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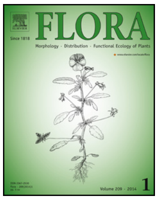

Reproductive biology of *Ziziphus mistol* Griseb. (Rhamnaceae), a wild fruit tree of saline environments

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ABSTRACT

Ziziphus comprises important fruit trees that inhabit arid environments of tropical and subtropical regions of the world and are able to adapt to drought and salt stress. Species of this genus are characterized by synchronous protandrous dichogamy and self-incompatibility; however, reduced seed set after hand self-pollination indicates that self-fertilization is possible and that it causes a strong inbreeding depression. We characterize the reproductive biology of *Ziziphus mistol*, a species typical of saline environments of Chaco American forests. For this purpose, we studied floral biology, observed foraging behavior and pollen load of captured floral visitors, and tested the breeding system through the assessment of fruit and seed set and pollen tube growth following self-, cross-, and open pollinations treatments. In addition, the dependence of a pollinator and autonomous self-pollination were evaluated by excluding floral visitors and airborne pollen. Cross-compatibility between individuals was tested through controlled hand pollinations. Results indicate that *Z. mistol* is predominantly outcrossing and depends on its pollinators to produce fruits and seeds. We observed high synchrony of flowering between individuals, as well as in floral anthesis between/within inflorescences within individuals. The flowers exhibit complete intrafloral dichogamy that fully prevents autonomous self-pollination, while sexual phases overlap within inflorescence; thus geitonogamy can easily occur. Manipulative experiments show that *Z. mistol* can set fruits and seeds after self- as well as cross-pollinations. However, reduction in fruit set and seed set after selfing and/or cessation of pollen tube growth in the style suggest a self-incompatible system. From reciprocal crosses tested ($N = 95$), we have identified only 32.6% compatible crosses via fruit and seed trials. We suggest that in *Z. mistol*, as well as in other species of the genus, both synchrony in floral development and partial self-incompatibility would be evolved to reduce the costs of inbreeding.

Introduction

Ziziphus Mill. comprises approximately 170 pantropical species, 25 of which are native to America and the Caribbean comprising the New World clade according to Islam and Simmons (2006). This genus contains trees and shrubs that inhabit arid environments on every continent due to their versatility in being able to adapt to drought stress (Arndt et al., 2001). *Ziziphus* species are important fruit trees with food and medicinal value and are commercially cultivated in hot and arid regions (Mizrahi et al., 2002). In China and India, species such as *Ziziphus mauritiana* Lam. and *Ziziphus jujuba* Mill. have a long tradition of selection and cultivation; as a result, species occurring in these countries are better known and more researched than those in other regions (Arndt et al., 2001). *Ziziphus mistol* Griseb., widely distributed in Brazil, Paraguay, and Argentina (Tortosa, 1995), is an important genetic resource of saline environments of Chaco forests (Ragonese, 1967). It fruits, prepared in different ways, have traditionally been used by humans as food and in popular medicine was mentioned the use of its bark and leaves (Scarpa, 2004). Currently, these are obtained in local markets extracted from natural populations, as there are no commercial orchards of the species.

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Studies on reproductive biology have shown an appreciable diversity in the breeding system of *Ziziphus*. Most species of this genus that were subjected to research are xenogamous and self-incompatible (SI) (Asatryan and Tel-Zur, 2013; Weekley and Race, 2001; Zietsman and Botha, 1992). Indeed, based on the presence of binucleate pollen grains and the cessation of pollen tube growth in the style, it has been suggested that SI system is gametophytically controlled (Asatryan and Tel-Zur, 2013). However, the strength of SI in this genus appears to be a variable trait because self-fertilization and parthenocarpy have been reported in cultivars of *Z. jujuba*, *Z. mauritiana* and *Ziziphus spina-christi* (L.) Desf. (Asatryan and Tel-Zur, 2013), and wild individuals of *Ziziphus celata* Judd. and D. W. Hall (Weekley and Race, 2001). Furthermore, the existence of self-compatible (SC) genotypes and varieties for *Z. jujube* (Ackerman, 1961) and *Z. mauritiana* (Mehrotra and Gupta, 1985; Rama Devi et al., 1989; Teaotia and Chauhan, 1964) were also mentioned. Finally, the failure in the style elongation in numerous flowers has led some authors to suggest the presence of andromonoecy (Galil and Zeroni, 1967; Tel-Zur and Schneider, 2009).

