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Original Article

Is there interspecific fruit set in distylous, synchronopatric species of *Erythroxylum* P. Browne (Erythroxylaceae)?

Há frutificação interespecífica em espécies distílicas e sincronopátricas de *Erythroxylum* P. Browne (Erythroxylaceae)?

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Abstract

Sympatric congeneric species that overlap the flowering period and have morphologically similar flowers tend to be pollinated by the same groups of pollinators, facilitating interspecific pollination and hybridization. If that situation involves distylous taxa, interspecific fruit set will theoretically rely on the match of sexual organs of the different species, because distylous plants only set fruits after crosses between floral morphs that are reciprocal herkogamous (RH) and are produced in different individuals. In a Caatinga forest in NE Brazil, there are three distylous species of *Erythroxylum* P. Br (*E. citrifolium* A.St.-Hil., *E. pauferrense* Plowman and *E. simonis* Plowman; Erythroxylaceae) that overlap their flowering period and have a high similarity in floral attributes. *Erythroxylum pauferrense* is a rare and endemic species of the region. This study aimed to describe the distylous pattern of those three *Erythroxylum* species (i.e., reciprocal hercogamy, proportion of morphs in the population and breeding system) and to evaluate the reproductive isolation between them. Flowering synchrony, interspecific RH and pollination were the pre-zygotic barriers investigated, and the post-zygotic one was the fruit set after interspecific pollination experiments. The species completely overlapped their flowering periods and were pollinated by the same group of species (*Apis mellifera*, *Tetragona* sp., and *Trigona spinipes*). In a general matter, a low level of both intra and interspecific RH was revealed. Interspecific pollination set fruits only between *E. pauferrense* and *E. simonis*. A low level of both intra and interspecific RH was revealed for all species, except for the low-level organs of *E. citrifolium*. The atypical distyly observed in *E. pauferrense* and *E simonis* indicates a collapse in the distylous system and may be related to a relaxation in the interspecific incompatibility mechanism.

Keywords: co-flowering, distyly, pollination, hybridization, pre-zygotic barrier, reciprocal herkogamy.

Resumo

Espécies sincronopátricas e congenéricas que têm flores morfologicamente semelhantes tendem a ser polinizadas pelos mesmos grupos de polinizadores, facilitando a polinização e a hibridização interespecíficas. Se tal situação envolver táxons distílicos, a frutificação interespecífica dependerá teoricamente da correspondência de órgãos sexuais das diferentes espécies, porque as plantas distílicas somente frutificam após cruzamentos entre morfos florais que possuem hercogamia recíproca (RH) e são produzidos em indivíduos diferentes. Em uma floresta de Caatinga no Nordeste do Brasil há três espécies distílicas de *Erythroxylum* P. Br (*E. citrifolium* A.St.-Hil., *E. pauferrense* Plowman e *E. simonis* Plowman; Erythroxylaceae) que se sobrepõem em seu período de floração e têm grande similaridade em atributos florais. *Erythroxylum pauferrense* é rara e endêmica da região. Este estudo teve como objetivo descrever o padrão distílico dessas três espécies de *Erythroxylum* (ou seja, hercogamia recíproca, proporção de morfos na população e sistema de reprodução) e avaliar o isolamento reprodutivo entre elas. Sincronia de floração, RH interespecífica e polinização foram as barreiras pré-zigóticas investigadas, e a pós-zigótica foi a frutificação após experimentos de polinização interespecífica. As espécies sobrepuseram completamente seus períodos de floração e foram polinizadas pelo mesmo grupo de espécies (*Apis mellifera*, *Tetragona* sp., e *Trigona spinipes*). Em geral, um baixo nível de RH intra e interespecífica foi revelado. A polinização interespecífica resultou em frutos apenas entre *E. pauferrense* e *E. simonis*. Um baixo nível de RH intra e interespecífica foi revelado para todas as espécies, exceto para os órgãos de nível inferior de *E. citrifolium*. A distilia atípica observada em *E. pauferrense* e *E simonis* indica um colapso no sistema distílico e pode estar relacionada a um relaxamento no mecanismo de incompatibilidade interespecífica

Palavras-chave: cofloração, distilia, polinização, barreira pré-zigótica, hercogamia recíproca.

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1. Introduction

Sympatric species that flowering in synchrony (i.e., synchronopatric species) and have similar flowers tend to share pollinators (Schemske, 1981; Castro et al., 2004; Arceo-Gómez and Ashman, 2014; Muchhala et al., 2014). When the species are congeneric there is more probability to form interspecific fruits, however, it will depend on the strength of pre-and post-zygotic barriers between these species (Scopece et al., 2010).

One of the main pre-zygotic barriers between species is the morphological in which the positioning of floral reproductive structures resulting in non-coincident areas of pollen deposition and capture on the body of shared pollinators (Barrett, 2002; Hopkins, 2013). Other barriers are temporal differences in flower resources supply (Stone et al., 1998), in the chemical composition of floral volatiles (which attract distinct groups of pollinators, ex. Salzmann et al., 2006), interspecific pollen-pistil incompatibility systems (Bradshaw Junior et al., 1995), ethological isolation of pollinators. Post-zygotic barriers include non-viability, sterility, and/or reduced reproductive performance of hybrids (Levin, 1971; Judd et al., 2009; Johnson, 2010; Greiner et al., 2011). Studies have shown that efficiency levels of pre-and post-zygotic barriers vary among species (e.g., Martin and Willis, 2007; Wendt et al., 2001, 2008). Therefore, it should not be considered as absolute (Wu, 2001) and can be differentially important depending on whether the species are rare or widespread (Baack et al., 2015).

Sympatric species of the tropical genus *Erythroxylum* P.Browne in the coca family (Erythroxylaceae) constitute excellent models for studies on morphological divergence and reproductive isolations, including pre-zygotic barriers driving interspecific fruit set. These species have similar flowers and consequently share pollinators, increasing the chances of interspecific pollen transfer. The genus *Erythroxylum* has about 230 species (Plowman and Hensold, 2004) and although flowers have traits similar to each other, several reproductive strategies had evolved in the genus, that exhibits dioecy (Bawa and Opler, 1975), gynodioecy and agamospermy (Domínguez et al., 1997; Avila-Sakar and Domínguez 2000).

