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## Reproductive biology of a rare cactus, *Opuntia spinosissima* (Cactaceae), in the Florida Keys: why is seed set very low?

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**Abstract** *Opuntia spinosissima* (Martyn) Mill. (Cactaceae) is an extremely rare taxon with a single remaining wild population of 13 plants located on Little Torch Key, Florida. The plants rarely set viable seeds and most ovaries abscise without initiation of fruits. Pollination and breeding system were examined in Little Torch Key and in plant accessions located at Fairchild Tropical Garden. Four pollination treatments were carried out to test for apomixis, self-pollination, outcrossing, and autogamy; a control treatment was also monitored. Additionally, pollen viability, pollinators, and seed set and viability were investigated. On selfed, crossed, and open (control) stigmas pollen grains germinated, but the tubes usually did not reach the base of the style. This suggests that *O. spinosissima* has pre-zygotic self-incompatibility. None of the pairwise crosses set seed, so the extant plants were apparently not inter-compatible. Out of 173 manipulated and control flowers, only one set fruit. Although this flower was outcrossed, no pollen tubes germinated on the stigma; this suggests agamospermy, a process common in the Cactaceae. In those flowers where pollen tubes did reach the ovary they failed to penetrate ovules, suggesting ovarian inhibition or that this taxon has lost the ability to be fertilized. Most field-collected seeds were viable, but there is no seedling recruitment under natural conditions, and vegetative reproduction is common. Based on these findings, I hypothesize that *O. spinosissima* is a sterile polyploid and that the 13 extant plants are asexually derived from a single lineage.

**Key words** Cactaceae · *Opuntia spinosissima* · Pollen tube · Apomixis · Polyploidy

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### Introduction

The reproductive biology of very few rare taxa has been studied in considerable detail (Karron 1991, Weller 1994). Karron (1991) suggested that many rare taxa are self-fertile and set seed in the absence of pollinators; other rare taxa require visitors to manipulate the flowers. Some taxa require cross-fertilization for seed production because they are self-incompatible. Species that became rare because of human activity, however, are unlikely to be selfing. For species that are not selfing, mating between close relatives is expected to reduce the number or vigor of offspring (inbreeding depression). The relative fitness of inbred versus outbred progeny should be determined to assess whether outcross pollination will be necessary for ex situ and in situ management programs.

The purposes of this work were to study floral biology to determine the nature of the breeding system of the rare Florida semaphore cactus, *Opuntia spinosissima* (Martyn) Mill., and to test for pollen viability, identify flower visitors or potential pollinators, and estimate seed set and viability. Information resulting from this study will allow us to ascertain if anything more than clones of this species should be preserved and will provide information useful for reintroduction (e.g., spatial distribution of plantings) and management to enhance seed production.

### Materials and methods

#### Study species

*Opuntia spinosissima* is a member of the family Cactaceae, subfamily Opuntioideae, within the subgenus *Consolea* (D. Austin, personal communication; Areces-Mallea 1996). It grows on bare rock with a minimum humus-soil cover in hammocks near sea level (Small 1930; Benson 1982). This taxon is polyploid and extremely rare, with a single remaining wild population of 13 plants located in Torchwood Hammock Preserve, Little Torch Key, Florida. The taxon formerly occurred on Key Largo and Big Pine Key; however the number and size of the populations in those areas were not described. Nine of the 13 plants are caged to prevent in-

festation of the cladodes by the cactus moth, *Cactoblastis cactorum* Berg. *O. spinosissima* is considered a G1S1 species and therefore a candidate for listing by the U.S. Fish and Wildlife Service under the Endangered Species Act. The plants rarely set viable seed and in most cases the ovary abscises before maturation.

The current specific epithet for this taxon is under investigation (D. Austin, personal communication). For now I will use the present epithet, *O. spinosissima*, until the new epithet is validly published.

#### Pollen viability

Mature anthers from flowers representing the whole population were stained with aniline blue in lactophenol to test for pollen viability (Hauser and Morrison 1964). Fresh anthers collected in Torchwood Hammock Preserve and accessions from Fairchild Tropical Garden (FTG) in Miami, Florida, were used in the study. Pollen grains were stained for a minimum of 24 h and then observed and scored under a light microscope.

#### Pollination studies

Hand pollination experiments were conducted with *O. spinosissima* on flowers of all plants producing flowers in Torchwood Hammock Preserve and with plant accessions located at FTG. At Torchwood Hammock Preserve, seven plants flowered and one to three flowers were manipulated per plant. At FTG, ten accessions (representing nine of 13 plants in the wild) flowered and one to 33 flowers were manipulated per plant.