Reports on phenology of *Ziziphus* stated that hermaphrodite flowers display synchronized protandry; that is, all open blossoms on a ramet are in the same phase and anther dehiscence precedes stigmatic receptivity (Lloyd and Webb, 1986). In fact, the presence of genetical morphs that differ in the timing of anthesis during the day (Asatryan and Tel-Zur, 2013; Galil and Zeroni, 1967; Lyrene, 1983; Zietsman and Botha, 1992) was reported. If there are two morphs at a 1:1 ratio, where each morph is SI and reproduction occurs between contrasting morphs, the phenomenon is called heterodichogamy (Lloyd and Webb, 1986; Renner, 2001) or temporal dioecism (Cruden, 1988). Pollination studies of cultivated species of *Ziziphus* mention honeybees (*Apis* sp.) as the most common visitors (Pham, 2012), while data for wild genotypes in *Z. mauritiana* (Mishra et al., 2004; Rama Devi et al., 1989) and *Z. joazeiro* (Nadia et al., 2007) suggest species of wasps, flies, and bees as their major pollinators.

Hermaphroditism provides advantages such as economy of resources because allocation to floral attraction and reward benefits both maternal and paternal function, and reproductive assurance through self-fertilization within mates and/or pollinators is rare or absent (Charlesworth and Charlesworth, 1987). However, it has negative consequences for individuals (Barrett, 2002). Costs of hermaphroditism, in SI and SC taxa (Culley et al., 1999; Dudash, 1990; Vogler et al., 1999), include sexual interference within as well as between flowers of the same plants (Lloyd and Yates, 1982), and consequently stigma clogging (Lloyd and Webb, 1986), pollen discounting (Harder and Wilson, 1998), and ovule discounting (Barrett et al., 1996); and inbreeding depression (Charlesworth and Charlesworth, 1987). Thus, mechanisms of temporal and physiological separation of sexual functions within the same individual are considered to have evolved to reduce the disadvantages associated with hermaphroditism.

Dichogamy, the temporal separation of male and female phases in hermaphrodite flowers, has been historically interpreted as a mechanism for avoiding inbreeding (Lloyd and Webb, 1986). Recently, correlation analyses (Bertin, 1993; Routley et al., 2004) and experimental works (Dai and Galloway, 2011; Harder et al., 2000; Jersáková and Johnson, 2007; Routley and Husband, 2003) have supported that protandry, the most common form of dichogamy, evolved through male fitness by interference avoidance between male and female functions, rather than female fitness. Moreover, other studies (Harder and Aizen, 2004; Narbona et al., 2011) have demonstrated that protandry may complement physiological SI by reducing selfing when the latter is incomplete (Becerra and Lloyd, 1992; Poehlman and Sleper, 1995). Therefore, in SC species or with incomplete SI, both mechanisms are involved in overcoming hermaphroditism cost of inbreeding depression (Charlesworth and Charlesworth, 1987).

Here, we present the reproductive behavior of *Ziziphus mistol*, and we document floral phenology, potential pollinators, and breeding system as a function of the following predictions: (1) *Z. mistol* is pollinated by a small–medium insect functional group, (2) *Z. mistol* exhibits intrafloral dichogamy, and (3) *Z. mistol* is an SI species but can set fruits and seeds through self-fertility.

Materials and methods

Study area

Fieldwork was conducted in the springs (September–December) from 2010 to 2013 in a natural population of *Z. mistol* located at "Colonia La Brava" (30°51′08.63″ S, 60°15′56.37″ W), Santa Fe Province, Argentina. Observations and experiments were repeated in four seasons. The study site is within ecotonal woods between Chaco and Espinal regions dominated by *Prosopis* L. spp., together with *Geoffroea decorticans* (Gillies ex Hook. et Arn.) Burkart, *Phytolacca dioica* L., *Vachellia caven* (Molina) Seigler and Ebinger, and *Erythrina crista-galli* L. (Hilgert et al., 2003). The chaquenean component is represented by Z. *mistol* and *Schinopsis balansae* Engl. populations. The average annual rainfall amounts to 1200 mm, and the average temperatures range from 16 to 24 °C in the autumn–winter and spring–summer seasons, respectively (INTA, 2013).

