

Pollinator dependency determines the susceptibility to habitat fragmentation of *Mucuna mutisiana* (Kunth) D.C. Fabaceae in the Tropical Dry Forest of the Colombian Caribbean

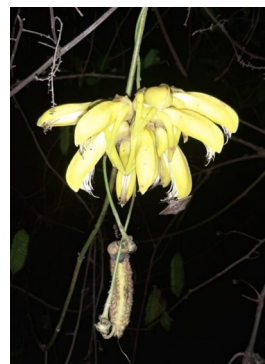
Dependencia de los polinizadores como determinante de la susceptibilidad a la fragmentación del hábitat de *Mucuna mutisiana* (Kunth) D.C. Fabaceae en el bosque seco tropical del Caribe colombiano

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Abstract

Plant responses to habitat fragmentation are determined by their reproductive system and the level of specialization in their pollination system. One way to understand possible responses

is through reproductive success, degree of self-compatibility, and susceptibility to pollination. *Mucuna mutisiana*, a liana typical of secondary forests and associated with the banks of rivers in the Tropical Dry Forests (BsT) of the Colombian Caribbean, flowers throughout the year, although it is more frequent in the rainy season, most visits to its flowers are caused by nectarivores bats, mainly *Glossophaga soricina* and *Glossophaga longirostris*. We describe that the specialization of pollination increases this susceptibility to fragmentation due to the negative effect it has on the reproductive success of plants by limiting the quantity or quality of the pollen they receive. Using controlled pollination methods, the reproductive susceptibility to pollen limitation was evaluated through the reproductive success of *Mucuna mutisiana*. We performed a consistent artificial pollination factorial experiment with different levels of pollen limitation that allowed us to estimate the production of fruits and seeds. We report that *M. mutisiana* is partially self-compatible, with an ISI = 0.776 and reproductive success of 34.04% when visited by its effective pollinators. An analysis of variance showed that there is differentiation in fruit production between the different levels of controlled pollination, being higher in natural pollination and pollination with own pollen, unlike cross pollination where the results were not significant. In addition, no differences were found between seed production by natural pollination and by self-pollination, with a higher seed production being observed in cross-pollination. This confirms that *M. mutisiana* has an affinity for its own pollen

depending on its pollinators, it is evident that they are facultative xenogamous with mixed mating, which gives them reproductive security with advantages for the fitness of their populations. This is a particularly clear focus for developing native habitat restoration and conservation strategies

Keywords: breeding system, habitat fragmentation, self-compatible, self-incompatible, pollen limitation.

Resumen

Las respuestas de las plantas a la fragmentación del hábitat vienen determinadas por su sistema reproductivo y el nivel de especialización de su sistema de polinización. Una forma de entender las posibles respuestas es a través del éxito reproductivo, el grado de autocompatibilidad y la susceptibilidad a la polinización. *Mucuna mutisiana*, una liana típica de bosques secundarios y asociada a las riberas de los ríos en los Bosques Secos Tropicales (BsT) del Caribe Colombiano, florece durante todo el año, aunque más frecuentemente en la época lluviosa; la mayoría de las visitas a sus flores son causadas por murciélagos nectarívoros, principalmente *Glossophaga soricina* y *Glossophaga longirostris*. Describimos que la especialización de la polinización aumenta esta susceptibilidad a la fragmentación debido al efecto negativo que tiene sobre el éxito reproductivo de las plantas al limitar la cantidad o calidad del polen que reciben. Utilizando métodos de polinización controlada, se evaluó la susceptibilidad reproductiva a la limitación de polen a través del éxito reproductivo de *Mucuna mutisiana*.

Realizamos un experimento factorial de polinización artificial consistente con diferentes niveles de limitación de polen que nos permitió estimar la producción de frutos y semillas. Reportamos que *M. mutisiana* es parcialmente autocompatible, con un ISI = 0,776 y un éxito reproductivo de 34,04% cuando es visitada por sus polinizadores efectivos. Un análisis de varianza mostró que existe diferenciación en la producción de frutos entre los distintos niveles de polinización controlada, siendo mayor en polinización natural y polinización con polen propio, a diferencia de la polinización cruzada, donde los resultados no fueron significativos. Además, no se encontraron diferencias entre la producción de semillas por polinización natural y por autopolinización, observándose una mayor producción de semillas en la polinización cruzada. Esto confirma que *M. mutisiana* tiene afinidad por su propio polen, dependiendo de sus polinizadores. Es evidente que son xenógamas facultativas con apareamiento mixto, lo que les da seguridad reproductiva con ventajas para la aptitud de sus poblaciones. Se trata de un objetivo especialmente claro para desarrollar estrategias de restauración y conservación de hábitats autóctonos.

