-sterility and xenogamous reproduction operating in *C. guianensis*, although inter-population variation of the breeding system in this species cannot be discharged in view of diverging results reported by Ormond et al. 1981. Our data also point to postzygotic LSI as the main cause for the self-sterility observed in *C. guianensis*.

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Author contributions All authors contributed to the study conception and design. Material preparation and data collection analysis were performed by Filipe Alisson Ribeiro, Ana Carolina Isepan-Motta and Edgar Ventura. Analysis was performed by Nelson Sabino Bittencourt Júnior, Filipe Alisson Ribeiro and Ana Carolina Isepan-Motta. The first draft of the manuscript was written by Nelson Sabino Bittencourt Júnior and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Data Availability No datasets were generated or analysed during the current study.

Declarations

Competing interests The authors declare no competing interests.

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