Studied species

Individuals of *Z. mistol* are large shrubs or trees, deciduous, 4–8 (15) m tall. Branches have spines in pairs and leafy short shoots at nodes. Leaves are 2–6 cm long, alternate, broadly elliptic or oblong, broadest at the middle, basally three-nerved: margin serrated, crenate, or entire. Inflorescences are axillary umbelliform cymes consisting of yellowish green flowers. Flowers are bisexual, 4–6 mm across, with five triangular sepals 1.5–2 mm long, five oblong-spatulate petals 1 mm long, and five petal-opposed stamens attached at the base to a yellowish disk. The ovary has 2–3 chambers, each with a single ovule, sunk in the disk and one 2- or 3-lobed style. The ovary develops into a drupaceous fruit with one to three seeds (Medan and Schirarend, 2004; Tortosa, 1995).

Floral phenology: population, inflorescence, and flower level

Flowering phenology at population level was described in three inflorescences from ten individuals, based on the number of scars of fallen flowers, buds, open flowers, and fruits present in selected trees. Sampling dates were evenly spread over each reproductive season in 2010 and 2011.

Flowering synchrony between individuals was estimated using Augspurger's index (Augspurger, 1983). This measure focuses on the between-individual synchronization through the number of days in which an individual overlaps flowering with the rest of the individuals of the population. The synchronization level (S_i) of individuals is calculated with the following formula: $S_\mathrm{i} = (1/-1)(1/f_\mathrm{i})\sum e_{j \neq \mathrm{I}}$, where *i* is a function of the number of individuals in the population *n*, the number of days the individuals is flowering f_i, and the number of days individuals i and j ($i \neq j$) are flowering simultaneously (e_i). S_i may vary between 0 and 1; when $S_\mathrm{i} = 0$, no synchrony occurs, and when $S_\mathrm{i} = 1$, perfect synchrony occurs. The index of population synchrony (Z) is the average of the S_i of all plants within the population.

To evaluate floral development within and between inflorescences, we used a modified method from Narbona et al. (2011).

In 2012 and 2013, we identified open flowers in three inflorescences of five individuals and observed the development of the flower phases (male phase, female phase, withering) in two successive cycles of floral dehiscence. Overlap between sexual phases within inflorescence was estimated by recording the proportion of inflorescences where at least once male phase (anthers in dehiscence) overlaps with a female phase (of flowers that opened in the previous cycle of floral dehiscence).

Floral development was determined by monitoring 100 randomly selected flowers (20 flowers from five individuals) from 06:00 to 19:00 h. The movement of the sepals, petals, and stamens, pistil development, as well as nectar production were closely monitored at 30-min intervals. Pollen availability was qualitatively determined by touching the anthers with a brush and observing the presence of pollen grains on it. Stigmatic receptivity was evaluated with a 0.1 M guaiacol and 0.1 M hydrogen peroxide solution, where a stigma color change to brown was indicator of receptive tissue (Tel-Zur and Schneider, 2009). Floral development observations were carried out in 2010 and 2011 during flowering peak.

Floral visitors

Foraging behavior and duration of visits were studied during 30-min observation periods conducted every 2 h, from 7:00 to 19:00 h over ten nonconsecutive days. Observations were made when weather conditions allowed moderate to high insect activity (temperature above 15 °C, null or moderate wind, sunny days). The censuses were carried out from 2010 to 2013 during flowering peak. Insects were captured when foraging on flowers using entomological nets, killed *in situ*, and preserved to be identified later. Insect determination was carried out at the lowest possible taxonomic level (i.e., species, genus, tribe, or family). Individuals that could not be identified at the species level were assigned to morph species. All captured specimens are preserved in the entomological collection of the Department of General Botany, Facultad de Agronomía, Universidad de Buenos Aires.

To analyze the pollen load in floral visitors, under a binocular microscope, a block of gelatin-glycerine with safranin was passed over the bodies of insects to extract the pollen grains adhered to them. A temporary pollen preparation on a glass slide was made with this gelatin cube, and the pollen grains were counted under the microscope at 200× magnification. Pollen grains observed were classified as cospecific (*Z. mistol*) and heterospecific pollen grains (other species).

