



Reproductive ecology of the prickly pear *Opuntia atropes*, a native plant subject to management

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ABSTRACT

Species of *Opuntia* (prickly pears), widely propagated for human use, are present in many agroforestry systems. Like other perennials, they are subject to environmental pressures that influence their reproductive ecology and reproductive output. Here, we studied floral biology, breeding system, and flower-bee interactions of *Opuntia atropes*, which is subject to management, to better understand its reproductive ecology. We documented floral phenology, morphology, and mating system by observing live flowers, measuring fixed flowers, and performing pollination treatments. We recorded frequency, behavior, and floral visitors' handling time to determine the most effective pollinator. *Opuntia atropes* has large, herkogamous flowers and high P/O ratios. These traits promote outcrossing, which was supported by the results of pollination treatments. Fruit weight and seed set exhibited moderate pollen limitation. Floral visitors comprised nine bee species. *Diadasia* sp. was the most effective pollinator according to frequency and behavior. *Opuntia atropes* shows preferential outcrossing through pollination by a specialist bee despite being in a modified habitat. Pollen limitation and variation in reproductive success may lead to mating system evolution in *O. atropes*. The observed bee diversity suggests that *O. atropes* is an important source of floral resources in the dry season in semi-arid environments under moderate human disturbance.

Keywords: *Opuntia*, native bees, effective pollinator, bee diversity, floral biology, cactus, tropical dry forest.

Introduction

In the face of global pollinator decline (Potts *et al.* 2010), maintaining native flora is a key factor for safeguarding the diversity of pollinators in natural and semi-natural habitats. Native flora provides high-quality resources for pollinators; in addition to assuring pollinator diversity,

this also maintains the interactions between plants and their pollinators (Carman & Jenkins 2016). An important plant element in the drylands of North America is the genus *Opuntia*—the prickly pears. Species of *Opuntia* may grow in several types of vegetation, with varying degrees of dominance. Vegetation that is dominated by one or several species of *Opuntia* is commonly called *nopalera* (Rzedowski

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1978; Rebman & Pinkava 2001). *Nopaleras* provide food and habitat resources for vertebrates, such as rodents, birds, and other mammals (González-Espinosa & Quintana-Ascencio 1986). Although less documented, *nopaleras* also harbor high insect diversity. For example, prickly pear crops have been shown to have bee and ant diversity that is on par with natural vegetation (Gómez-Otamendi *et al.* 2018; Ávila-Gómez *et al.* 2019), suggesting that *Opuntia* plants provide high quality resources such as food and habitat.

Several species of the genus *Opuntia* also represent an important food resource for humans, since their mature fruits and their shoots, called cladodes, are edible (Pimienta-Barrios 1990; Fernández-Montes *et al.* 2000). *Opuntia* species are also used as fodder for livestock and as living fences. Due to these multiple uses of *Opuntia*, the species of this genus have been subject to management for centuries (Bravo-Hollis 1978), and some species are completely domesticated. The most important domesticated species is *O. ficus-indica*, cultivated in several countries worldwide (Griffith 2004; Reyes-Agüero *et al.* 2005b). Other species have been subject to a less intense selection since plants are tolerated within agroforestry systems rather than actively cultivated, and they are only moderately selected for desirable traits, such as fleshy cladodes or lower spine density (Reyes-Agüero *et al.* 2005a). For instance, the species *O. atropes* is endemic to Mexico and is at an incipient stage of domestication, as evidenced by differences in vegetative traits between natural and managed *nopaleras* (López-Gutiérrez *et al.* 2015). In the tropical dry forest of Morelos state in central Mexico, *O. atropes* is used as a food resource by humans, as the young cladodes are highly appreciated (Maldonado-Almanza 2013). In this area, *O. atropes* is tolerated within agroforestry systems, and *nopaleras* are frequently found at the intersection of native tropical dry forests and lands that have been transformed for agriculture and livestock uses (Arias-Medellín *et al.* 2014).

Like many clonally propagated plants, prickly pears are perennial, and hence, they are expected to have preferential outcrossing or even completely self-incompatible mating systems (Barrett *et al.* 1996; McKey *et al.* 2010). Because of the disadvantages of clonality (*e.g.*, high inbreeding depression and mate limitation), many clonal domesticated plants still retain sexual recruitment (McKey *et al.* 2010). Furthermore, repeated outcrossing with their wild counterparts (a frequent phenomenon in *Opuntia*) precludes the fixation of sterility genes (McKey *et al.* 2010). Although outcrossing mating systems are expected in *Opuntia*, there have been documented several *Opuntia* species with mixed mating systems (Mandujano *et al.* 2010), as well as species with variation in their mating systems across populations (Ortiz-Martínez *et al.* 2022). This diversity of mating systems in prickly pears could have evolved in response to environmental pressures such as pollen limitation, resource limitation, and/or the frequency of clonal propagation. High rates of clonal propagation could reduce effective mate availability because many plants are the same genetic

individual, leaving few plants with which to outcross; this makes strict outcrossing a less advantageous strategy compared to mixed systems in which selfing also leads to some degree of successful reproduction (Mandujano *et al.* 2010). In other words, in highly clonal plants, mixed mating systems could be advantageous because geitonogamy (selfing) ensures some reproductive success, while outcrossing promotes recombination (Mandujano *et al.* 2010). Phenomena such as pollen limitation may also influence the evolution of plants' sexual expression by favoring the evolution of floral traits that allow self-pollination (Ashman *et al.* 2004). Pollen limitation has several causes, the most common of which includes invasive plants that interfere with pollination networks, habitat fragmentation, low plant population sizes, and loss of native pollinators (Ashman *et al.* 2004). These factors are common in disturbed habitats, and hence may ultimately affect plant reproductive systems in those habitats.