Four pollination treatments were carried out to test for apomixis, self-pollination, outcrossing, and autogamy; a control treatment was also monitored (Table 1). All possible pairwise crosses between plants were performed to determine which pairs of plants are inter-compatible. The largest flower buds (0.9–1.0 cm long) were selected because these will generally open within 1 or 2 days. For treatments requiring flower emasculatation (apomixis and outcrossing), upper petals of flower buds were removed with a sharp razor blade, then upper whorls of stamens were removed. Paper pollination bags were used for all treatments except the control in order to prevent pollen contamination transfer by animals or wind (Faegri and Pijl 1979; Richards 1986). Pollen collected from flowers in Torchwood Hammock Preserve was also used to pollinate the plant accessions located at FTG. Prior to flower anthesis, Tanglefoot was used around the basal portion of the ovary to exclude non-flying insects from the flowers.

For each treatment, abscised flowers with intact styles were collected and fixed with 70% ethanol. The styles were then examined for pollen tube growth in order to test for possible prezygotic self-incompatibility mechanisms. The entire style was softened in 8 N NaOH for 24 h at room temperature. The material was rinsed with distilled water for a minimum of 1 h, and then stained with aniline blue in 0.1 M  $K_3PO_4$  for a minimum of 4 h. The styles were squashed under a cover slip and observed for pollen tube growth under UV light using an epifluorescence microscope (Schou and Philipp 1982).

To measure seed set on monitored flowers (selfing, outcrossing, and control treatments), fruits were left to mature on the

**Table 1** Breeding system treatments

Treatment	Emas- culation	Bagged	Hand Pollination
Apomixis	Yes	Yes	No
Control (open)	No	No	No
Selfing	No	Yes	Yes (pollen from same plant)
Outcrossing	Yes	Yes	Yes (pollen from different plant)

plants. Mature fruits were collected, dissected in the lab, and the seeds were counted. Aborted abscised ovaries from manipulated flowers were collected and fixed with 70% ethanol. These ovaries were later dissected longitudinally, and the ovules and surrounding tissues were stained (as above) and examined for ovule abortion and pollen tubes with an epifluorescence microscope.

At Torchwood Hammock Preserve, floral visitors were collected for identification and their activities were studied during the flowering season in 1996 and 1997.

#### Seed germination experiments

To test for seed viability, mature fruits were collected from both manipulated and non-manipulated flowers. Seeds with the funiculus attached, seeds with the funiculus removed, and excised embryos were sterilized by placing them in a mixture of 5–10% Clorox and one drop of Tween 20 (a detergent) for 10 min. The seeds were then rinsed in sterile water for 10–15 min and placed in Murashige and Skoog medium (a plant cell culture media) with 1.5% sucrose and 0.22% Phytigel. The samples were allowed to grow at room temperature. Additionally, another group of seeds with the funiculus attached and others with the funiculus partially removed exposing one side of the seed were placed in petri dishes and kept moistened in wet filter paper until germination occurred; the seedlings were then transferred to a soil medium.

## Results

### Floral behavior

Flower production occurred throughout the year but reached a peak during the dry months of December through April. Flowers opened (anthesis) when they reached 0.9–1.0 cm in length, 14–20 days after flower buds emerged. For the majority of flowers, stigmas were receptive at anthesis, as indicated by opening of the stigmatic lobes. Receptive stigmas were a light reddish color, while pollinated stigmas were distinguished by a dark red coloration.

Stamens are sensitive when touched, each filament twisting around the style, and consequently, facilitating deposition of self-pollen. In most flowers, anthers dehisced synchronously with, or within minutes after flower anthesis, promoting autogamy (selfing). In some flowers, only the lower whorl of anthers dehisced and this occurred within 1–2 days after flower anthesis. Pollen mass was bright yellow. Of the 4559 pollen grains scrutinized for viability, 85–92% were viable as assessed by stainability.

Flowers produced a copious, sticky nectar. Nectar quantity and availability appeared to coincide with rainy periods.

### Floral visitors

Flowers were visited only by a species of ant, *Crematogaster* near *ashmeadi* (Hymenoptera, Formicidae), which may have been attracted by a faint rotten meat-like odor produced by the opened flowers (Fig. 1). The ants were observed feeding on pollen, and while doing this they passively loaded the stigmatic lobes with self-pollen.



**Fig. 1** Flower of *Opuntia spinosissima* visited by a species of ant, *Crematogaster* near *ashmeadi*, in Torchwood Hammock Preserve, Florida

### Controlled pollinations

A total of 181 flowers were manipulated, 24 at Torchwood Hammock Preserve and 157 at FTG (Table 2). Styles of 173 flowers were collected 3–4 days after polli-

nation was performed. Ovaries of 112 of these flowers were collected and used in the analyses; 111 ovaries abscised before maturation, and one ovary matured and developed seeds. The other nine flowers and 69 ovaries were not included in the analyses because they did not retain their tags when they fell to the ground.