Palabras clave: sistema de reproducción, fragmentación del hábitat, autocompatibilidad, autoincompatibilidad, limitación del polen.

Introduction

Pollinators as pollen vectors allow reproduction in angiosperms (Ashman

et al., 2004; Knight et al., 2005). Ensuring effective reproduction requires biological attributes that allow them to reproduce effectively, which could determine differential ecological responses to the effects of habitat fragmentation (Da Silva Elias et al., 2012; Dainese et al., 2019). Certain adaptations of the reproductive biology of plants could be associated with the degree of dependence and/or specialization of their pollinators (Faegri & Pijl, 1979; Johnson & Steiner, 2000; Willmer, 2011). This could explain the susceptibility that these species may have at the reproductive level (Aizen et al., 2002). Other observations suggest that plants can ensure sexual reproduction by producing seeds through autonomous self-fertilization (Hokche & Ramírez, 2016). Consequently, the reproductive system is considered an important trait to evaluate the degree of dependence of the mutualistic relationship with its pollinators, its possible consequences on the reproductive success of plants and the possible responses to the effects of habitat loss (Aizen & Feinsinger, 1994; Wilcock & Neiland, 2002).

The species are usually obligate outbreeders that depend on pollinators for their sexual reproduction, since they use cross-pollination with pollen from other individuals for seed production, which could result in a high dependence on their pollinators. Authors have suggested that some species of the genus *Bauhinia* a Caesalpinioideae pollinated by bats, are self-incompatible, so their pollinators ensure the formation of fruits and seeds as crossings increase (Hokche & Ramírez, 2016). The tropical tree species of the genus *Inga* with

massive flowering typical of the Montane Cloud Forests of Costa Rica are mostly self-incompatible and have evolved in response to excessive geitonogamy (Barros et al., 2013; Koptur, 2022). Alternatively, species with the possibility of forming fruits by self-pollination could be favoring their reproductive success in the absence of pollinators (Ashman et al., 2004; Aguilar et al., 2006). The authors have documented that *Sophora fernandeziana*, *Crotalaria retusa* and *Crotalaria micans* are self-compatible legumes, although they do not reproduce by agamospermy or spontaneous self-pollination, indicating that these species depend on pollinators for fruit formation (Brito et al., 2010).

It is currently inconclusive to generalize about the reproductive susceptibility of plants to habitat loss as a function of compatibility with pollination systems (Aizen & Feinsinger, 1994; Aguilar et al., 2006). However, the relationship between the pollination system and the reproductive performance of plants can be estimated (Johnson et al., 2003; Knight et al., 2005; Diego et al., 2019). Through the proportion of fruits and seeds in simulations of the absence or presence of the pollinator according to each pollination syndrome, which allows estimating whether the plant is self-compatible or self-incompatible (Saborío & De la Costa, 1992). It would be expected that, in self-incompatible plant species, self-pollination produces a self-incompatibility reaction that blocks the possibility of fruit and seed production (Aguilar et al., 2006). In contrast, self-compatible plants depend on animal pollinators to transport

pollen through cross-pollination for fruit formation. Authors suggest that the flowers of the species that are pollinated by bats receive more visits due to cross-pollination, which allows a greater production of fruits, however, the possibility of forming fruits by self-pollination (geitonogamy) through agents to promote reproductive success (Hokche & Ramírez, 2016).

In this study we focused on evaluating the compatibility system of *Mucuna mutisiana* to understand the degree of specialization and/or dependence of mutualism on pollination through reproductive effectiveness and how their reproductive success might be affected by habitat loss. The reproductive biology of the oxeye *Mucuna mutisiana* in the tropical dry forest (TDF) of the Colombian Caribbean allowed us to explore how the reproductive system can explain the specialization of the chiropterophily syndrome that these species present. Few studies have focused on the study of the reproductive system of the species of this genus. Through a factorial design that allowed us to evaluate the susceptibility of reproductive success and the possible dependence of its pollinators to understand the compatibility system of *M. mutisiana*. We consider the following hypotheses: (1) *M. mutisiana* can be self-compatible (2) Pollinators are necessary to produce fruits and seeds (3) Pollination of *M. mutisiana* is susceptible to processes of habitat loss and fragmentation. We expect this species to be self-compatible, with a degree of reliance on pollinators to produce fruit and seeds that provide fitness benefits to the plant.