Pollination ecology includes such crucial topics as the interactions of the flowers with their visitors and the degree to which plants depend on their pollinators for successful reproduction. *Opuntia* flowers are diurnal, short-lived, brilliant in color, radially symmetric, and bowl-shaped (Reyes-Agüero *et al.* 2006; Mandujano *et al.* 2010). These flowers are mainly bee-pollinated, and their rewards are nectar and pollen. *Opuntia* flowers are visited by a variety of bee species (mean \pm s.d. = 9.4 ± 10.2 ; data from Tenorio-Escandón *et al.* 2022), but bee species differ in their effectiveness as pollinators (De Jesús-Romero 2021), and some of them may even act as nectar or pollen robbers (Cota-Sánchez *et al.* 2013).

Although there have been previous studies of the reproductive biology of *Opuntia* (Reyes-Agüero *et al.* 2006; Mandujano *et al.* 2010), detailed studies of flower-bee interactions would contribute a better understanding of the environmental pressures that may shape successful sexual reproduction and the degree of specialization of these plant species to their pollinators (Tenorio-Escandón *et al.* 2022). Furthermore, the reproductive system of *Opuntia atropes* is particularly interesting given that it is a used species subject to management. Specifically, in this study, we sought to determine the mating system of this species and whether their flowers are generalists or specialists in terms of pollination. These data permitted us to discuss the pollination ecology of *O. atropes* as a native, managed plant in an agroforestry system and reflect on its role in conserving native bee diversity.

Materials and methods

Study sites

We conducted this study in three localities of Morelos state within the municipality of Tlaquiltenango: Valle de Vazquez (18°31'44.66"N, 99°3'51.05"W), Quilamula (18°29'38.94"N,



99° 0'19.38''W), and San Jose de Pala (18°33'3.71'' N, 99° 0'57.23'' W) (Fig. S1). Tlaquiltenango has a warm, subhumid climate with summer rains and a mean annual temperature of 22–24 °C. Annual precipitation varies between 800 and 1,000 mm. The dominant vegetation in Tlaquiltenango is tropical dry forest (46.7%), followed by agricultural lands (31.5%), oak forest (11.3%), pasturelands (9.6%), and urban zones (0.8%) (INEGI 2010). Livestock grazing and agriculture are the predominant economic activities in the area, which have resulted in some degree of disturbance in all three of these localities. The main locality of the study was the village of Valle de Vázquez, where we conducted most of the measurements in a *nopalera* of *O. atropes* located along the border between the village and the surrounding tropical dry forest in 2019. The other two localities, Quilamula and San José de Pala, were included to conduct pollination experiments during 2020. In these two latter localities, we worked in *nopaleras* along the roadsides. In all three *nopaleras*, we worked with plants of moderate height (between 1–2 m) on which flowers could be manipulated.

Study species

Opuntia atropes Rose is a Mexican endemic species distributed in the West-Central states of Morelos, Mexico, Michoacán, and Guerrero (Britton & Rose 1908; Bravo-Hollis 1978). It inhabits mainly tropical dry forests, pine-oak forests, and xerophytic scrubland (Bravo-Hollis 1978). It has a highly ramified scrub-like life form that reaches up to 3 m in height. Its cladodes are 17–30 cm long and 8–11 cm wide. The cladodes are light green with a pubescent epidermis. The young cladodes collected for human consumption are usually brilliant green with reddish tips. The flowers reach 5 cm in diameter during anthesis. They are yellow, with reddish parts in the most external perianth segments. The pericarpel—the structure that embodies the ovary and later becomes the fruit—is 2 cm wide and 2 cm long. The fruits are globular in shape and green, varying in diameter from 1.5 to 3 cm. Flowering has been recorded in March, and fruiting in April (Bravo-Hollis 1978). *Opuntia atropes* is appreciated for the quality of its *nopalitos* (young cladodes used for human consumption) (Reyes-Agüero *et al.* 2005a; López-Gutiérrez *et al.* 2015). It is widely used as fodder and living fences (Maldonado-Almanza 2013; Arias-Medellín *et al.* 2014; Pérez-Sánchez *et al.* 2015). Like many *Opuntia* species, *O. atropes* is clonally propagated, mainly when used for living fences (personal observation). Given the management derived from these uses, *O. atropes* is at the early stages of an ongoing process of domestication, in which cultivated plants have been found to have larger cladodes, more areoles, and fewer spines than their wild counterparts (López-Gutiérrez *et al.* 2015).

Reproductive phenology and floral longevity

The flowering and fruiting periods were characterized by visiting Valle de Vázquez every two weeks from February to

August 2019. During these visits, we recorded the presence of flower buds, open flowers, and fruits on 50 individuals of *O. atropes*.

To document individual floral longevity and sexual functions, we selected 20 flowers of *O. atropes*, each from a different plant. Every two hours, from 10:00 a.m. to 4:00 p.m., we measured flower opening and evaluated the functionality of stigmas and anthers (following Martínez-Peralta *et al.* 2014b). The flower opening was measured as the diameter of the perianth. The functionality of the stigmas was evaluated as the opening of the stigma lobes (diameter) (Matías-Palafox *et al.* 2017). Anthers were qualified as functional or dehiscent when their surface was grainy, indicating pollen presentation (Martínez-Peralta *et al.* 2014b). Dichogamy—the temporal separation of female and male functions—was determined if the stigma and anthers were functional at different intervals of the floral life.

Floral morphology and P/O ratio

We collected 20 flowers in pre-anthesis, each from a different individual, and fixed the flowers in 96% ethanol. Fixed flowers were longitudinally dissected and manipulated under a stereomicroscope to measure the floral traits (Fig. 1B) (Martínez-Peralta *et al.* 2014b). Before fixation in ethanol, we separated one indehiscent anther per flower and placed it in a 1.5 ml vial. To each vial, we added 1 mL of water and vortexed the vial to facilitate the liberation of the pollen grains from the anther into the water. Immediately after agitation, we used a stereomicroscope to count the pollen grains contained in a 100 µL aliquot of the liquid. The number of pollen grains per aliquot was multiplied by 10 (dilution factor), and this quantity was then multiplied by the total number of anthers of each flower to estimate the number of pollen grains per flower (Martínez-Peralta *et al.* 2022). We then used this estimated number of pollen grains and the number of ovules per flower to calculate the pollen/ovule ratio (P/O) per flower.