Although the atypical distyly was recorded in *E. campestre* (Barros, 1998), most species of genus *Erythroxylum* are reported as typically distylous and have self- and intra-morph-incompatibility (Ganders, 1979; Richards and Koptur, 1993; Pailler and Thompson, 1997; Silva et al., 2007). Therefore, it is necessary to investigate the possible sources of variation that cause deviations from distyly in synchronopatric *Erythroxylum* species and the possible consequences of interspecific fruit set.

Distylous species have self- and intramorph-incompatibility and the positioning of sexual organs is more specialized (Webb and Lloyd, 1986). The morph ratio is 1:1, called isoplethy; in atypical cases that ratio is different from 1:1, and is called anisoplety (Ganders, 1979; Barrett and Shore, 2008). The stamens and the carpel's heights are at a similar level to the opposite morph, what is called reciprocal herkogamy (hereafter RH; Ganders, 1979; Barrett and Shore, 2008). In these species it is expected that interspecific crosses can be more critical to occur since reciprocal herkogamy is expected for the opposite morph of the same species.

In a fragment of Brazilian dry forest occurs populations of three species of *Erythroxylum* (*E. citrifolium* A.St.-Hil., *E. pauferrense* Plowman, and *E. simonis* Plowman). These species have overlapping flowering periods, strong similarities in floral traits and share the floral visitors. Thus, we investigate the phenology and reproductive biology of those three species to test the following premise: in populations of distylous, synchronopatric, and congeneric species and that share pollinators, the rate of fruiting from interspecific crosses will depend on the level of compatibility and reciprocal herkogamy between species.

This study aimed to Brazilian species of *Erythroxylum* and to answer the following questions: 1) What is the period of flowering overlap between species? 2) Are the *Erythroxylum* species typically distylous? 3) Do the species share pollinators? 4) Is there interspecific RH? 5) Is there fruit set after interspecific crosses?

2. Materials and Methods

2.1. Study area and species

We carried out our study in natural populations of *E. citrifolium*, *E. pauferrense,* and *E. simonis* in a fragment of Caatinga (Brazilian dry forest). That is a physionomy of the Atlantic Forest recognized in Brazilian literature as "Brejos de Altitude". Those areas have Atlantic Forest vegetation under a strong influence of Caatinga, within which it is geographically inserted (Andrade-Lima, 1982). This configuration leads to high endemism and the coexistence of species from both vegetation types, which characterizes those areas as having floristic and vegetation peculiarities (Veloso et al., 1991; Myers et al., 2000).

The area is located at State Park Mata do Pau-Ferro, (6°58'12'S; 35°42'15'W), Paraiba, Northeast Brazil. It has approximately 600 ha, and the predominant vegetation type is open ombrophylous forest (Barbosa et al., 2004). The populations of the three species are comprised of shrubs to small trees, being distributed throughout the study area. *Erythroxyllum citrifolium* is apparently less abundant than the other two species. The species have wide distributions in most of NE Brazil, except *E. pauferrense*, which is endemic to Paraíba State (Plowman and Hensold, 2004).

2.2. Flowering phenology, morph ratio, floral biology, and morphometrics

To evaluate the overlap of flowering periods, we monitored 20 clusters (from two to 16 individuals per cluster) of marked individuals of the three species in intervals of 15 days for three years (2014-2016) and recorded the presence of flowers using the Activity Index (Bencke and Morellato, 2002). We marked 20 pre-anthesis buds on 20 individuals per morph per species, which were monitored from flower anthesis until flower senescence. In another ten flowers per morph (ten individuals/species), we tested the period of stigmatic receptivity in the early morning, at midday, and late afternoon, using hydrogen

peroxide (Dafni et al., 2005). To estimate the floral morph ratio (i.e., the proportion of thrum (T) and pin (P) individuals of each species), we classified the floral morph of all individuals distributed along a transect of approximately 1000m (n = 65 individuals of *E. citrifolium*, 312 individuals of *E. pauferrense* and 347 individuals of *E. simonis*). Buds (n=10) distributed in ten individuals/morph/species were preserved in 70% ethanol and used in calculations of pollen/ovule ratio (Cruden, 1977).

We collected 20 flowers from 20 individuals of each morph of each species, preserved in a 70% alcohol solution to carry out morphometric analyses of the height of anthers and stigmas, which are used in calculation regarding RH (Figure 1). We performed the generalized linear model (GLM) analysis and the calculation on floral inaccuracy to evaluate the intra- and interspecific reciprocal hercogamy. Differences in stigma and anther heights between floral morphs and species were analyzed with GLM using the "glm" function in the "lme4" package (Bates et al., 2015) after detecting that there was no data overdispersion. The models included sexual organs (stamen and stigma height), floral morph, species, and the interaction between factors. Then we tested the significance of the model with a type II ANOVA using "Anova" functions. Subsequently, we used the "Lsmeans" function with Tukey adjustment (emmeans package; Lenth, 2016) to compare differences between height of sexual organs. We carried out all analyses in R statistical environment (version 3.6.2; R Development Core Team, 2019).