Materials and Methods

Study area

The Caribbean Region of Colombia has the largest coverage of tropical dry forest (Minambiente-IAvH 2014). Its plant

formation is frequently distributed in gallery forests, primary forests and forests disturbed by habitat loss and transformation (IDEAM, 2013). We conducted this study in the northern region of Colombia, where BD-T is considered one of the most threatened ecosystems in the world. For Colombia

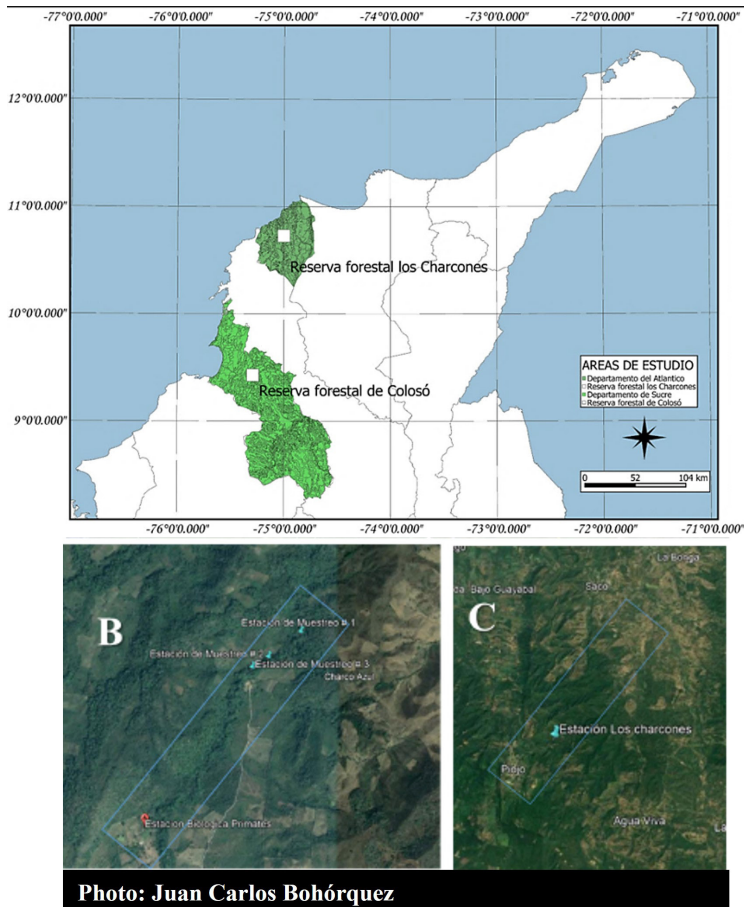


Fig. 1. Maps of the study sites: (A) Caribbean region of Colombia, (B and C) map of the natural reserves used as study sites in the Caribbean region of Colombia. Sites located in two geographical areas in the Serranía de la Coraza–Montes de María in the department of Sucre and in the Serranía de Piojó in the department of Atlántico. Within each region, different sampling points were chosen to evaluate the reproductive system of *Mucuna mutisiana*.

in 2010 it was estimated that there was a remnant of 735,514 ha, distributed mostly in the Caribbean plain. According to IGAC reports, in the Atlantic, 31.6% of the territory is destined for agroforestry (non-arable) and conservation. *Mucuna mutisiana* occupies a restricted area in habitats that are not considered fragmented (Moura et al., 2018). We selected two places in the region with similar landscape features located along water sources or streams. Specifically, the Primate Protective Forest Reserve in the Serranía de Coraza - Montes de María, in Colosó (9° 30' north latitude and 75° 21' west longitude). This area has an extension of 6,730 hectares with a reserve category through Executive Resolution No. 204 of October 24, 1984. Charcones Nature Reserve located in the department of Atlántico (10° 75' north latitude and 75° 09' west longitude) in the Serranía de Piojó, which has 42.98 hectares, Resolution 031 of March 6, 2018 (Fig.1). The climate in this area is typically tropical, influenced by the topography and the action of northeasterly winds, which vary in temperature, relative humidity, and precipitation (Cuervo, A; et al; 1986). It has an average monthly temperature of 27.45°C, a relative humidity of 77% (IDEAM, 2013).