We used the P/O ratio approach to infer the breeding system (Cruden 1977). Plant species with the highest P/O ratios are expected to have an obligate xenogamous mating system (i.e., require strict outcrossing to set fruits). In contrast, those with the lowest P/O ratios have cleistogamous flowers (i.e., flowers that do not open at all and do not require pollen transfer for setting fruits) (Cruden 1977). The following cutoff values were used to define breeding system categories: P/O of 5859 ± 936 = xenogamy, 796 ± 87 = facultative xenogamy, 169 ± 22 = facultative autogamy, 28 ± 3 = obligate autogamy, and 5 ± 0.1 = cleistogamy. We also calculated an outcrossing index (OCI) based on three floral traits: perianth diameter, herkogamy and dichogamy. OCI values ranges from 0 to 4 points, assigned as follows: 1 point for the presence of herkogamy, 1 point for the presence of dichogamy, and between 0 and 3 points according to perianth size (perianth size < 1mm



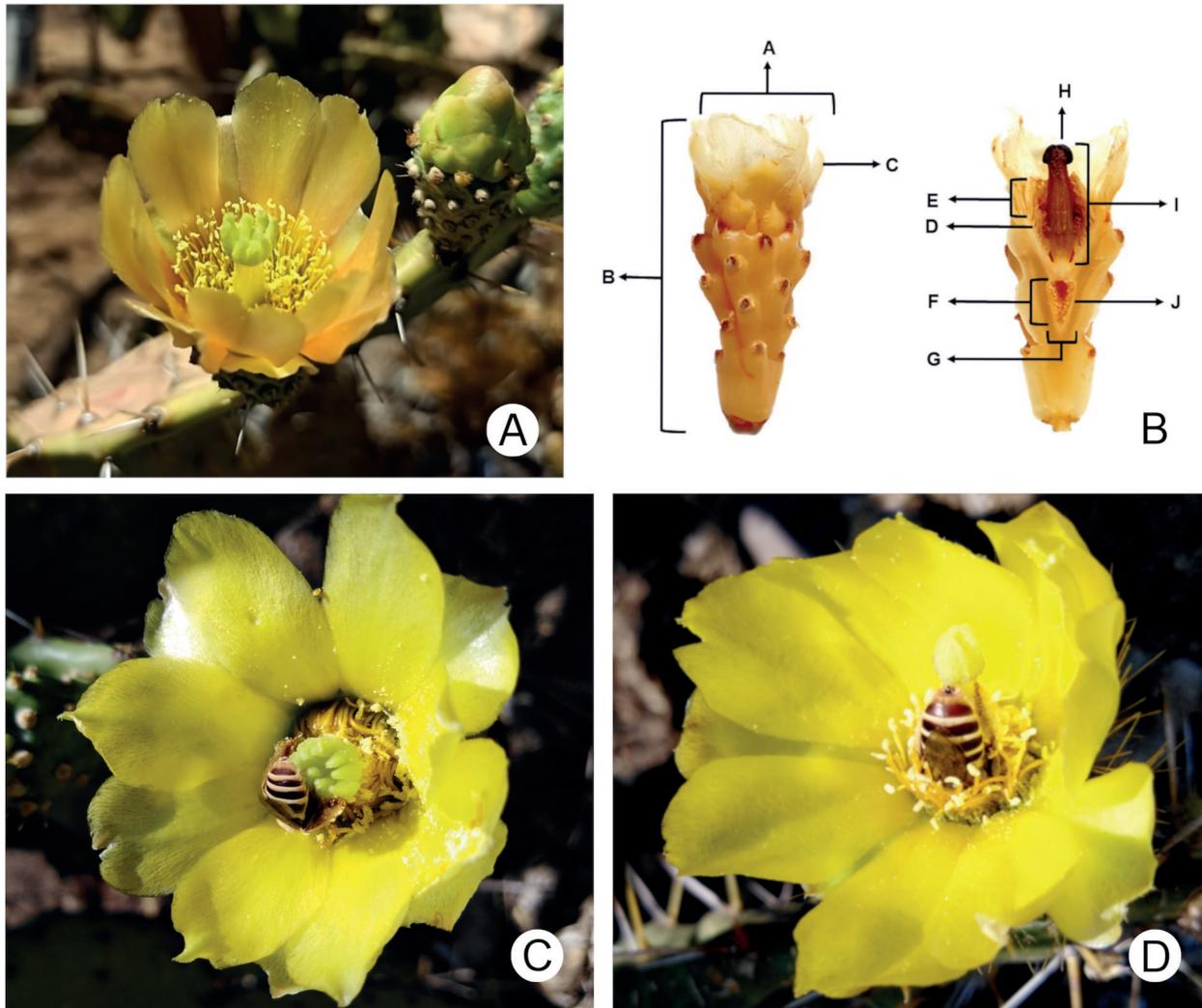


Figure 1. Flowers of *Opuntia atropes* in a tropical deciduous forest of central Mexico. **A.** Open flower and flower bud on the cladode. **B.** Floral measurements considered for floral morphology. **C.** The solitary bee *Diadasia* sp. after landing on the flower's stigma, and then (D) enters the male whorl. Floral measurements in panel **B**: (A) Perianth width, (B) floral length, (C) Number of perianth segments, (D) Number of stamens, (E) Stamen length, (F) Polar diameter of ovary chamber, (G) Equatorial diameter of ovary chamber, (H) Number of stigma lobes, (I) Style-stigma length, (J) Number of ovules.

= 0, 1-2 mm wide = 1, 2-6 mm = 2, and wider than 6 mm = 3) (Cruden 1977). Based on this punctuation, breeding system is classified as follows: cleistogamy (0), autogamy (1), facultative autogamy (2), facultative xenogamy (3), and obligate xenogamy (4) (Cruden 1977).