To test the intra and interspecific RH we used the floral adaptative inaccuracy method (Armbruster et al., 2009, 2017; Li et al., 2018). This index estimates the reciprocity between the higher (pin stigmas and thrum anthers) and lower (thrum stigmas and pin anthers) floral organs of distylous species and considers the midpoint of the correspondent floral organ as the flower optimum (Armbruster et al., 2017). The optimum is described as the most reciprocal value (a perfect RH) and represents the highest chances of legitimate (intermorph) pollination (Armbruster et al., 2017). We used the mean and the variance of stigma and anthers' heights to calculate the values of the adaptive inaccuracy of the higher and lower organs (higher: Equation1; lower: Equation 2, 3, and 4). Because pin flowers of *E. citrifolium* and *E. pauferrense* have two anther heights (hereafter referred to taller and shorter anthers, Figure 1), we performed three calculations for the lower organs in those flowers: one for each anther level (Equation 2 and Equation 3) and another using the mean of those two levels of anthers (a and Aa; Equation 4), as follows:

$$\text{Inaccuracy}_{\text{higher organs}} = (\bar{A} - \bar{S})^2 + VA + VS \quad (1)$$

$$\text{Inaccuracy}_{\text{lower organs}} = (\bar{a} - \bar{s}) + Va + Vs \quad (2)$$

$$\text{Inaccuracy}_{\text{lower organs}} = (\bar{A\bar{a}} - \bar{s})^2 + VAa + Vs \quad (3)$$

$$\text{Inaccuracy}_{\text{lower organs}} = (\bar{A}\overline{aa} - \bar{s}) + Va + Vs \quad (4)$$

In which: $\bar{A}$, $\bar{A\bar{a}}$, and $\bar{a}$ represent, respectively, anther height of thrum flowers (all species), anther height of the taller and shorter anthers of pin flowers of *E. citrifolium* and *E. pauferrense*. $\bar{A\bar{a}a}$ is the mean height between A and a. S and s are, respectively, stigmas' height of pin and thrum flowers and V is the variance (Matias et al., 2020).

The inaccuracy is calculated and presented as units squared (here we used mm; (Armbruster et al., 2017). An innacuracy of zero represents the flower optimum and values higher than that are interpreted as deviations from the optimum, and consequently a lower degree of RH (Armbruster et al., 2017).

We also calculated the standardized inaccuracy, which is a percentage that represents the extension to which the

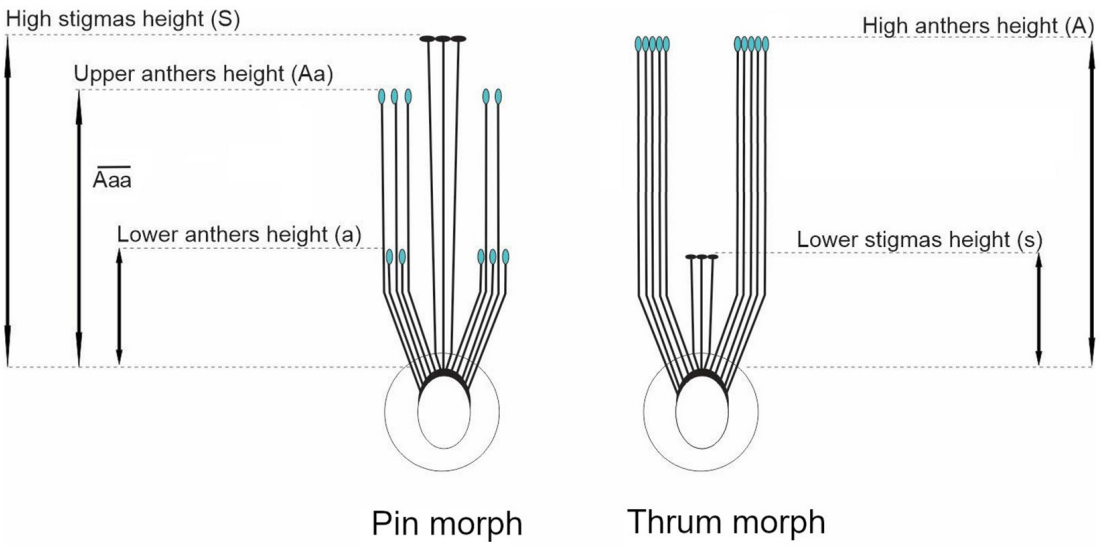

Figure 1. Schematic representation of anthers and stigma arrangement on pin and thrum flowers of three synchronopatric, distylous species of *Erythroxylum* (Erythroxylaceae) in a Caatinga Forest of NE Brazil. Pin flowers of *E. citrifolium* and *E. pauferrense* have two anther levels [(taller (Aa) and shorter (a)], and *E. simonis* has only one. $\bar{A\bar{a}a}$: average of Aa and a anthers.

estimated inaccuracy deviates from zero, or the flower optimal (i.e., perfect RH). It is calculated by dividing the estimated inaccuracy by the average height of all organs squared (Armbruster et al., 2017).

Although the inaccuracy index and the standardized innacuracy were originally developed for the evaluation of intraspecific RH, we used those calculations to evaluate the interspecific RH of the studied *Erythroxylum* species. We aimed to check if anthers and stigma' heights of the three *Erythroxylum* species studied here allow (and in which extension) pollen deposition and receipt in coincident areas of shared pollinators' body and, consequently, interspecific pollination.

2.3. Floral visitors

We conducted direct observations of floral visitors in 10 focal plants of each species from 5:00 h to 18:00 h, on different days, in a total of 36 h per species, homogeneously distributed along the day and among plants. In each visit we recorded the time, the visitor species, the number of flowers visited, and floral visitor's behavior (contact with the anthers and stigma, the floral resource collected). We classified floral visitors as effective pollinators (EP; contact anther and stigma in a frequency ≥ 10 visits.hour^{-1}), occasional pollinators (OP; contact anther and stigma in a frequency < 10 visits.hour^{-1}), or robbers (RO; explore floral resource but do not contact reproductive structures; Dafni et al., 2005). Floral visitors were collected, identified by specialists, and deposited at the Plant Ecology and Reproductive Laboratory at Federal University of Paraíba (UFPB).

To evaluate if floral visitors' species have preferences for *Erythroxylum* species, we compare the number of visits of each visitor to *Erythroxyllum* species using a generalized linear model (GLM; *glm* function and *lme4* package; Bates et al., 2015). We used as fixed factors pollinator, time of visit, and species of *Erythroxylum* visited, as response variables interaction between these factors and number of visits. We modeled the data with a negative binomial distribution. We performed the calculation using the software R (version 3.6.2; R Development Core Team, 2019).