Reproductive system and effective pollination of *M. mutisiana*

We performed five hand pollination treatments to determine the reproductive system of *M. mutisiana*, and whether there is a dependency on pollinators to produce fruits and seeds. The inflorescences were bagged before the opening of the flowers to avoid natural pollination. We applied five pollination treatments to 124 and 138 flowers in four sampling stations. Subsequently, the selected flowers received the following treatments: (1) Emasculation of flower buds: the buds were bagged before the floral opening; (2) Flowers that were bagged avoiding natural pollination; (3) Manual self-pollination induced with own pollen; (4) Manual cross-pollination with pollen from other plants; (5) Natural pollination (Fig. 2). These treatments were carried out at three times during the night: 6:00 pm, 10:00 pm and 12:00 am, taking flowers with receptive stigma and viable pollen in mature flowers. The proportion of fruits produced per flower was measured in each treatment in which reproductive success was then measured through the self-incompatibility indices (ISI) and the reproductive success index (RRS) (Hokche & Ramírez, 2016).

$$ISI = \frac{\# \text{ average number of fruits produced per flower by manual self - pollination}}{\# \text{ average number of fruits produced per flower by manual cross - pollination}}$$

$$RRS = \frac{\# \text{ fruits produced}}{\# \text{ flowers produced}} * \frac{\# \text{ seeds produced}}{\# \text{ ovules produced}}$$

In plants, the compatibility system with values equal to one indicates that it is self-compatible, greater than zero and less than one the self-compatibility is partial, finally when it is equal to zero it is total self-incompatibility (Zapata & Arroyo, 1978; Hokche & Ramírez, 2016). Subsequently, reproductive efficiency was evaluated by comparing the proportion of fruits by cross fertilization with respect to the proportion of fruits under natural conditions (Khorsand & Awolaja, 2020). Therefore, it allowed to directly infer whether *M. mutisiana* is self-compatible or incompatible through the effect of the production of fruits per flower in the different reproductive success treatments (Hokche & Ramírez, 2016).

Analysis of results

To validate our hypotheses about the susceptibility of reproductive success with the possible dependence on their pollinators. The reproductive system of *M. mutisiana* was determined through a program of controlled crossings in flowers isolated from natural pollination activity. The number of fruits and seeds produced by the pollination treatments carried out was quantified, the data obtained in the experiment showed a normal distribution according to the Shapiro-Wilks test, for the homogeneity of the variance the Bartlett test was carried out. We performed a one-way ANOVA to test the effects of treatment on fruit set. Tukey's HSD with a Bonferroni correction was used to determine meaningful pairwise comparisons. All analyzes were performed using the Rstudio base package (R Core Team 2018; auto package v. 3.0-2, following

Roxaneh S. Khorsand, Olufisayo Awolaja 2020).

Discussion

Mucuna mutisiana includes lianas with glabrous, welded staminal filaments 3.5–5.0 cm long; the gynoecium is between 4.5 and 6.0 cm long; style 3.5–5.0 cm long, hairy except at apex; ovary 5.0–10 × 2.0 mm, sericeous (Moura et al., 2018). We observed flowering at the sampling points between the months of October and November with an approximate duration of 1.4 months, each individual flower lasting less than a week if it is not visited by a potential pollinator. The flowers treated during the pollination experiments showed senescence between 3 and 5 days when the flowers were observed with withered petals and no pollen.

Experimental tests of controlled crosses on 262 flowers isolated from natural pollination activity showed that many selected flowers aborted during the experiment. It is possible that this event could have occurred due to internal mechanisms of the *Mucuna* flowers, given that the results of greater reproductive success are related to pollination treatments with pollen from flowers of the same individual, as well as natural pollination when legitimate pollinators act. Experimental results indicate that this species is self-compatible, although they usually depend on the bats *Glossophaga longirostris* and *Glossophaga soricina* as floral visitors (Rebolledo Contreras, 2021). Natural pollination produced a higher percentage of fruits per flower, with a reproductive efficiency of 34.04% compared

to self-pollination with flower pollen from the same individual, where a reproductive efficiency of 26.97% was obtained. Fruit set differed significantly between treatments (ANOVA; $F_{9.643}$: 0.00643, $P < 0.01$) in relation to the number of seeds produced per fruit (ANOVA; $F_{1.104}$: 0.30813, $P < 0.01$). Fruit production was from highest to lowest: (1) emasculated flower treatment (mean 0.5, Sd

± 0.0 , n: 124), (2) self-pollination treatment without manipulation (mean 0.5, Sd ± 0.0 , n: 135), (3) cross-pollination (mean 1.50, Sd ± 1.29 , n: 132), (4) pollination treatment with own pollen (mean 10.75, Sd ± 4.57 , n: 138), (5) natural pollination (mean 12.00, SD ± 4.32 , n: 127) (Fig. 2).