Mating system and pollen limitation

To determine the mating system of *O. atropes*, we conducted five pollination treatments (Kearns & Inouye 1993; Martínez-Peralta & Mandujano 2011) in Valle de Vazquez through March 2019. For the Autonomous Selfing (ASELF) treatment, flowers were covered and otherwise unmanipulated throughout the anthesis to test for autonomous intraflower self-pollination. In the Manual Selfing (SELF) treatment, flowers were covered during the pre-anthesis and then manually pollinated with pollen

from the same flower. The Outcrossing (CROSS) treatment consisted of covering the flowers during pre-anthesis to prevent insect visitation and manually cross-pollinating them using a mixture of pollen from five donor plants. In the Natural Pollination (NAT) treatment, flowers were left uncovered and available to pollinators throughout the anthesis. Finally, in the Supplementary Pollen (SUPL) treatment, flowers were left available to pollinators and were also manually pollinated once with a mixture of pollen from five donor plants. Each treatment comprised 20 flowers, each from a different individual. For the treatments SELF, CROSS, and SUPL, manual pollination was conducted between noon and 14:00, the time of maximum stigma receptivity. Pollen donors for the CROSS and SUPL treatments were plants located in the surroundings of the *nopalera*, away from the recipient plants, to minimize the risks of inbreeding. At the



end of the anthesis, we covered each flower with a mesh bag that was tied to the cladode to prevent any harm to the developing fruits from cattle, herbivores, or humans. During July 2019 many plants in the *nopalera* were cut down by local people before some of the fruits were collected, and the SUPL treatment was completely lost. To overcome this situation, we repeated the NAT and SUPL treatments in 2020, using plants from San José de Pala and Quilamula. These two pollination treatments were conducted in the same year to be able to test for pollen limitation.

Fruits were considered to be mature when their coloration was reddish; they were collected between May and July 2019 and during July 2020. For each fruit we recorded weight (g), length (mm), width (mm), and number of seeds. We evaluated fruit set across treatments using a generalized linear model (GLM) with binomial error and logit link function and seed set using a GLM with Poisson error and log link function (Dunn & Smyth 2018). After conducting these two GLMs, we conducted orthogonal contrasts to determine pairwise differences among treatments (Mangiafico 2015). Fruit weight, length, and width were analyzed using ANOVA, then post-hoc Tukey comparisons were conducted to determine differences among treatments. All analyses were conducted in the program R (R Core Team 2020).

As an indicator of the mating system, we obtained the outcrossing index $t_e = w_x / (w_x + w_s)$, where w_x is the progeny from outcrossing and w_s is the progeny from selfing (Mandujano *et al.* 2010). For this index, progeny means the proportion of fruits (fruits/flowers) and proportion of seeds (seeds/ovules) obtained from pollination treatments CROSS and SELF (for w_x and w_s , respectively). This index ranges from 0 to 1, expressing the relative contribution of the progeny from outcrossing in the total plant fitness. An outcrossing index of 0 refers to strict selfing, 1 to strict outcrossing, and values between 0.2 and 0.8 represent mixed mating systems (Mandujano *et al.* 2010).

To determine the extent of pollen limitation, we used the index of pollen limitation, L , proposed by Larson and Barrett (2000): $L = 1 - (P_o / P_s)$, where P_o is the relative success of natural pollination, and P_s the relative success of supplemental pollination. This index was calculated using the fruit and seed set from the pollination treatments conducted during 2020.

Pollinators

To determine the identities of floral visitors of *O. atropes*, we conducted direct observations of 20 flowers from 5 plants (4 flowers per plant). These 20 flowers were observed for 45 min, followed by a 15 min break for each hour between 10:00 a.m. and 3:00 p.m., resulting in a total sampling time of 270 min (4.5 h) per flower. During these observations, we considered a floral visitor as any insect or vertebrate that made physical contact with the flower. We recorded the taxonomic identity of the floral visitor,

the part of the flower it had touched (stamens, stigma, both organs, or the perianth segments), the handling time (the time that a visitor spends in a flower) (Ne'eman *et al.* 2006), and its behavior on the flower. For each species of visitor, we calculated the contact rate as the percentage of visits in which it contacted stamens, stigma, or both organs, considering that pollinators contact both organs in a single visit (Sigrist *et al.* 2021). One specimen of each species of floral visitor was photographed under a stereomicroscope; these images were analyzed with ImageJ (Rasband 2018) to determine the body length of floral visitors to the nearest mm. Body size was used to discuss the effectiveness of each floral visitor as a pollinator. To identify the insect floral visitors, we collected some individuals directly from flowers by sacrificing them in ethyl acetate killing jars, and then pinning the specimen. Bees were identified by comparing with bees that were previously collected and identified from the same study sites (Martínez-Peralta *et al.*, in preparation) and from the Colección Entomológica del Centro de Estudios en Zoología (CZUG) at the University of Guadalajara. We calculated the visitation rate of each species of visitor as the total visits/total observation time. Species with less than 10 total visits were pooled into the category "Other". To determine whether the species differed in handling time, we conducted a non-parametric Kruskal-Wallis test (Mangiafico 2015); pairwise differences between species were identified using a post-hoc test with Holm-Bonferroni correction for multiple comparisons (Mangiafico 2015).

Results

Reproductive phenology and floral longevity

We found *O. atropes* buds beginning in early February and flowers in anthesis from the last week of February to the last week of May. During May, the number of open flowers decreased markedly, while some floral buds remained unopen. Immature fruits were found beginning in May, and the fruiting period lasted from May to July.

Opuntia atropes had hermaphroditic, actinomorphic flowers (Fig. 1) that were similar to other *Opuntia* flowers in morphology and size. They were bowl-shaped, with a perianth width of 29.7 ± 10.5 mm (mean \pm s.d.) and a floral length of 44.2 ± 16.0 mm (this length includes perianth and pericarpel length). The pistils protruded from the center of the male whorl (Fig. 1A, B) and measured 20.2 ± 7.0 mm in length. The ovular chamber measures 5 ± 2.2 mm long and 3.9 ± 1.5 mm wide, containing 152 ± 133 ovules. There were 430 ± 195 stamens per flower, measuring 14.8 ± 5.2 mm in length. The number of pollen grains per flower was $177\ 760 \pm 90\ 766$.