Breeding system and cross-compatibility trials

Pollination experiments were conducted during the peak of flowering of four consecutive years (2010–2013). Five manipulative treatments were performed on a known number of virgin flowers in ten individuals: (a) *open pollination* (*control*): not manipulated flowers exposed to free pollination; (b) *autonomous self-pollination* (*spontaneous within-flower selfing*)/*apomixis*: flowers bagged with voile bags and absence of hand pollination; (c) *anemophily*: flowers bagged with 1 mm mesh, excluding floral visitors and allowing the passage of airborne pollen; (d) *hand cross-pollination (xenogamy)*: emasculated flowers bagged with voile bags and subsequently hand-pollinated using a mix of pollen from several other individuals; and (e) *hand self-pollination* (*self-compatibility*): flowers bagged with voile bags and subsequently hand-pollinated using pollen from the same flower or other flowers of the same plant. The fruit set (percentage of fruits/flower) and seed set (percentage of viable seeds/fruits) were variables used to compare the treatments. Hand-pollinations were performed after 18–24 h from anthesis when stigmas were most receptive (stigmas clearly expanded and styles fully elongated). Pollinations were made by brushing fresh pollen across a stigma until observing the deposition of pollen grains.

Samples of pollen used in hand-pollinations were removed at anthesis and placed in different germination media (10, 20, and 30% sucrose solution) to test their viability. Percentage of pollen germination was evaluated using an Olympus CH30 microscope.

With values obtained in some treatments, a self-compatibility index (SCI) was calculated (Becerra and Lloyd, 1992), where average fruit set or seed set after self-pollination (autogamy more geitonogamy) is divided by fruit set or seed set after the cross-pollination (xenogamy) treatment. According to Lloyd and Schoen (1992), species with an SCI between zero and 0.75 are considered self-incompatible, and an SCI higher than 0.75 is considered self-compatible species.

Cross-compatibility between individuals was tested by experimental hand-pollinations on flowers bagged with voile bags, in a design that took into account the reciprocal crosses between all studied individuals. Pollen-receiving flowers were emasculated after bud break to prevent contamination with self pollen. Pollen donor compatibility was assessed by fruit set.

In vivo ***pollen germination***

Pollen tube growth in styles was evaluated for open pollination and hand self- and cross-pollinations. Subsamples of flowers were fixed in FAA (formalin: acetic acid: alcohol, 1: 1: 3) at 18 and 28 h after floral anthesis and pollination for open pollination and hand-pollination treatment, respectively; subsequently, these flowers were subjected to aniline blue staining protocol (Martin, 1959). The tissues were observed with a fluorescence microscope (Leica DM1000), and pictures were taken with a Canon EOS Rebel T2i (DS126271) camera.

Statistical analysis

Overlap of sexual phases within inflorescence was evaluated, applying a *t*-test to independent samples (normality and homogeneity of variances were previously tested), using InfoStat software: 2011 version (Di Rienzo et al., 2011). Differences in fruit set and seed set among treatments were analyzed by the adjustment of general lineal models with the **lme** function of the **nlme** package (Pinheiro et al., 2011) of the R statistical language (R Development Core Team, 2011), using the interface provided by InfoStat (Di Rienzo et al., 2011). Means of statistically significant factors obtained with mixed models were compared using the Di Rienzo, Guzman, and Casanoves (DGC) test (Di Rienzo et al., 2002). Normality and homoscedasticity were tested graphically (Q–Q plot and residuals vs. predictors plot, respectively). Variance structure was modeled with *varexp* function for fruit set variable and *varIdent* function for seed set of the *nlme* package of R (Pinheiro et al., 2011).

Results

Floral phenology: population, inflorescence, and flower level

The flowering of *Z. mistol* occurred between the last week of October and the second week of December. The mean flowering duration of the individuals was 47.5 ± 3.5 days. The blooming period of individuals showed a high population synchrony ($Z = 0.82 \pm 0.12$), which was not statistically different between sampled years ($t = 1.16$, $p = 0.26$).

Each inflorescence produces 29 ± 11 flowers, and 2 ± 1 flowers open up each day. Flowers of the same inflorescence bloomed at the same time. This synchronous anthesis – and further development – occurs between flowers that open at one time of a given day in different inflorescences of a plant.