2.4. Breeding system

We studied the breeding system of the three *Erythroxylum* species employing controlled cross and natural pollination (control), following the protocol adopted by Bawa and Beach (1983). We performed each one of the following treatments in 60 bagged, pre-anthesis buds, distributed in ten individuals/morph/species: spontaneous self-pollination (bagged control; SS), hand self-pollination (HS), and intermorph manual-cross pollination (IM). We emasculated all pre-anthesis buds used in the intermorph pollination experiment. All flowers were remained bagged during anthesis to avoid contact with floral visitors. We marked the other 50 flowers of ten individuals/morph/species and we monitored their natural fruit formation (natural pollination or control, hereafter referred to as NP). We recorded the number of ripe fruits for all treatments. Fruit formation lasted around 30 days.

To test for interspecific fruit set between the species studied, we conducted interspecific manual cross-pollination. Each interspecific test between two species included four treatments: a) thrum pollen of species 1 on pin stigma of species 2; b) pin pollen of species 1 on thrum stigma of species 2; c) thrum pollen of species 2 on pin stigma of species 1; d) pin pollen of species 2 on thrum stigma of species 1. Before being used in controlled crosses, we removed pin pollen grains of *E. citrifolium* and *E. pauferrense* from the two sets of anthers, from flowers of around five individuals, mixed them in a petri dish, and then deposited them onto receptor stigmas. All hand crosses were conducted approximately one hour after anthesis.

To compare fruit set among the treatments, we performed a GLM analysis with binomial distribution using the R Program (version 3.6.2; R Development Core Team, 2019).

3. Results

3.1. Flowering phenology, morph ratio, and floral biology

The flowering period of the three species lasts from two to three months (Figure 2). The species showed similar variation in the flowering period between years. In 2014 no species bloomed, in 2015 they only bloomed from Apr to May, and in 2016 from Feb to Apr (Figure 2).

Erythroxylum citrifolium had similar proportion of morphs or isoplethy (thrum: 30; pin: 35; χ^2 =0.38; p = 0.53), whereas *E. pauferrense* (thrum:183; pin: 129; χ^2 = 9.3; p = 0.0022) and *E. simonis* (thrum: 195; pin: 152; χ^2 = 5.3; p = 0.0209) had more thrum than pin individuals (anisoplethy).

The species have similar floral attributes. Flowers have five sepals that connate at the base, and a pentamerous, white, actinomorphic corolla of similar size (Table 1), and nectar as floral resource. All species are hermaphrodite, with ten stamens distributed in two arrangements: one set of five stamens is opposite to the sepals and another set of five stamens is opposite to the petals. Stamens of thrum flowers of all species and pin flowers of *E. simonis* are at the same level within the flower. Pin flowers of *E. citrifolium* and *E. pauferrense* have two heights of stamens, resulting in two anthers' levels (hereafter referred to as taller and shorter anthers; Figure 1). The gynoecium includes a superior ovary and three styles (fused in *E. pauferrense* and free in the other two species) with capitate stigmas. In *E. pauferrense* and *E. simonis*, the stigmas of pin flowers are positioned in a downwards direction, touching the dehiscent anthers during the bud phase. Pollen-ovule ratio ranged between 2,800 (thrum flowers of *E. pauferrense*) to 8,600 (pin flowers of *E. citrifolium*, Table 1).

All species showed diurnal anthesis that started between 6:00am to 09:00am. Stigmatic receptivity occurred approximately one hour after the beginning of the anthesis. The flowers of all species remained receptive throughout the day. On the second day, the petals become beige and felt down.

The species did not exhibit intraspecific reciprocal hercogamy, except for the low organs of *E. citrifolium* (i.e., thrum stigma and pin anthers; Table 2, Figure 3). The lowest values of intraspecific inaccuracy (which indicate the

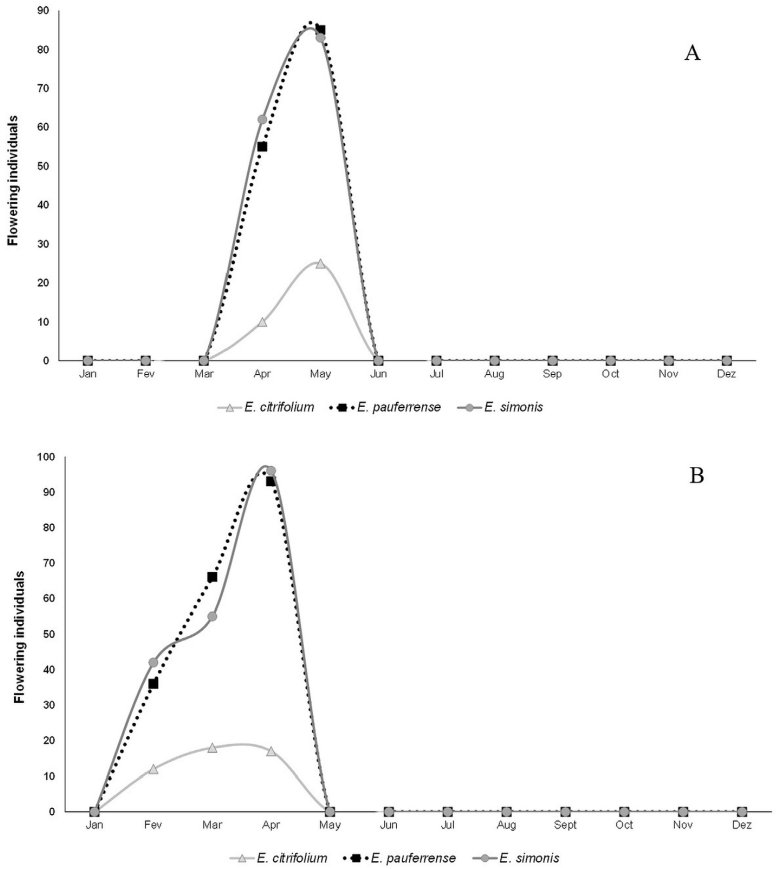

Figure 2. Flowering period of three synchronopatric, distylous species of *Erythroxylum* (Erythroxylaceae) in a Caatinga Forest of NE Brazil. (A) 2015; (B) 2016.