Opuntia atropes flowers were diurnal, with a longevity of 6 h. They opened from 10:00 a.m. to 4:00 p.m., with a maximal opening at 12:00 p.m. Stigma opening, a proxy of



stigma receptivity, reached the widest diameter at 2:00 p.m. The anthers began to shed pollen before the stigma became receptive, around 10:00 a.m.; however, both functions occurred simultaneously around 12:00 p.m., so these flowers are homogamous. There was a significant difference between the mean length of stamens *versus* stigmas ($t = 11.45$, d.f. = 19, $P = 5.73e-10$); therefore, flowers of *O. atropes* were herkogamous.

Flowers of *O. atropes* showed changes in color throughout their life. At the anthesis beginning, flowers were categorized within a single category of the Munsell system (5Y/8/10) (Fig. S2). At 12:00 p.m., aside from the 5Y hue, the hue 2.5Y was also recorded. Flowers in color categories that included hues between yellow and red were recorded from 2:00 p.m. to 4:00 p.m. In summary, these results indicate that floral color changed from a highly uniform yellow hue at the beginning of anthesis to more heterogeneous categories of hue, chroma, and value.

The flowers of *O. atropes* were wider than 3mm and thus received a flower size score of 3 in the OCI (Cruden 1977); they are herkogamous, which was scored as 1 point, and homogamous (with sexual functions occurring simultaneously), scored as 0 points. Thus, the overall OCI score was 4, corresponding to obligate xenogamy (Cruden 1977). The pollen/ovule ratio (P/O) obtained was $1,418 \pm 873$ (mean \pm standard deviation), which corresponds to a facultative xenogamous system according to the categorization proposed by Cruden (1977).

Mating system and pollen limitation

There were significant differences among pollination treatments in fruit and seed production ($Z = -2.87$, d.f. = 139, $P = 0.0041$ and $Z = 21.034$, d.f. = 76, $P = 2e-16$, respectively). All treatments that involved natural and cross-pollination set significantly more fruits than the two selfing treatments (Fig. 2). Fruit weight, length and width also differed significantly among treatments ($F = 18.71$, d.f. = 5, $P = 4.49e-14$; $F = 34.14$, d.f. = 5, $P = 2e-6$; and $F = 30.8$, d.f. = 5, $P = 2e-016$, respectively). Due to collinearity in these three fruit measurements, only fruit weight is shown. Natural pollination from 2019 (NAT) and supplemental pollination had the heaviest fruits, followed by natural pollination from 2020 (NAT2) and the cross-pollination treatment; finally, both the manual and autonomous selfing treatments set the lightest fruits (Fig. 2). The outcrossing rate obtained indicates a prevalence for outcrossing in fruit set ($t_e = 0.95$), but outcrossing is not as strict for seed set ($t_e = 0.66$); however, the number of seeds to obtain the t_e comes from a unique fruit. These results indicate that in *O. atropes*, outcrossing is more effective than selfing.

Natural pollination and supplementary pollen conducted in 2020 (NAT2 and SUPL2, respectively) were statistically indistinguishable in the percent fruits set. However, the supplementary pollination treatment (SUPL2) set significantly more seeds than the natural pollination

treatment (NAT2), suggesting pollen limitation for the seed set. The pollen limitation index value suggested a moderate pollen limitation for fruits ($L = 0.15$) and seeds ($L = 0.17$).

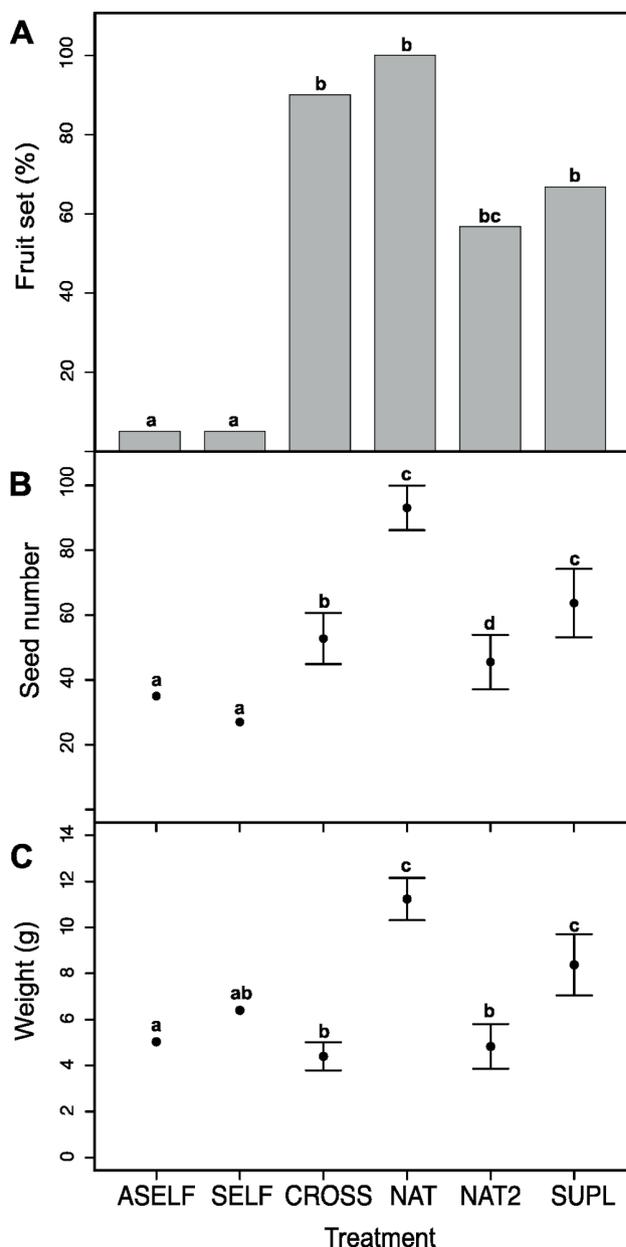


Figure 2. Fruit set (A), seed set (B), and fruit weight (C) following the pollination treatments conducted in *O. atropes*.