Pollen germinated in all treatments; however, germination did not occur on all flowers within a treatment. At least some pollen grains germinated on the stigmas of 62% of the flowers in the control treatment, 70% pollinated with self pollen, 53% in the outcrossing treatment, and 76% of the flowers bagged to test autogamy. The ratio of pollen tubes on the stigma to pollen grains on the stigma was higher for flowers testing autogamy (0.33) than in the other treatments, suggesting that the plants are autogamous. For each treatment, more pollen tubes were present on the stigma than the middle of the style (Table 2). Very few pollen tubes reached the base of the style, and this occurred only in the selfing (0.5% reached the base) and outcrossing (1.7%) treatments. Seed set occurred in only one of 181 manipulated flowers (Table 3). Although this flower was from the outcrossing treatment, the fact that pollen failed to germinate on the stigma of this flower suggests that these seeds were produced by agamospermy.

**Table 2** Pollen grain deposition and pollen tube growth reaching the stigma, the middle, and the base of each style in hand pollination experiments on *Opuntia spinosissima*. *n*=Number of flowers

Treatment	<i>n</i>	Pollen grains deposited per stigma			Pollen tubes									Number of seeds
		Mean	Range	SD	Stigma			Middle			Base			
					Mean	Range	SD	Mean	Range	SD	Mean	Range	SD	
Apomixis	3	0			0			0			0			0
Self	20	118.0	2–410	121.3	13.63	0–98	22.36	0.44	0–6	1.36	0.25	0–5	1.12	0
Autogamy	21	78.50	5–168	51.34	28.30	0–89	30.77	0.33	0–3	0.91	0			0
Control	29	73.00	2–249	63.56	22.00	0–89	25.05	2.20	0–30	7.19	0			0
Outcrossing	99	104.50	7–312	64.76	10.96	0–90	17.29	1.32	0–84	9.0	0.86	0–58	6.18	0

manipulated and examined. Five outcrossing treatments of accession number 200 by pollen of accession number 199 were not included

**Table 3** Number of seeds developed from outcrossing treatment of accession number 200 (Little Torch Key plant 9) by pollen of accession number 199 (Little Torch Key plant 8)

Recipient plant number, flower number	Donor plant number, flower number	Number of pollen grains deposited per stigma	Pollen tubes			Number of seeds
			Stigma	Middle	Base	
200,4	199	43	0	0	0	0
200,7	199,9	21	0	0	0	3
200,15	199,19	76	0	0	0	0
200,16	199,24	41	0	0	0	0
200,26	199,29	130	0	0	0	0

**Table 4** Number of seeds germinated per treatment from mature unmanipulated fruits collected in Torchwood Hammock Preserve (Little Torch Key). – Represents no data

Germination treatments	Number of seeds treated	Number of seeds germinated	Days between treatment and germination
Petri dish – funiculus attached	4	2	100, 119
Petri dish – funiculus removed	5	3	5, 11, 16
Agar – funiculus attached	7	0	–
Agar – funiculus removed	1	1	9
Agar – embryo excised	2	1	9

Ovaries of *O. spinosissima* contained an average of 50.64 ovules ( $n=36$ ,  $SD=13.50$ , range 29–85). The ovules from these manipulated flowers were not fertilized. In most cases, pollen tubes observed in the tissue surrounding the ovules, clearly visible by observing stained callose, were twisted and/or stopped growing near the micropyle, and hence did not penetrate ovules, suggesting a mechanism that prevents fertilization.

Seeds from non-manipulated fruits collected in the field were viable, as indicated by their ability to germinate in agar and in wet filter paper (Table 4), and by the presence of one seedling growing inside a mature, abscised fruit (personal observation). Germination occurred faster when the funiculus was removed (Table 4).

## Discussion

The pollination data suggest that *O. spinosissima* possesses self-incompatibility (SI), a mechanism that prevents fertilization when a plant is pollinated with its own pollen or pollen from another plant that shares the SI allele. None of the pairwise crosses set seed, thus these plants are not inter-compatible. Even though the pollen grains were able to germinate, pollen tubes usually did not reach the base of the style. Inhibition for incompatible pollen tubes occurred in the stigma, in the style or, occasionally, in the ovary. The presence of SI has been documented for several Cactaceae genera (Boyle et al. 1994; Boyle 1996; Ganders 1976), and it has been suggested that this family evolved from a primarily outcrossing ancestor with a functional SI system (Boyle 1996).

In those flowers where pollen tubes did reach the ovary tissue they failed to penetrate the ovules. This finding suggests that ovules do not release a hormonal signal to the pollen tubes; thus the tubes grow randomly without contacting the ovules. It is therefore possible that this taxon has lost the ability to be fertilized. This could be explained by ovarian inhibition (Nettancourt 1977), a SI system exhibited by *Theobroma cacao* where levels of ethylene and indole-3-acetic acid increase prior to pollen tube-ovule contact in incompatibly pollinated flowers, whereas these hormones decrease in compatibly pollinated flowers (Baker et al. 1997).