Table 1. Means (variance in parentheses) of floral parts (mm) and pollen/ovule ratio (P:O) of three synchronopatric, distylous species of *Erythroxylum* (Erythroxylaceae) in a Caatinga Forest of NE Brazil.

Species	Morph	Stigma	Anther	Taller anthers	Shorter anthers	P:O
E. citrifolium	Thrum	3.65 (0.18)	9.21 (0.01)			5.700
	Pin	17.75 (4.28)	8.72 (24.39)*	13.64 (0.5)	3.81 (0.01)	8.600
E. pauferrense	Thrum	4.20 (0.08)	5.60 (0.05)			2.800
	Pin	13.50 (1.64)	7.90 (3.86)*	9.83 (0.01)	5.9 (0.05)	4.000
E. simonis	Thrum	4.19 (0.08)	4.59 (0.01)			3.500
	Pin	12.25 (1.64)	8.29 (0.01)			3.800

Stigma, anther, taller and shorter anthers represent the heights of those organs. *Mean and variance of taller and shorter anthers.

Table 2. Comparisons (using generalized linear model analysis, GLM) of heights between sexual organs, morphs, and between three synchronopatric, distylous species of *Erythroxylum* (Erythroxylaceae) in a Caatinga Forest of NE Brazil.

Source of variation	÷2	d.f	*p*
Organ	484.7	2	<0.001
Morph	1620	1	<0.001
Species	262.2	2	<0.001
Organ:Morph	1580	1	<0.001
Organ:Species	97	3	<0.001
Morph: Species	127.2	2	<0.001
Organ:Morph: Species	510.2	2	<0.001

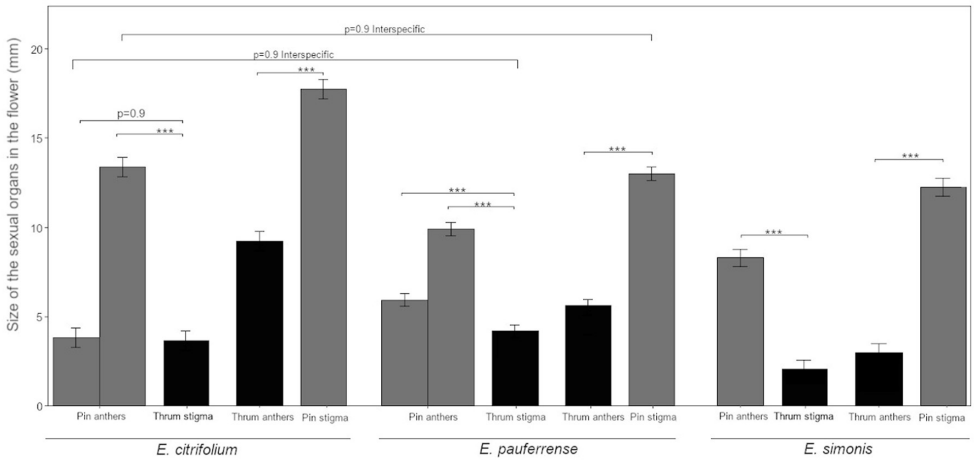

Figure 3. Intra and interspecific comparisons between anthers' and stigmas' heights of distylous species of *Erythroxylum* (Erythroxylaceae) in a Caatinga Forest of NE Brazil. In interspecific comparisons, only the organs that did not show significant differences are shown. ***Significant differences.

Table 3. Intra and interspecific estimates of inaccuracy and standardized inaccuracy (in parenthesis, given in %) of three synchronopatric, distylous species of *Erythroxylum* (Erythroxylaceae) in a Caatinga Forest of NE Brazil.

Inaccuracy	Inaccuracy by organ type		Total innacuracy	Inaccuracy of the lower organs considering the different heights of anthers in pin flowers	
	Higher (S-A)	Lower (s-Aaa)		(s-Aa)	(s-a)
Intraspecific					
E. pauferrense	57.00 (85.4)	17.90 (26.8)	74.90 (112.2)	31.90 (47.8)	**1.10 (1.9)**
E. citrifolium	77.20 (63.11)	50.30 (41.1)	127.50 (104.2)	100.50 (82.1)	**0.20 (0.3)**
E. simonis	61.70 (50.4)	39.40 (32.2)	101.10 (89.8)	-	-
Interspecific	Higher (stigma x anther)	Lower (anther x stigma)			
E. pauferrense x E. simonis	73.30 (132.4)	38.90 (70.3)	112.22 (171.3)	61.03 (111.9)	15.90 (38.64)
E. simonis x E. pauferrense	47.20 (82.0)	16.90 (29.4)	64.10 (111.4)	-	-
E. simonis x E. citrifolium	12.30 (17.6)	21.72 (31.1)	34.02 (48.8)	-	-
E. citrifolium x E. simonis	177.50 (259.0)	69.40 (101.0)	246.90 (360.0)	135.30 (149.6)	**3.60 (7.24)**
E. pauferrense x E. citrifolium	16.40 (22.9)	22.40 (31.3)	38.80 (54.2)	38.40 (48.1)	5.60 (8.8)
E. citrifolium x E. pauferrense	151.70 (184.8)	45.00 (54.7)	196.70 (239.5)	89.90 (84.8)	**0.20 (0.3)**

Total inaccuracy: the sum of innacuracies of higher and lowers organs; Aa and a: taller and shorter anthers of pin flowers of *E. citrifolium* and *E. pauferrense*. Values in bold represent the lowest values of inaccuracy.

highest chances of intraspecific pollen flow) were found in the shorter anthers of pin flowers and low stigmas of thrum flowers of *E. citrifolium* and *E. pauferrense*, which were remarkably close to the optimal (Table 3). Regarding interspecific inaccuracy, the lowest values (which indicate the highest chances of interspecific pollen flow) were found between the shorter anthers of pin flowers of *E citrifolium* and the low stigmas of thrum flowers of *E. pauferrense* (i.e., pollen flow from *E. citrifolium* pin anther to *E. pauferrense* thrum stigma). It is interesting to note that this inaccuracy value was similar to that described above for the highest intraspecific inaccuracy value of *E citrifolium* (Figure 3).