Pollinators

The observed floral visitors of *O. atropes* comprised nine species of bees, one wasp, and one hummingbird species. The solitary bee *Diadasia* sp. accounted for most visits, with 74.2% of relative frequency (RF). Other visiting bees were *Augochlorella* sp. (6.3%), *Lithurgopsis* (6.3%), one morphospecies of Halictidae (that was not collected and referred to as Halictidae) (4%), *Apis mellifera* (2.6%),

Xylocopa sp. (2.6%), *Ashmeadiella* sp. (1.1%), *Euglossa* sp. (not collected) (0.6%), and *Ceratina* sp. (0.15%). The wasp (Vespidae) and the hummingbird (Trochilidae) represented 0.5% and 1.7% of the visits, respectively. Flowers were visited between 10:00 am and 4:00 pm, with a peak activity between 12:00 p.m. and 1:00 p.m. (26% of the total visits). The number of visits per flower during the observation period ranged from 15–67, with a mean visitation rate of 3 ± 7.6 per hour (mean \pm s.d.). During our observations of floral visitors, we detected that stamens have thigmonastic movements: they move towards the style when touched by floral visitors.

During most of the visits, floral visitors made contact with at least one sexual organ. The overall contact rate was 44.5% for stamens, 16.2% for stigmas, and 35.8% for both organs. Of the total visitors recorded, only 1.8% touched only the perianth segments. All of the visits conducted by the hummingbird (1.7%) were focused on searching for nectar, and we did not observe clear contact with the flower's sexual organs. When visiting a flower, the solitary bee *Diadasia* sp. most frequently landed on the stigma and then descended to the male whorl, often reaching the inner stamens (Fig. 1). This species had the highest contact rate of both organs during a single visit (Table 1). *Lithurgopsis* sp. landed on the stigma in some visits, but this was not as consistent as in *Diadasia* sp., and the rate of contact with the stamens was higher than with the stigma or both organs. In contrast, the most frequent contact of *Ashmeadiella* sp., Halictidae, and *Xylocopa* sp. was with the stamens, suggesting they primarily collected pollen. Despite being able to contact both organs at a similar rate as *Diadasia* sp., *Apis mellifera* did not show the characteristic pattern of landing on the stigma shown by *Diadasia* sp. Due to the remaining visitors' low frequency, it was impossible to describe clear behavior patterns.

The handling time of all visitors together ranged from 1 to 120 s with a positive-skewed distribution. The non-parametric Kruskal-Wallis test indicated significant differences in handling times among groups (chi-square = 18.8, d.f. = 7, $P = 0.0088$). According to the pairwise comparison with multiple comparison correction, *Diadasia* sp., Halictidae, and *Lithurgopsis* sp. had similar handling times to *A. mellifera* and *Augochlorella* sp. but spent significantly more time in a single visit than Trochilidae, *Xylocopa* sp., and the low-frequency floral visitors grouped as "Other" (Table 1). These data of floral visitors indicate that *Diadasia* sp. is the species that more effectively pollinates the flowers of *O. atropes* based on frequency and behavior.

Discussion

Flowering and fruiting in *O. atropes* occur simultaneously, a phenological reproductive pattern described in other cacti as asynchronous (Pimienta-Barrios & Nobel 1998; Bustamante 2003; Razo-León *et al.* 2022). Flowering of *O. atropes* occurs simultaneously with *O. decumbens*, another species of prickly pear that occurs in the same area (Gómez-Aguilar 2022). Flowering of *O. atropes* occurs in the middle of the dry season of the tropical dry forest, when floral resources from native vegetation are limited (Hernández-Galindo 2016). It thus may represent an essential source of floral resources (pollen and nectar) for native bees. Fruiting also occurs during the dry season and, hence, could constitute an important food resource for vertebrate fauna, such as rodents and birds (González-Espinosa & Quintana-Ascencio 1986).

Floral longevity of *O. atropes* is relatively short (6-7 h), a feature that can be considered advantageous since it limits the loss of water from transpiration in a dry environment (Primack 1985; Pimienta-Barrios 1990).

Table 1. Rate of visitation, contact rate, handling time, and body size of floral visitors of *O. atropes* in March 2019 ($n = 20$ flowers) in Tlaquiltenango, Morelos, a locality of tropical dry forest in central Mexico. Different letters in Handling time column indicate significant differences among groups following the pairwise comparisons after the Kruskal-Wallis test. Species with an asterisk in Handling time column were grouped as the category "Other" due to low frequency of visit.

Floral visitor	Rate of visitation (mean \pm SD) (visits/hour)	Stamens contact rate (%)	Stigma contact rate (%)	Stamens and stigma contact rate (%)	Handling time (s)	Body size (length) (mm)
<i>Diadasia</i> sp.	5.36 \pm 2.31	37.3	19.9	41.1	13.3 \pm 19.3 a	12
<i>Augochlorella</i> sp.	0.48 \pm 0.74	51.2	7	39.5	15.9 \pm 24 ab	6
<i>Lithurgopsis</i> sp.	0.44 \pm 0.11	82.5	2.5	12.5	9.75 \pm 12.3 a	11
Halictidae	0.29 \pm 0.10	84.6	0	15.4	14.1 \pm 23.1 a	NA
<i>Apis mellifera</i>	0.20 \pm 0.05	55.6	5.6	38.9	7.5 \pm 6.4 ab	11
<i>Xylocopa</i> sp.	0.17 \pm 0.09	80	13.3	6.7	6.13 \pm 5.4 bc	21
Trochilidae	0.12 \pm 0.05	0	0	0	3.55 \pm 2.5 bc	NA
<i>Ashmeadiella</i> sp.	0.08 \pm 0.04	85.7	0	0	6.1 \pm 6.2* bc	7
<i>Euglossa</i> sp.	0.04 \pm 0.03	75	25	0	3.25 \pm 1.9* bc	NA
Vespidae	0.03 \pm 0.02	33.3	33.3	33.3	4.67 \pm 0.6* bc	9
<i>Ceratina</i> sp.	0.01 \pm 0.01	0	0	0	2* bc	7