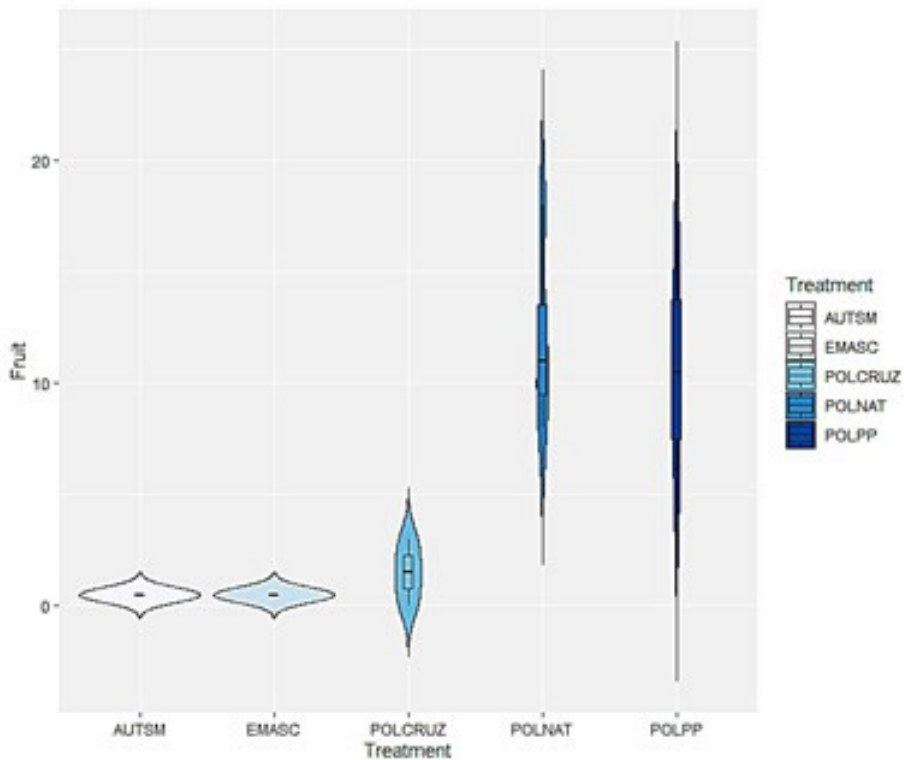


Fig. 2. Mean fruit set given by the proportion of fruits per flowers per treatment. Treatments include (1) emasculacion of flower buds; (2) Self-pollination without manipulation (Autogamy); (3) Manual self-pollination (Facilitated Autogamy) and (4) Manual cross-pollination with pollen from other plants (xenogamy); (5) Pollination natural in flowers exposed to pollen vectors. significant ($P < 0.05$).

The set of pairwise comparisons between the means for each level of pollination treatments ($P < 0.05$) showed that there are differences between the levels of pollination treatment. The mean values of fruit set are significantly higher in natural pollination and pollination with own pollen than the mean values of self-pollination without manipulation and by emasculation. Although the cross-pollination treatment

was expected to yield high mean values, the results were not significant (Fig. 3). The proportion of seeds per ovule produced by natural pollination did not differ from that produced by self-pollination, while the proportion of seeds per ovule produced by cross-pollination was significantly lower than that produced by self-pollination; These results confirm that *Mucuna mutisiana* appears to be a species that has an affinity for its own pollen.