3.2. Floral visitors

We observed the bee *Apis mellifera* L., *Tetragona* sp., and *Trigona spinipes* Fabr randomly visiting both floral morphs of the three species, from which they collected nectar and pollen. We recorded a significantly higher number of visits

between 9:00h and 12:00h (Table 4, Figures 4 and 5). The bees visited all open flowers of an inflorescence. We considered all visitors as effective pollinators since they carried pollen all over their bodies. *Tetragona* sp. and *Trigona spinipes* had the highest visiting rates in both morphs of all plant species in the entire flowering period (Figures 4 and 5). There was a significant difference in the number of visits between *Erythroxylum* species: *E. simonis* was the most visited species (503 visits), being followed by *E. pauferrense* (468) and *E. citrifolium* (321; Table 4; Figure 5). However, we recorded no significant differences in the frequency of each pollinator visit between the three plant species.

Table 4. Comparisons (using generalized linear model analysis, GLM) of the visitors' frequency of the three synchronopatric, distylous species of *Erythroxylum* (Erythroxylaceae) in a Caatinga Forest of NE Brazil.

Source of variation	÷2	d.f	P
Frequency of visits			
Erythroxylum species	21.35	2	<0.001
Day time	422.34	3	<0.001
Pollinators	4.68	2	0.096
Erythroxylum species x day time	26.38	6	<0.001
Erythroxylum species x pollinators	3.97	4	0.41
Day time x pollinators	13.12	6	0.04
Erythroxylum species x day time x pollinators	8.99	12	0.70

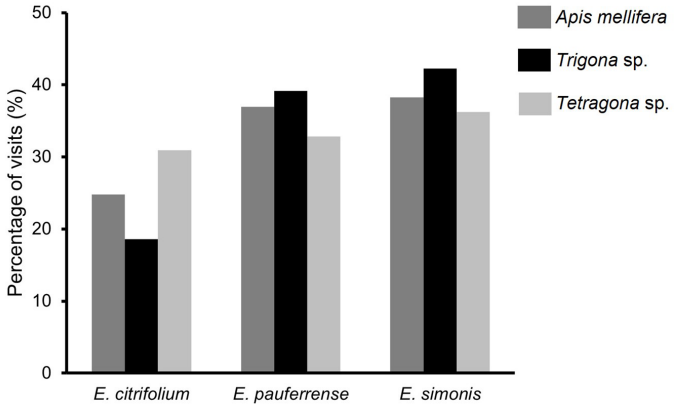

Figure 4. Pollinators' percentage of visits to three synchronopatric, distylous species of *Erythroxylum* (Erythroxylaceae) in a Caatinga Forest of NE Brazil.

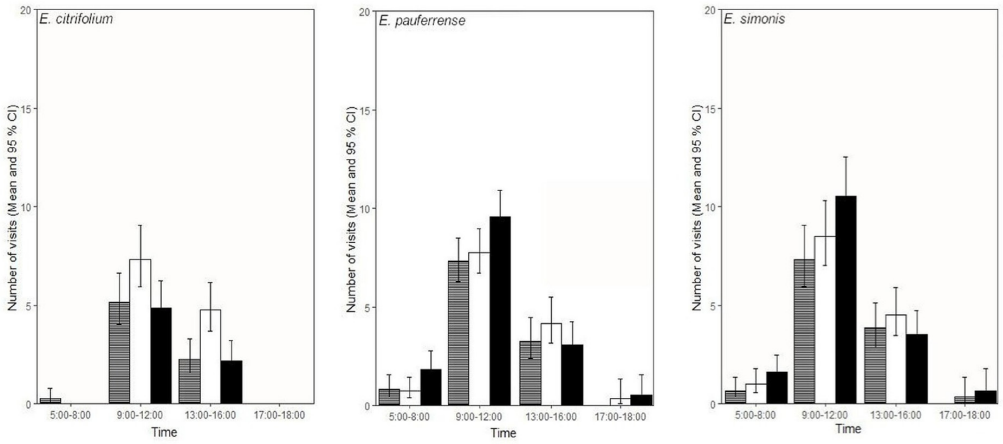

Figure 5. Pollinators' frequency of visits to three synchronopatric, distylous species of *Erythroxylum* (Erythroxylaceae) in a Caatinga Forest of NE Brazil.

3.3. Breeding system

Erythroxylum simonis formed fruits in all treatments, *E. pauferrense* formed fruits in all treatments except after hand self-pollination (HS, or bagged control) in the pin morph, and *E. citrifolium* only formed fruits after natural pollination (NP) and intermorph pollination (IM) (Table 5, Figure 6). The fruit set after natural pollination (NP) and intermoph pollination (IM) was higher than that after

Table 5. Percentage of fruit set after intra- and interspecific pollination experiments on three synchronopatric, distylous species of *Erythroxylum* (Erythroxylaceae) in a Caatinga Forest of NE Brazil.

Crosses	Species	Morph	Natural (50)	Intermorph (60)	Spont. self (60)	Self-pollination (60)
Intraspecific	*E. citrifolium*	P	24	33.3	0	0
		T	56	81.7	0	0
	E. pauferrense	P	82	73.3	1.7	0
		T	54	43.3	6.7	5
	E. simonis	P	68	90	10	3.3
		T	94	5	6.7	6.7

Interspecific (60 for all)	Morph	EC x EP	EP x EC	EC x ES	ES x EC	EP x ES	ES x EP
	P	0	0	0	0	13.3	5
	T	0	0	0	0	21.7	10

Numbers in parenthesis represent the number of flowers used in each treatment. P: pin morph; T: thrum morph; Natural: natural (control); Intermorph: intermoph manual cross-pollination;: spontaneous self-pollination; EP: *E. pauferrense*; ES: *E. simonis*; EC: *E. citrifolium*. The first species in the column of interspecific manual cross-pollination is the pollen donor, and the second is the pollen receptor.