Color variation throughout the floral lifespan could be linked to either the age of the flower *per se* or to the process of pollination (Erickson & Pessoa 2022), which have both been documented in other *Opuntia* (Pimienta-Barrios 1990; Piña *et al.* 2007). Our observations indicate that pollination would be a plausible mechanism underlying floral color variation since bagged flowers of the autonomous selfing treatment remained closer to yellow hues (personal observation). In the genus *Opuntia*, whose fruits are edible and constitute an important economic resource, it would be helpful to determine whether flower color changes after pollination, therefore, could be used as an easy proxy of successful pollination.

We found that in *O. atropes*, sexual reproduction is an important mode of propagation in the agroforestry system where the populations were located. Sexuality can be lost in clonally propagated plants due to a negative feedback loop that diminishes the frequency of fertile clones (McKey *et al.* 2010). However, such a loss of sexuality in *Opuntia* has been rarely documented (Negrón-Ortiz 1998). According to the pollination experiment, *O. atropes* has a predominantly outcrossing mating system and may express some degree of self-incompatibility, given the low success of the selfing pollination treatments (ASELF and SELF). These results indicate that this species depends on native bees to set fruits. Across *Opuntia*, species with mixed mating systems are more common than those with strict or predominantly outcrossing systems (Mandujano *et al.* 2010). Floral attributes that promote this outcrossing mating system are floral herkogamy, large flowers, and the high pollen/ovule ratio. Although flowers are homogamous, herkogamy could be sufficient in promoting outcrossing and avoiding self-interference (Barrett & Harder 2006). The fact that there was a minimal but non-zero success of selfing indicates a slight departure from strict outcrossing in *O. atropes* and could indicate mating system evolution (Vogler & Kalisz 2001; Goodwillie *et al.* 2005). For example, a population of *O. macrocentra* that occurs in an environment with pollinator availability was found to be completely self-incompatible. In contrast, a different population in a site with poor pollinator availability had a mixed mating system (Ortiz-Martínez *et al.* 2022). Partial self-incompatibility, in which some individuals are self-compatible while others are self-incompatible in the same population (Levin 1996; Ferrer *et al.* 2009), has been reported in the cactus family and was linked to pollen limitation (Martínez-Peralta *et al.* 2014a; Martínez-Peralta & Mandujano 2016). These two cases of cacti that slightly deviate from strict outcrossing may represent a transition to mixed mating systems. Mixed mating systems are thought to evolve as a strategy for reproductive assurance (Goodwillie *et al.* 2005) in poor pollinator environments or when compatible mates are absent. In the studied populations of *O. atropes*, the high levels of human disturbance from livestock and agriculture may lead to unpredictable pollinator availability. Outcrossing

in a species with clonal propagation and a large floral display could face processes that decrease effective pollination, such as intraplant or intraflower pollination due to recurrent visits (i.e., geitonogamy). The high success rate of natural pollination in this study suggests that native bees, in addition to having good within-flower performance, also transport pollen effectively among flowers due to their foraging pattern (Schlindwein 2004).

Although there were no statistically significant differences in fruit sets among treatments that involved outcrossing (Natural Pollination (NAT, NAT2), Outcrossing (CROSS), and Supplementary pollen (SUPL)), fruit sets tended to be lower in both treatments conducted during the second year (NAT2 and SUPL). However, there were differences in seed set and fruit weight between Natural pollination treatments conducted in different years (NAT and NAT2). Despite the mixed effect of year and site, these comparisons suggest variability in reproductive success in time and/or space. The variability of reproductive success is multifactorial and could involve maternal effects, availability of pollinators, stigmatic pollen load, and climatic variation, among other factors (Burd *et al.* 2009), and it may affect floral traits. Among the floral traits we measured, there was notably wide variation in the number of ovules per flower (coefficient of variation = 82.4). Ovule number variation has been reported to be related to an unpredictable pollination environment; when ovule fertilization is highly variable among flowers within a population, the high variation in ovule number permits some flowers to be very successful, even when though some resources were wasted on the numerous non-pollinated flowers (Burd *et al.* 2009). The high variability in fertilization could also be related to stigmatic pollen loads, which reflect the efficacy of pollen transfer in the population. A better understanding of the relationship between ovule number variation and stigmatic pollen loads would be informative of the extent of reproductive success in the population and how reproductive success affects the evolution of floral traits in *O. atropes*.

We found moderate pollen limitation in *O. atropes*, as seed set and fruit weight were higher for the supplemental pollination treatments (conducted in 2020). Our observations of floral visitors conducted in 2019 and the success of all naturally pollinated flowers during that year suggest that pollinators had an optimal performance for fruit and seed formation in that particular year and site. However, the supplementary pollen treatment, conducted in a different year in another locality, indicated that conditions for successful pollination are likely to change and that pollen limitation may arise in *O. atropes*. Pollen limitation has been documented in many angiosperms (Ashman *et al.* 2004), including cacti (Piña *et al.* 2007; Martínez-Peralta & Mandujano 2016). Pollen limitation is linked to low mate availability, with two leading proximate causes: a low number of plants to outcross and low pollinator abundance or performance (Ashman *et al.* 2004). In this species, low mate



availability could result from the typical clonal propagation of *Opuntia*. Clonal propagation occurs naturally through cladode fragmentation and human management, as this species is widely used. Suboptimal pollinator performance cannot be ruled out since pollinator assemblages fluctuate in time and space (Herrera 1988). Based on these results, we recommend a more detailed analysis of the extent of pollen limitation in *O. atropes*, including temporal and spatial variability in reproductive success, to disentangle which factors account for pollen limitation.