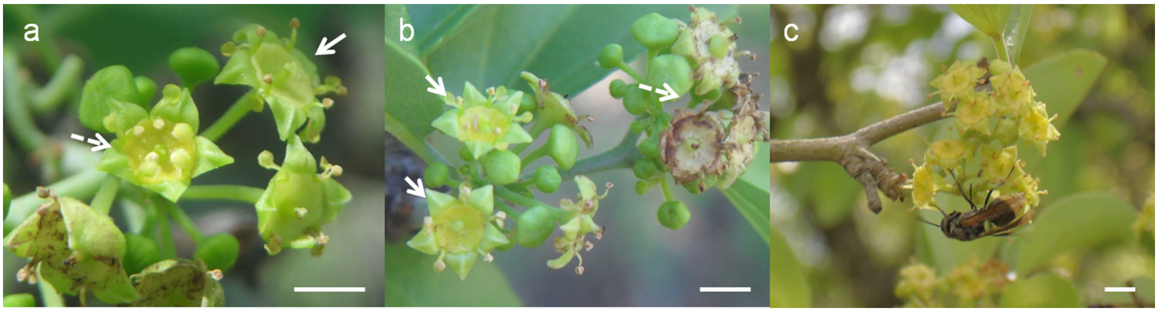

Fig. 1. Inflorescence of *Ziziphus mistol*. (a) Flowers in female (unbroken arrow) and male phase (dashed arrow) in the same inflorescence. (b) Inflorescences with only female flowers (unbroken arrow); dashed arrows indicate withered flowers. (c) *Brachygastra lecheguana* (Vespidae) visiting flowers. Scale bars: 5 mm.

Overlap between sexual phases within inflorescence occurred at least once in 82% of evaluated inflorescences; that is, the male phase of flowers in dehiscence (new floral dehiscence cycle) overlaps with the female phase of flowers, which opened in the previous cycle of floral dehiscence (Fig. 1a–b). In 56% of the inflorescences, this overlap occurred in two consecutive cycles of anthesis. The overlap between sexual phases occurred during 0.7 ± 0.6 h and was not statistically different between years ($t = 0.79$, $p = 0.43$).

Flowers exhibit protandry (i.e., anther dehiscence precedes stigmatic receptivity). Along the flower life span, we identify three floral phases (Table 1). In the male phase, the flower starts to separate the tip of the sepals and the anthers of the five stamens are dehiscent. This phase lasts 6.4 ± 0.55 h. During the male phase, the stamens suffer centrifugal position changes, accompanied by a slow development of stigma (Figs. 2, stage A1–2 and 3a, b). Nectar is produced during this male phase. In most flowers, nectar was rapidly removed by flower visitors. At the end of this phase, petals are lightly expanded (Fig. 2, stage A2). The neutral phase, when there is little pollen available in anthers and stigmas, is not yet totally elongated (Fig. 2, stage B), lasts 1.5 ± 0.58 h. We did not observe nectar in this phase.

Finally, in the female phase, the style achieved its maximum elongation and acquired a bilobed appearance (Fig. 2, stage C1–2 and 3c–d); it lasted 22.0 ± 1.2 h. There is little quantity of nectar in this female phase. Floral senescence is characterized by stigmas withered (brown color) and a white nectar disk with dark spots.

Anthesis at population level occurred throughout the day. Different individuals opened their flowers consecutively from 7:00 to 18:00 h so that pollen supply was constant. Some individuals open flowers once a day, while other individuals do it twice daily.

Floral visitors

Sixty-nine insects belonging to Diptera (12 species), Hymenoptera (8 spp.), Coleoptera (6 spp.), and Lepidoptera (1 sp.) were captured as flower visitors (Table 2). Vespid wasps made the highest number of visits to the flowers during the years of observation. The most abundant individuals were *Brachygastra lecheguana* and *Polybia sericea* (Vespidae), which accounted for 38% of all visits to flowers (Fig. 1c). These wasps visit a large number of flowers throughout the day, from sunrise to sunset, contacting anthers and stigma during the gathering of nectar. Insects sequentially visit many flowers when foraging in cymes and spend several seconds on each flower. All the floral visitors captured carried pollen grains of *Z. mistol*. Diptera were the richest group, but all species in this group

Table 1
Description of *Ziziphus mistol* floral phases. Numbers between brackets indicate the approximate time in hours of each stage.

Phase	Stage	Description
Male phase	A 1 (2 h)	Floral anthesis. Sepals, petals and stamens perpendicular to nectar disk. Anthers dehiscents
	A 2 (4 h)	Flowers fully opened. Sepals entirely expanded. Stamens perpendicular to receptacle pollen loaded, but still covered by petals
Neuter phase	B (1 h)	Petals begin to diverge. Stames with little pollen. These form an angle less than 90° with the receptacle. Styles start to elongate. Nectariferous disk bright yellow
Female phase	C1 (12 h)	Stigmatic lobules attached. Petals parallel to disk. Stamens with little pollen and oblique position relative to the receptacle
	C2 (10 h)	Stigmas fully development with separate lobules. Petals recurved. Stamens form an angle less than 180° with the receptacle. Nectariferous disk pale yellow

Table 2
Floral visitors sampled on flowers of *Ziziphus mistol*. T = number of individuals captured.