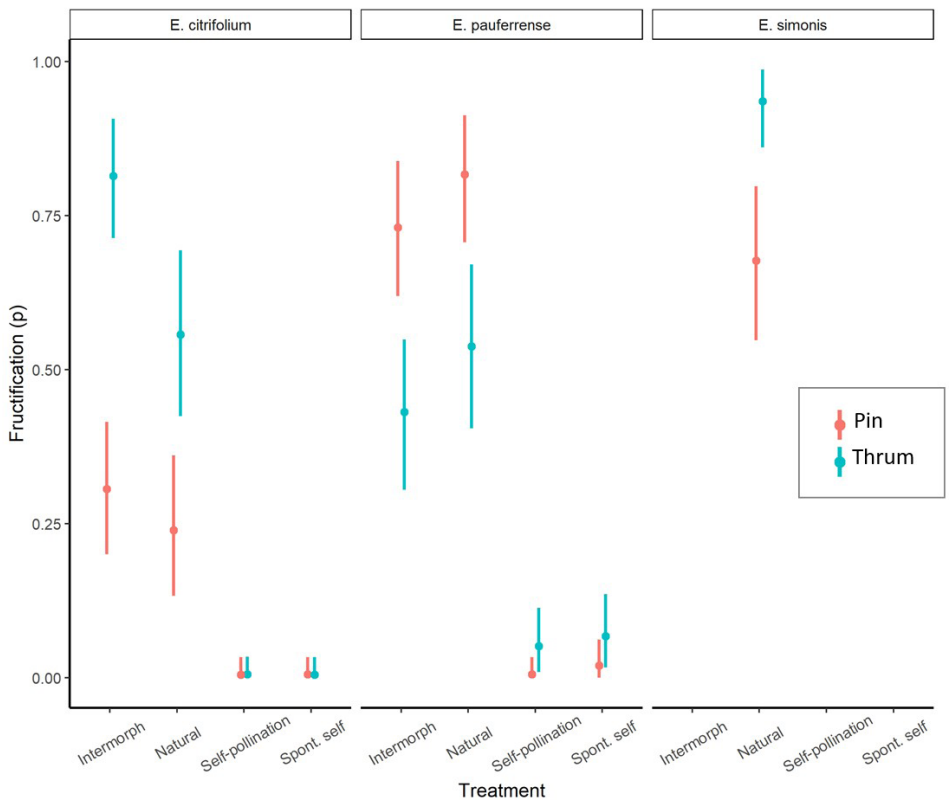

Figure 6. Comparisons of mean fruit set (using generalized linear model analysis, GLM) resulted from pollination experiments on three synchronopatric, distylous species of *Erythroxylum* (Erythroxylaceae) in a Caatinga Forest of NE Brazil. Ec: *E. citrifolium*; Ep: *E. pauferrense*; Es: *E. simomis*; NF: natural fruit set; TxP: thrum pollen on pin stigma; PxT: pin pollen on thrum stigma; PxP: pin manual self-pollination; TxT: thrum manual self-pollination; TS: thrum spontaneous self-pollination; PS: pin spontaneous self-pollination.

HS and SS for all species and morphs, except for thrum individuals of *E. simonis*, whose fruit set after intermoph pollination (IM) was similar to hand self-pollination (HS) and spontaneous self-pollination (SS) (Table 5, Figure 6). Interspecific crosses set fruits only after manual cross-pollination between *E. pauferrense* and *E. simonis* (Table 5).

4. Discussion

The flowering period of the three studied species seems overlapped completely, and their responses to climate variations suggest a similar phenological pattern across these species. Despite this overlap, interspecific pollination was largely ineffective, as no fruit set occurred from interspecific crosses, underscoring the critical role of floral morphology and hercogamy in reproductive isolation. Only *E. citrifolium* exhibited typical distyly with strong reciprocal hercogamy (RH), while the other species displayed atypical patterns. Additionally, self-compatibility in *E. pauferrense* and *E. simonis* appears to be associated with deficiencies in effective distyly and insufficient pollination services. Despite high visitation rates, particularly for *E. citrifolium*, fruit set did not significantly increase, highlighting the complex interactions between floral morphology, pollinator behavior, and reproductive success.

The variation in the flowering period among the three consecutive years probably is partially a result of a strong drought that occurred in 2014. The similarity of variations in flowering phenology between species suggests that they respond in a similar way to climate variations. Flowering overlap of *Erythroxyllum* distylous species of the same section of the species studied here (Plowman and Hensold, 2004) with similar flowers and shared pollinators was already observed in the Cerrado (tropical savannah of Brazil), Barros, 1998). Some studies report the effect of co-flowering on the attraction of a higher number of floral visitors in other plant families, such as in *Primula* (Gurung et al., 2018) and *Psychotria* (Mesquita-Neto et al., 2018). The flowering phenology constitutes a critical factor for co-flowering, incompatible species that share pollinators, because it may influence plant reproductive success (Ghazoul, 2006; Yang et al., 2007; Grab et al., 2017). Strong similarities may lead to competition when pollinators are limited (in which species may reduce their fitness) or facilitation (in which reproduction is enhanced because of a higher attraction of pollinators; Moeller, 2004; Ghazoul, 2006; Mitchell et al., 2009; Yang et al., 2013).

Stamens of different sizes observed in pin flowers of *E. citrifolium* and *E. pauferrense* have already been recorded in other species of *Erythroxylum* by Amaral Jr. (1980) in both morphs of the *E. coca* and *E. novogranatense* (Ganders, 1979). The species exhibited different patterns related to the distylous syndrome, i.e., the presence of RH, isoplethy, and/or heteromorphic incompatibility system (Ganders, 1979; Pailler and Thompson, 1997; Sá et al., 2016). It is generally accepted that if the species lack one of those characteristics, it should be classified as atypically distylous (Hamilton, 1990). Our results indicate that only *E. citrifolium* is typical distylous since it exhibited those three characteristics when considering the shorter anthers in the pin morph (taller anthers of pin morph did not exhibit reciprocity with thrum stigma). Typical distyly constitutes a primitive character in the genus *Erythroxylum* (Payens, 1958; Robson, 1963), but atypical conditions were already reported (Barros, 1998). Matias et al. (2020), for example, also observed variations from the distylous pattern in *Erithroxylum* species of the Cerrado: in a general matter, a 1:1 morph ratio was observed in self-incompatible species. Conversely, populations of self-compatible species, such as *E. campestre,* showed an excess of pin or thrum individuals or were thrum-monomorphic. Thrum flowers of *E. havanense* had low pollen viability, being considered as a gynodioecious species.