Floral visitors to *O. atropes* were mainly bees, although a hummingbird and a wasp were also observed. This diversity of visitors indicates that *O. atropes* has the potential to attract a broad assemblage of floral visitors from different taxonomic groups. These observations and the floral morphology could suggest an apparent and phenotypic generalization of the flower based on floral traits (*sensu* Ollerton *et al.* 2007). In other words, the apparent generalized floral morphology (large, radially symmetrical flowers) would permit a high diversity of floral visitors to interact with the flower. However, our findings also suggest that *O. atropes* flowers may be more specialized than they appear since most pollination (both in terms of rate of visitation and pollinator efficiency) could be attributed to a single bee species, *Diadasia* sp. This species accounted for the majority of visits (74%) and touched both sexual organs at a higher rate than the other visitors. The high contact rate of both organs could be promoted by its behavior of landing on the stigmas (Fig. 1) and its size (Table 1), as described for other *Opuntia* species in which effective pollinators are medium to large bees (Fachardo & Sigrist 2020). This solitary bee belongs to a genus whose preferred host is *Opuntia* (Sipes & Tepedino 2005; Ortiz-Martínez *et al.* 2022). In *O. atropes*, the halictids (*Augochlorella* sp. and the morphospecies of Halictidae) seem inefficient pollinators, since they spend long handling times on the flower, even though they do not reach the inner stamens to collect pollen. The small body size of these halictids could also be linked to the low contact rate of both organs. Similar to the halictids, *A. mellifera* and *Lithurgopsis* sp. spent substantial time on the flowers, and despite their size, their contact rates suggest that their behavior does not favor pollination. The fact that *Diadasia* sp. had similar handling times to other visitors that were less frequent and with low rates of contact with both sexual organs, indicates that handling time does not necessarily correlate to performance as a pollinator. In addition, thigmonastic stamens could prevent inefficient pollinators from accessing the nectar chamber and the innermost and most rewarding anthers (Cota-Sánchez *et al.* 2013). These results indicate that *O. atropes* is specialized for bee pollination, conducted by an *Opuntia* specialist bee, as has been documented for other species of prickly pears (Ortiz-Martínez *et al.* 2022). Floral traits, like the thigmonastic stamens, provide further support for the idea of phenotypic specialization of the flower (Ollerton *et*

al. 2007) as documented in the epiphytic cactus *Rhipsalis neves-armondii* (Martins & Freitas 2018).

Although the other visiting bees were less effective pollinators than *Diadasia* sp., their interactions with *O. atropes* flowers would provide them with floral resources such as pollen and nectar. For instance, as the contact rates revealed, all bees except *Diadasia* sp. and *A. mellifera* touched only the stamens on most of their visits, which suggests that these bees visit *O. atropes* mainly to collect pollen. Our observations indicate that the hummingbird would visit *O. atropes* flowers for nectar consumption, rather than pollinating. The flowering period of *O. atropes* occurs during the dry season when other floral resources are scarce, and bee abundance is low (Martínez-Peralta *et al.*, in preparation); this scenario makes *O. atropes* an important floral resource for native bees and possibly other nectar consumers, because of their abundant pollen, nectar, large floral display, and extended flowering period. When abundant floral resources appear, or their preferred hosts are scarce, bees may abandon their usual foraging patterns to take advantage of the available resources (Waser 1986).

It is noteworthy that even though the main study site was located at the edge of the tropical dry forest and a village and is subject to chronic disturbance from agriculture and cattle grazing, the most frequent visitor to *O. atropes* flowers was a native *Opuntia* specialist bee, *Diadasia* sp. (Sipes & Tepedino 2005). Overall, the exotic bee *Apis mellifera* accounted for a meager percentage of the visits (2.6%), which indicates that the pollination system occurs mainly between native bees and *O. atropes*, a native plant. Other studies in the area have shown that although present due to apiculture, *A. mellifera* is not a frequent visitor of cactus flowers (Martínez-Peralta & Martínez-Zavala 2021; Gómez-Aguilar 2022). Thus, although cactus species are not dominant in the tropical dry forest, they constitute valuable floral resources for native bees. The main rewards that *Opuntia* species offer to visiting bees are nectar and pollen. Nectar is often produced copiously (Reyes-Agüero *et al.* 2006; Mandujano *et al.* 2010) and actively consumed by adult bees. Pollen is abundant and highly nutritious (Osborn *et al.* 1988); it is collected by female bees to provide food to their larvae (Michener 2007). Therefore, aside from the management of *O. atropes* for human consumption, this plant is ecologically relevant since it is visited by an important number of native bee species and offers them pollen, nectar, and even refuge. Other studies have documented that bee diversity is high in vegetation where *Opuntia* is dominant (Sánchez-Echeverría *et al.* 2016; Ávila-Gómez *et al.* 2019), suggesting that *nopaleras* may act as reservoirs of wild bee diversity.

We found that *O. atropes* has an outcrossing mating system, which is promoted by floral size and herkogamy, and may have a self-incompatibility system (Goodwillie *et al.* 2005). Despite its clonal growth and human management, sexuality in this species is fundamental to the maintenance



of populations. Our data on seed number and fruit weight indicate that reproductive output is pollen-limited, and the extent of this pollen limitation may be variable in time and/or space. The most effective pollinator is the native bee *Diadasia* sp., which, together with the floral morphology and the thigmonastic stamens, indicates a functionally specialized pollination. This study highlights the importance of studying the pollination ecology of *Opuntia* species. In addition to their cultural and economic significance, *Opuntia* species have a critical role as a source of floral resources for native bees, which further increases their ecological importance in the context of pollinator declines.

Supplementary material

The following online material is available for this article: Figure S1. Study sites of *Opuntia atropes* were located at the south of Morelos state, in central Mexico. Figure S2. Floral color variation during anthesis of *Opuntia atropes*. At the beginning of the anthesis, there was found only one category of color; as flowers age, they turned to more reddish and darker categories of color, according to the Munsell color system.

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