Order	Family	Species/morpho-species	T
Coleoptera			
	Staphylinidae	Staphylinidae sp. 1	1
	Cerambycidae	Cerambycidae sp. 1	1
		Cerambycidae sp. 2	1
	Coccinellidae	*Harmonia axyridis*	1
	Scarabaeideae	*Macrodactylus* sp. 1	1
	Lampyridae	Lampyridae sp. 1	1
Diptera			
	Syrphidae	*Nausigaster* sp.	1
		Palpada furcata	1
		Palpada elegans	2
		Syrphidae sp. 1	2
	Sarcophagidae	*Oxysarcodexia thornax*	1
		Ravinia advena	2
	Muscidae	*Bithoracochaetha* sp.	1
		Limnophora sp.	1
		Muscidae sp.1	2
		Muscidae sp. 2	3
	Tabanidae	Tabanidae sp. 1	2
	Stratiomyidae	Stratiomyidae sp. 1	1
Hymenoptera			
	Apidae	*Apis mellifera*	1
	Vespidae	*Brachygastra lecheguana*	15
		Plagiolabra nigra	1
		Polistes sp.	1
		Polybia occidentalis	4
		Polybia sericea	11
		Polybia sp.	3
	Formicidae	Formicidae 1	5
Lepidoptera	Riodinidae	*Riodina lysippoides*	3

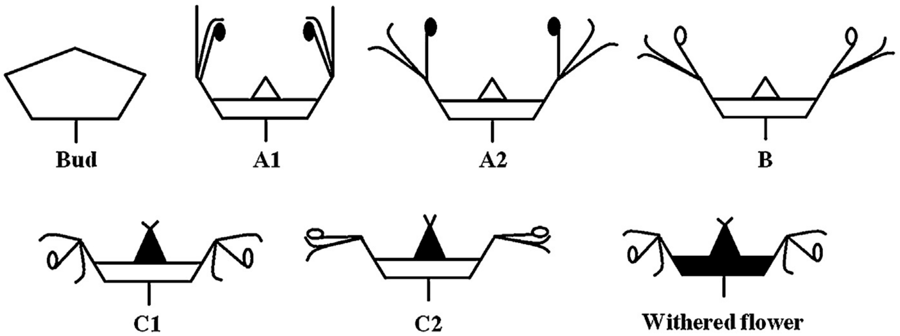

Fig. 2. *Ziziphus mistol* floral schemes showing morphological changes experienced by calyx, corolla, androecium and gynoecium during anthesis. (A) male phase; (B) neuter phase; (C) female phase.

Table 3
Percentage of fruit-set and seed-set (mean ± SD) in different treatments used to study the breeding system of *Ziziphus mistol*. Different letters represent significant differences between treatments by Fisher test LSD ($p < 0.05$). n = number of treated flowers.

Treatments	n	Fruit-set	Seed-set
Open-pollination	800	8 ± 1^b	65 ± 6^a
Hand cross-pollination	851	14 ± 3^a	39 ± 6^c
Hand self-pollination	569	6 ± 2^b	22 ± 7^c
Anemophily	800	3 ± 1^c	49 ± 16^b
Autonomous self-pollination	800	0^c	–

were infrequent and probably played a secondary role in pollen transport.

Breeding system and cross-compatibility trials

Fruit set ($F = 13.35$; $p < 0.0001$) and seed set ($F = 140.46$; $p < 0.0001$) varied among treatments (Table 3). Hand cross-pollinated flowers produced 60 and 40% more fruits than self-pollinated and open-pollinated flowers, respectively. Seed set was higher in open pollination than in other treatments. The absence of fruit initiation in unpollinated flowers indicates that autonomous self-pollination and/or apomixis did not occur. The low fruit set in anemophily treatment (3%) suggests that natural fruit set was pollinator-mediated. SCI was 0.40 for fruit set and 0.56 for seed set.

The pollen grains viability was similar in the three levels of sucrose analyzed. The average viability was 32.8%, and it ranged from 15.0 to 46.0%.