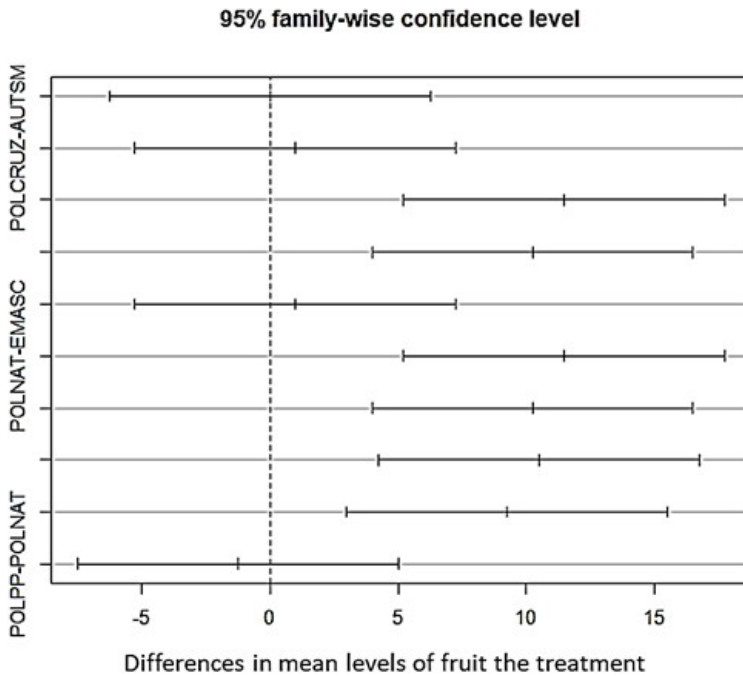


Fig. 3. Multiple comparisons for means that are significantly different between pollination treatments ($P < 0.05$). Self-pollination without manipulation (AUTSM), emasculated flowers (EMASC), pollination with own pollen (POLPP), natural pollination (POLNAT), cross-pollination (POLCRUZA).

Discussion

The specialization of pollination through the reproductive system of *M. mutisiana* was studied, as well as the degree of dependence of its mutualistic relationship with its pollinators. Studies suggest that plant responses to habitat fragmentation are determined by their reproductive system, which in turn is related to the level of specialization in their pollination system (Aizen & Feinsinger, 1994; Ashman et al., 2004). The results of the analysis show that the flowers of the genus *Mucuna* are hermaphrodite and predominantly geitonogamic, confirming that *M. mutisiana* presents functional self-fertilization in chiropteran pollination systems when visited by its legitimate pollinators. Studies on some species indicate that both self-compatibility and self-incompatibility are present in *Mucuna* (Agostini, 2004; Kobayashi et al., 2019).

The results indicate that *Mucuna mutisiana* is a self-compatible species, with an ISI = 7.75% and a reproductive success of 34.04% when *G. soricina* and *G. longirostris* visit the flowers (Rebolledo Contreras, 2021). Studies on *Mucuna macrocarpa* confirm that they do not form fruits in pollination treatments by emasculation or by spontaneous self-pollination for experiments carried out in different locations in Kyushu, Japan (Kobayashi et al., 2018). Their results were like this study, in cross-pollination, manual self-pollination, and natural pollination experiments. The rate of proportion of fruits per flower and per inflorescence were significantly higher in the cross-pollination

treatments than in the spontaneous self-pollination treatments (Kobayashi et al., 2018). Confirming that fruiting only occurs when effective pollinators visit and open the flowers, it is not reported whether the compatibility system confirms that *M. macrocarpa* depends on the mutualistic relationship by its pollinators *C. caniceps* and *C. finlaysonii* to achieve reproductive success (Kobayashi et al., 2018). It seems that the absence of fruits due to spontaneous self-pollination is probably related to the non-rupture of the stigmatic cuticle, like what happens with *C. juncea* (Roberts, 1971), indicating that these species depend on pollinators for the formation of fruits. As seems to happen in *M. mutisiana* whose own pollen deposition on the stigma, which occurs only when visiting bats succeed in pressing their snouts into the cleft on the wing petals causing the keel to burst and the column of stamens under it to burst. pressure releases the pollen (Rebolledo Contreras, 2021).

Facultative xenogamous plant species are usually self-compatible with adaptations for cross-pollination, considered as a mixed mating system where the level of activity of pollinators could be determining the balance between self-pollination and cross-pollination (Cruden, 1979). Here we consider that *M. mutisiana* is facultatively xenogamous because it is self-compatibility is partial with flowers adapted for cross-pollination, where the behavior of the pollinators, the functional traits of the flowers, as well as the size of the inflorescence affect self-fertilization, mainly resulting in geitonogamy. Empirical

evidence suggests that many plants have evolved this mixed mating as a stable evolutionary strategy that confers fitness benefits and provides reproductive security if pollinators are limited (Cruden, 1979; Gallardo et al., 1994; Etcheverry et al., 2011; Khorsand & Awolaja, 2020).