In general, the values of adaptive inaccuracy observed here were much higher when compared to other species of *Erythroxylum* (10 and 43%, Matias et al., 2020) and in other heterostylous groups (e.g., Armbruster et al., 2017; Jacquemyn et al., 2018), indicating the absence of reciprocity. The only exception was the shorter anthers of pin flowers and low stigmas of thrum flowers of *E. citrifolium*, which showed the lowest inaccuracy values (i.e., the highest degree of RH). Curiously, it was the only species whose fruit set of thrum flowers were two times that observed in pin flowers, which may indicate a higher efficiency in pollen transfer between low-level organs when compared to the high-level ones. Also, this efficiency may explain the higher fruit set of thrum morph (56%) when compared to the pin morph (24%). Matias et al. (2020) also recorded the association between low inaccuracy values and high fruit set after natural pollination in *E. campestre*, *E. deciduum*, *E. suberosum*, *E. tortuosum* in the Cerrado of central Brazil. *Erythroxylum deciduum* and *E. suberosum* are included in the same section of the species studied here (Plowman and Hensold, 2004). The association between low inaccuracy values and high fruit set after natural pollination was also observed in *Pulmonaria* (Boraginaceae, Jacquemyn et al., 2018). The greater reciprocity of the lower organs was also reported in Primulaceae (Armbruster et al., 2017; Jacquemyn et al., 2018; Li et al., 2018).

The high P:O ratio observed in all species indicates facultatively xenogamy, i.e., they are usually self-compatible but xenogamic crosses through morphological and/or physiological adaptation occur, with self-pollination restricted to the absence or in addition to pollination (Cruden, 1977; Barros, 1998). The high P:O was favored by the presence of a single ovule and a high number of pollen grains (Cruden, 1977; Barros, 1998).

The self-compatibility observed in *E. pauferrense* and *E. simonis* was also reported in other *Erythroxylum* species (Ganders, 1979; Barros, 1998; Pailler and Thompson, 1997; Silva et al., 2007) and in some distylous groups of Boraginaceae and Rubiaceae (Faria et al., 2012; Ferrero et al., 2017). Self-compatibility in distylous taxa indicates a failure in distyly functioning (Matias et al., 2020), and maybe associated both with the lack of reciprocal hercogamy and inefficient or insufficient pollination services (Armbruster et al., 2009). Since there were no differences in the number of visits among plant species, is possible that the pollinators' behavior combined with HR is exerting selective pressure influencing the evolution of atypical

distyly in those species (Arroyo et al., 2002; Barrett and Hodgins, 2006). Pollinators are considered important agents for the maintenance of polymorphic species because they promote the flow of disassortative pollen (Armbruster et al., 2006; Armbruster et al., 2009). The higher visitation rates observed in *E. citrifolium*, for example, did not result in higher fruit set after natural pollination when compared to the other two species, reinforcing the idea that floral morphology, breeding system, and pollinators play an integrative influence on fruit set. On the other hand, the high number of visits within a single inflorescence by pollinators may impact differently the three species, since *E. citrifolium* is self-incompatible and a high degree of RH between pin anthers and thrum stigmas, and the other two are self-compatible and RH is almost absent. Therefore, further studies are needed to evaluate the pollen transfer rate by pollinators in those species.

It is interesting to note that, even *E. pauferrense and E simonis* being capable of self-pollinating during the bud phase, fruit set after spontaneous self-pollination was relatively low when compared to natural and intermorph pollinations. Thus, apparently, this self-pollen deposition does not cause stigma clogging, allowing pollen grains deposited by pollinators to germinate.

The lower value of interspecific inaccuracy observed for the shorter anthers of pin flowers of *E. citrifolium* with the thrum stigmas of *E. pauferrense* is similar to the values of the intraspecific inaccuracy of *E. citrifolium*, indicating that the chances of this interspecific pollination (i.e., from *E. citrifolium* anthers to *E. pauferrense* stigma) are similar to intraspecific (for *E. citrifolium*) pollination. Considering that *E. citrifolium* and *E. pauferrense* grow closely intermixed, overlap their flowering periods, and share pollinators, interspecific pollination may occur (Thomson, 1982; Chittka et al., 1997; Seifan et al., 2014). However, interspecific pollinations set no fruits. On the other hand, although manual interspecific pollinations among *E. pauferrense* and *E. simonis* set fruits, the high inaccuracy values indicate low chances of natural interspecific pollination. Moreover, it is not possible to state if the resulting hybrid seeds germinate, grow, and reproduce satisfactorily. It is important to note that, if the hybrids grow, their flower morphometrics may be different from the non-hybrid individuals. Therefore, it is also not possible to state if our data on floral morphometrics of *E. pauferrense* and *E. simonis* was collected from hybrids or not. These issues can be elucidated by studies including production of plants and genetics.

5. Conclusions

A low level of both intra and interspecific RH was revealed for all species, except for the low level organs of *E. citrifolium*. The high levels of reciprocal herkogamy observed in *E. citrifolium* were associated with a high fruit set, similarly to what was observed in other distylous species. The floral morphometric analysis also showed that the chances of intraspecific pollination are higher when compared to interspecific ones, except for the pollination between pin flowers of *E. citrifolium* and thrum flowers of *E. pauferrense*. However, the manual interspecific pollination between these two species did not form fruits, conversely to what was observed between *E. pauferrense* and *E. simonis*, whose all interspecific intermorph pollinations set fruits. The atypical distyly observed in *E. pauferrense* and *E simonis* indicates a collapse in the distylous system and may be related to a relaxation in the interspecific incompatibility mechanism. More field, experimental and molecular studies are needed to understand the reproductive relationships between the species studied, as well as whether these relationships influence the local population dynamics of these species.

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