Thirty-one of 95 crosses (32.6%) yielded fruit ($n = 851$ hand-pollinated flowers). On average, each individual was compatible with other three ($\dot{x} = 2.9 \pm 1.3$) individuals. However, this compatibility was variable among years. Fruit set did not vary among pollen donors ($H = 2.89$; $p = 0.9$); however, seed set differs significantly ($H = 14.56$; $p = 0.02$) between pollen donors.

In vivo pollen germination

Observations *in vivo* of pollen germination and pollen tube growth showed pollen tubes growing through the style and reaching the ovary in 68% of analyzed open-pollinated flowers ($n = 28$)

Fig. 3. Stigmatic receptivity changes in flowers of *Ziziphus mistol*. (a) Flowers at onset of anthesis (calyx, corolla and androecium were removed), (b), (c) and (d) 2, 6 and 20 h after anthesis, respectively. Whole flower was stained with a drop of 0.1 M guaiacol and 0.1 M H_2O_2, and brown color in anthers (b) and stigmas (c, d) is the indicator of peroxidase activity. Scale bars: 1 mm.

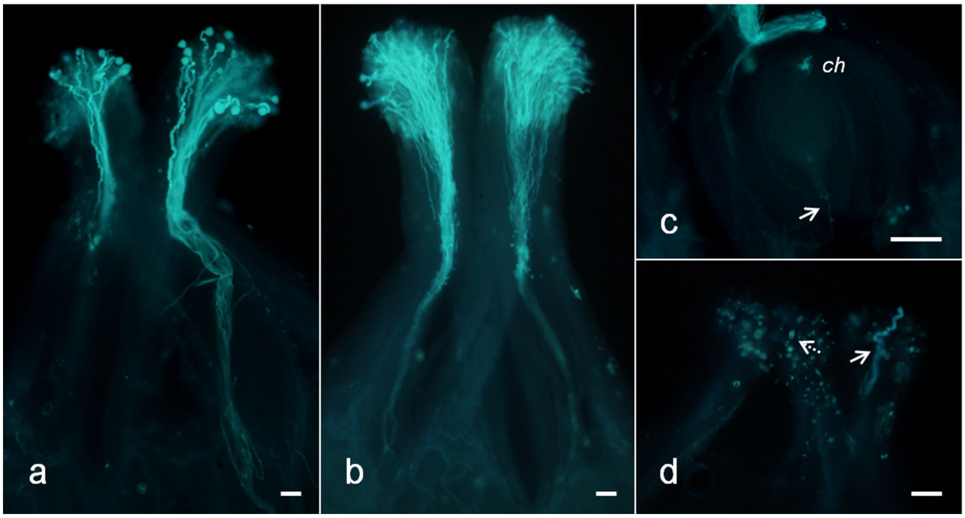

Fig. 4. Pollen tube growth in open- and hand-pollinated flowers of *Ziziphus mistol*. (a) Pollen tubes in stylar tissue in open-pollinated flowers, 24 h after floral anthesis. (b) Pollen tube in stylar tissue and ovary 18 h after hand cross-pollination. (c) Pollen tube (arrow) into the ovule, *ch* chalazal callose. (d) Callose plugs (dashed arrow) and non-germinated pollen (unbroken arrow) on stigma, after 18 h of hand self-pollination. Scale bars 0.2 mm.

(Fig. 4a). In 56% of hand cross-pollinations ($n = 35$), pollen tubes grew through the style and reached the ovary (Fig. 4b–c). Pollen tube growth in hand-self-pollinated flowers ($n = 17$) was arrested on the stigmas and/or showed abnormalities as short pollen tubes (Fig. 4d); only 13% of flower pollen tubes reached ovary. Pollen tube reached the ovary after 18 h of floral anthesis and pollination in open- and hand-pollinated flowers, respectively.

Discussion

Our results suggest that *Ziziphus mistol* is essentially an obligate outcrosser and dependent on pollen vectors. However, the geitonogamy can easily take place at the level of inflorescence and the entire plant.

Results obtained in this study are consistent with those that suggest synchronous dichogamy in the *Ziziphus* species (Asatryan and Tel-Zur, 2013; Galil and Zeroni, 1967; Tel-Zur and Schneider, 2009), as anthesis – and floral development – in *Z. mistol* occurs in synchrony between flowers that open on a given moment of the day. However, heterodichogamy (Renner, 2001) and flowering types described for *Ziziphus* species (Asatryan and Tel-Zur, 2013; Galil and Zeroni, 1967; Zietsman and Botha, 1992) were not observed in *Z. mistol*. Instead, each individual of *Z. mistol* opens its flowers at different moments in a day. This pollen presentation distributed over the day, rather than at two discrete periods, was also observed in *Z. celata* (Weekley and Race, 2001).