In the experiments, the natural pollination treatment exhibited significantly higher fruit formation than the xenogamy pollination treatment (RRS: 34.04% vs. 26.97%, respectively), suggesting that our *M. mutisiana* populations are not limited by quality of pollen. However, the degree of dependence on pollinators for the release and transfer of pollen from anthers to stigmas is evident. Therefore, the susceptibility of pollination of *M. mutisiana* by habitat fragmentation processes is favored given the dependence on biotic agents for this process. Other experimental studies on the reproductive biology and floral visitor interactions of insect-pollinated *Sophora tomentosa* and *Crotalaria vitellina* (Fabaceae) are also self-compatible, with fruit and seed production dependent on their pollinators (Brito et al., 2010). In contrast, our study showed a lower production of fruits by cross-pollination compared to natural pollination and pollination with own pollen. A possible explanation is that it could be due to the technique, some authors describe that it is difficult to reach the stigma of papilionaceous flowers because they are protected by a fringe of erect hairs and some stamens are not completely exposed (Khorsand & Awolaja, 2020).

Alternatively, it is possible that the low production of fruits by cross-pollination is due to the availability in the stigmatic receptivity (Cruden, 1979), however, when making the comparison between the production of fruits of the treatments by manual self-pollination and natural pollination we found higher values. Authors suggest that changes in floral traits associated with the reproductive system from xenogamy (crossbreeding) to autogamy (selfing) are possibly associated with flower size (Diego et al., 2019), which influences the energy cost per flower (Breed et al., 2012). Empirical studies have shown that self-incompatible and xenogamous species produce more pollen grains than self-compatible and/or closely related autogamous species (Fernández et al., 2009). We consider that *M. mutisiana* has a mixed reproductive system in which case fruits are formed by natural pollination and by self-pollination depending on a pollinating agent. The count of pollen tubes in 62 flowers shows that the proportion of flowers that developed complete pollen tubes was 0.79, higher than the proportion of flowers with pollen tubes reaching the base of the style with 0.22, the number of pollen tubes per flower varied between 1 to 7 tubes. Unlike other floral characteristics, the viability in obtaining optimal pollen for the development of pollen tubes and the subsequent formation of seeds in the fruits would be determined by the number of ovules per ovary, which varies depending on the species under study (Mione & Anderson, 2022). In the grass known as Christmas bells, *Blandfordia grandiflora* (Liliaceae), in addition to being self-compatible under

natural conditions, autogamous pollen preempts the ovules, making them inaccessible for cross fertilization (Ramsey et al., 2003). Self-compatible species may not necessarily produce higher fruit production when successfully crossed given the amount of pollen produced (Zapata & Arroyo, 1978; Cruden, 1979; Gibbs et al., 1999). However, it is possible that in many self-compatible species, some degree of interbreeding occurs through dichogamy (Bawa, 1974). Although a deeper study is suggested for this topic, the formation of the pollen tubes that reach the ovary, the production, and characteristics of the fruit, after self-pollination or cross-pollination, could be evaluated in populations with self-compatible heterozygous genotypes in contrast to homozygous genotypes. self-compatible to know how the effect of consanguinity manifests itself (Dicenta et al., 2002).

In general, knowing the reproductive system of *M. mutisiana* in the regions of the tropical dry forest in the Colombian Caribbean suggests that, in the face of a mismatch in the pollination system of this species, fruiting would be affected given the close relationship for pollination by nectivorous bats (Faegri & Pijl, 1979; Moura et al., 2018; Rebolledo Contreras, 2021). Along with self-compatibility, it is possible that imbalances caused by changes in climatic conditions or habitat loss and fragmentation in local areas would imply a decrease in pollen deposited on the stigma of flowers, reducing reproductive success (Ashman et al., 2004; Porcher & Lande, 2005; Eckert et al., 2010). This pollen limitation would be seen mainly in late reproductive stages, mainly in the production of flowers of the progeny (Husband & Schemske, 1996; Aguilar et al., 2019). Authors suggest that species occurring in early successional habitats tend to be self-compatible and/or autogamous (Cruden, 1979). Different from those plants that are usually established in habitats with later successions, including tropical forest trees that are usually xenogamous (Zapata & Arroyo, 1978). Thus, the variability of the reproductive system of plants probably has a direct effect on the population dynamics of forests (Ashman et al., 2004; Knight et al., 2005; Bennett et al., 2018).

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