I hypothesize that when seed set does occur, it is due to agamospermy. Agamospermy is not rare in plants (Asker and Jerling 1992) and it is common in the Cactaceae (Cota 1993; Naumova 1992). Vegetative reproduction is also common in this taxon and in general in the family (Benson 1982; Small 1930). This taxon can be easily propagated using cladodes. Also, the abscised ovaries often develop a branch which in some cases grows by reabsorbing the nutrients from the hypanthium before or while producing a root system.

## Floral behavior and visitors

Features such as a bright red perianth, a stylar nectar chamber and copious sticky nectar, and a faint meat-like odor indicated that these flowers were adapted for pollination by animals. One species of ant was observed visiting the flowers, but this finding does not mean that this ant was the pollinator of *O. spinosissima*, because it occurred under very artificial conditions (the plants were caged). Bees, hawkmoths, hummingbirds and mammals (bats) are pollinators reported for the Cactaceae (Cota 1993; Parfitt 1985). Bees and hummingbirds are likely to be potential visitors to some of the Caribbean *Opuntia* flowers. For instance, *O. rubescens* Salm-Dyck flowers from Guánica Forest, Puerto Rico, were visited by bees and hummingbirds (personal observation). The flowers of *O. rubescens* share morphological features with *O. spinosissima* flowers, except that they are larger in size. Therefore, bees which have been reported visiting *Opuntia* species (Grant and Hurd 1979; Mandujano et al. 1996; Schlindwein and Wittmann 1997), and perhaps hummingbirds, are potential pollinators of *O. spinosissima*.

Stamen movement and bright pollen mass also play roles in pollination of *O. spinosissima*. Several studies suggest that stamen movement causes insects to depart from the stigma (Porsch 1938), facilitates deposition of pollen on the insect body (Rauh 1979), and promotes outcrossing (Vogel 1983) and self-pollination (Grant and Hurd 1979). Schlindwein and Wittmann (1997) showed that stamen movement can be an adaptation to hide pollen, offering it only to specialized pollinators. In the case of *O. spinosissima*, stamen movement facilitates deposition of self-pollen on the stigma, while the bright yellow pollen mass guides pollinators.

## Seed germination

Seeds were observed to germinate while still inside the fruit, after the fruit fell off the mother plant. This survival mechanism allows seedlings to avoid desiccation during the dry months, just before the onset of the rainy season. This germination strategy has not been previously reported in Cactaceae, except as a suggestion by Conde (1975). Several cases of viviparous seed (seed germination while the fruit is still on the plant) have been reported for the family (Conde 1975; Lombardi 1993), but this is not the case for *O. spinosissima*.

Seeds are surrounded by a funiculus, which appeared to delay germination (Table 4). If this is the case, under natural conditions the delayed germination is an adaptation because seeds take approximately 4 months to germinate, avoiding the dry months. In general, most of the field-collected seeds were viable; however, there is no seedling recruitment under natural conditions.

In summary, I propose two explanations for high fruit abortion and low fruit set in *O. spinosissima*. First, this species may be unable to reproduce sexually due to meiotic problems resulting from polyploidy. Seeds occasion-

ally form by agamospermy, so they are genetically identical to the parent plant. Polyploidy and asexual reproduction of zygotes and embryos have long been correlated (Stebbins 1979). Different ploidy levels occur in the Cactaceae, and it has been suggested that both polyploidy and hybridization events have led to speciation in this family (Cota and Philbrick 1994; Pinkava et al. 1985; Remski 1954). Relative to the basic chromosome number for the family and for *Opuntia*,  $x=11$ , *O. spinosissima* is considered an hexaploid species ( $2n=66$ ; Cota, unpublished data; Pinkava et al. 1985). According to J.H. Cota (personal communication), the chromosomes are very small and morphologically uniform in shape and size. Additionally, Cota suggested that this taxon is a consequence of a recent polyploidization event; therefore, low genetic diversity is expected. Electrophoretic data showed that this population is genetically monomorphic for 12 of 13 loci, and at the remaining locus (malate dehydrogenase-1) all individuals are heterozygous, suggesting this genotype is fixed (Hamrick and Godt 1996). Evidence implies that these 13 extant plants are clones from a single lineage.

The alternative explanation is that *O. spinosissima* failed to reproduce sexually because the plants are self-incompatible. In this hypothesis, all plants from the extant population are close relatives and share the same *S* alleles preventing fertilization. This alternative is supported by the findings that pollen grains were viable and capable of germination. The loss of *S* alleles would have been due to genetic drift caused by small population size. In this model, human-caused reduction in population size would be the indirect cause of low seed set.

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