The pollination experiments showed that *Z. mistol* is capable of producing fruits and seeds after cross- and self-pollination. However, based on the values of SCI calculated for fruit set and seed set and the cessation of pollen tubes growth in selfing flowers, we suggest the possible presence of gametophytic self-incompatibility (GSI) system (Lloyd and Schoen, 1992; Richards, 1986) in our *Z. mistol* population. Variable selfing rates have been observed in many self-incompatible species (Busch, 2005; Mena-Ali et al., 2008; Vogler et al., 1999), caused by a weak self-incompatibility reaction (Lloyd and Schoen, 1992). In most of these species, inbreed progeny commonly has lower fitness than outbreed progeny, and self pollen growth rate is smaller than cross-pollen. This phenomenon is termed as pseudo-self-fertility (Levin, 1996; Poehlman and Sleper, 1995), and could be related to a weak self-incompatibility reaction (Lloyd and Schoen, 1992). These SI breakdowns in nature have been documented for populations within species or species within genus (Igic et al., 2006; Stone, 2002; Stone and Pierce, 2005; Tsukamoto et al., 2003) and have been attributed to all types of mutations conferring SI, as the suppressed expression of the S-RNase gene, duplication of S-locus, absence of S-protein product in pollen, or production of a defective S-protein product (Igic et al., 2008). Moreover, variation in the amount of selfing differs according to environmental conditions such as pollinator abundance and temperature, and physiological conditions such as relative competitive abilities of self and cross-pollen, growing season, and flower age (review in Levin, 1996; Lloyd and Schoen, 1992; Mena-Ali et al., 2008; Vogler et al., 1999).

Different studies have demonstrated that SI often evolves self-compatibility (Goodwillie, 1999; Nasrallah et al., 2004) and have suggested that the maintenance of SI and outcrossing is caused by a combination of high inbreeding depression and pollen discounting (Busch, 2005; Culley et al., 1999; Good-Avila et al., 2003; Porcher and Lande, 2005; Vogler et al., 1999). In *Z. mistol*, as in other *Ziziphus* species (Asatryan and Tel-Zur, 2013; Liu et al., 2008; Lyrene, 1983; Weekley and Race, 2001), reduction in fruit set and seed set upon selfing suggested the presence of inbreeding depression. Furthermore, selfing because of pollinator-mediated geitonogamous fertilization in *Z. mistol* is virtually inevitable because of the overlap of flowering and foraging behavior of floral visitors. While in *Z. mistol*, as in other species of the genus, the presence of SI could be associated with high levels of selfing, further studies are needed to clarify the dynamics of SI in species of *Ziziphus*.

Experimental crosses showed incompatibility between some of the individuals of *Z. mistol*, as it was observed in other species and cultivars of *Ziziphus* (Asatryan and Tel-Zur, 2013; Lyrene, 1983; Weekley and Race, 2001; Weekley et al., 2002). Similarly suggested by Weekley et al. (2002) for *Z. celata*, the most likely explanation for the cross-incompatibility of *Z. mistol* individuals could be the presence of a multiallelic gametophytic self-incompatibility system, where cross-incompatible individuals share SI alleles and SI mating types (same SI genotypes) (Richards, 1986). Differential success in seed set after cross-pollinations in the studied population of *Z. mistol* could be the result of paternal and maternal effects on seed yield. Studies on pollen performance demonstrate that some pollen grains sire more seeds (Marshall, 1991; Mitchell and Marshall, 1998) and display higher growth rate than others across a range of maternal plants (Lankinen et al., 2009; Stephenson et al., 2001). Added to this, it was demonstrated the effect of the maternal component on the quantity and quality of seeds produced in different populations of cross-pollinated plants (Carlson et al., 2013; Waser et al., 1995).

In summary, the present study demonstrates that fruit set and seed set in *Z. mistol* is affected by mechanisms of self-pollination and incompatibility between individuals. As in other *Ziziphus* species, in *Z. mistol*, floral development and physiological incompatibility are insufficient to reduce selfing by themselves, but both mechanisms may act complementarily to avoid a probable future inbreeding depression. Further work is needed to elucidate evolutionary pathways of selfing avoidance in *Ziziphus*.

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