Reproductive Isolation in Fragmented Wild Populations of *Opuntia streptacantha*♦

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ABSTRACT

During pre-Hispanic times, there were extensive *Opuntia* forests in the semiarid regions of central Mexico. In the colonial period the migration of the Spanish to these regions promoted land clearing for agricultural activities, a tendency that continues in modern times, ultimately leading to deforestation and fragmentation of wild *Opuntia* populations. The main objective of this work is to evaluate the effects of fragmentation of *Opuntia streptacantha* populations on the reproductive success. Pollen-grain germination, rates of pollen-tube growth, fruit and seed set were evaluated in cross-pollinations between isolated populations and compared to within-population crosses. The activity of insects was recorded during the blooming period and chromosome number was recorded in the isolated populations under study.

Cross-pollinations between populations significantly reduced the number of pollen tubes that reached the base of style and the ovules, affecting the values of fruit and seed set. This seemed to explain the differences obtained between the control (open pollination) and cross-pollination within population. The two populations under study are octaploid. Cross-pollinations between individuals of isolated populations of *O. streptacantha* showed low performance of out-crossing as indicated by the low number of pollen tubes that reached the base of the style, the ovules, and the low values of fruit set and seed set.

Key words: Fragmented population, fruit set, *Opuntia streptacantha*, pollen-tube growth, seed set, sexual incompatibility

INTRODUCTION

During pre-Hispanic times relatively dense stands of wild prickly pear (*Opuntia* spp.) occurred in continuous populations on the semiarid lands of the southwestern corner of the Chihuahuan Desert (Rzedowski, 1978; Janzen, 1986). Colonization of these semiarid lands by Spanish immigrants, and

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subsequent agricultural development, resulted in fragmentation of this cactus forest (Simonian, 1999). Deforestation and fragmentation restricted wild populations of *Opuntia* to environments unsuitable for agricultural activities, such as rocky environments (Serna-Perez, 1984; Janzen, 1986; Simonian, 1999).

The development of isolated populations and reduced population size as result of fragmentation, caused a degree of reproductive isolation among remaining wild populations (Dudash and Fenster, 2000; Young and Clarke, 2000; Stockwell *et al*., 2003). Effects of distance on mating between populations have been studied in terms of progeny fitness and reproductive variables (e.g., seed set, fruit set, seed germination or seedling survivorship) (Sobrevila, 1988; Fischer and Matthies, 1997; Hauser and Siegismund, 2000; Fenster and Galloway, 2000; Stacy, 2001). In most cases, such studies do not show to what extent differences in crossing success are caused by prezygotic versus postzygotic barriers (Waser and Price, 1991). In a few cases, for example *Phlox drummondii,* a prezygotic barrier has been demonstrated that affects reproductive success in isolated populations. Such barriers are expressed as reductions in pollen-grain germination and in pollen-tube development in the stylar tissue, restricting gene flow by alien germoplasm (De Nettancourt, 1977; Levin, 1989; Cruzan, 1990; Waser and Price, 1991; 1993).

We explored cross-pollinations within and between isolated *Opuntia streptacantha* populations for their effect on pollen-grain germination, pollen-tube growth, fruit set, and seed set.

We hypothesized that the fragmentation of *O. streptacantha* populations created the stimulus for the development of reproductive prezygotic barrier, which ultimately will affect their reproductive success.

MATERIALS AND METHODS

Plant Material, Site Description, and Experimental Design

Opuntia streptacantha Lem. is a perennial arborescent cactus 0.5–2.5 m tall, with a defined trunk made up of thick, obovate, green cladodes (Figures 1 through 5). It is widely distributed on the semiarid lands of central Mexico. It is a self-compatible species (Bravo, 1978; Pimienta, 1993). At the study sites, *O. streptacantha* is the dominant perennial species. It grows isolated or in association with other species, and always in a patchy distribution. Vegetation at these sites is "crassicauleous shrubland" (Rzedowski, 1978), consisting mainly of fleshy succulent plants; platyopuntias, barrel cacti, agaves, and *Yucca* species (Pimienta-Barrios *et al.,* 2002).

The study was conducted in two rocky communities located in the Llanos de Ojuelos (INEGI, 1981) in highlands of the southwestern corner of the Chihuahuan Desert, Mexico. One of the study sites is located near El Rayo, Zacatecas, (21° 58' N, 101° 35' W, 2190 m), *Opuntia streptacantha* density at this site was 1852 plants ha^{-1}. The second study site is located near Laguna de Guadalupe (21° 49' N, 101° 22' W, 2180 m) *Opuntia streptacantha* density had 216 plants ha^{-1}. The two populations are separated by 32 km of agricultural land. The climatic conditions of the two sites are comparable, with 450–500 mm rain annually and average temperature that varies from 16 to 18°C (Pimienta-Barrios *et al.,* 2002).

Pollination Treatments

The study was conducted from April to September 2003. Two hundred and forty flowers were pollinated by hand (60 per treatment). To avoid self-pollination, flower buds were emasculated and stigmas were hand-pollinated with fresh pollen, and bagged immediately with glassine bags, except for the control treatment, which received no manipulation (Rosas and Pimienta, 1986). We compared four pollination treatments: a) pollination of El Rayo plants with pollen from Laguna de Guadalupe (hereafter "*ERB*" for "*El Rayo between*"); b) pollination in the reciprocal direction, Laguna de Guadalupe plants with pollen from El Rayo ("*GB*" for "Guadalupe between"); c) pollination of plants within the El Rayo population with pollen from other plants in the same population (*"ERW" for "El Rayo within"*); and; d) control;

flowers of *O. streptacantha* in the El Rayo population were left to receive open pollination by natural insect visitors. We were unable to perform hand pollinations within the Laguna de Guadalupe population, because the blooming peak was later than in the El Rayo population.

In cross-pollination treatments between populations, pollen-donor flowers were transported in paper bags. The time to transport pollen from donor to recipient populations was 30 min. To standardize handling of flowers across treatments, we also held flowers for pollinations within the El Rayo population in paper bags for 30 minutes before using them. We selected plants separated from each other by at least 10 m as pollen donors to ensure that a wide range of genotypes was included.

Pollen-Grain Germination and Pollen-Tube Growth

To evaluate pollen-grain deposition, we collected 15 pistils from each treatment 24 h after pollination. To evaluate pollen-tube growth in styles and pollen tubes approaching the ovules, we collected 15 pistils and 15 ovaries from each treatment 72 h after pollination. Finally, to evaluate fruit and seed set we collected 15 fruits per treatment 80 days after pollination. The pistils and ovaries were frozen with dry ice (CO_2) to avoid removal of pollen grains during fixation of the tissue (Jensen, 1962). Styles were squashed and stained with I_2KI and numbers of pollen grains were evaluated by ocular field and calculated to mm^2. To observe pollen-tube growth, styles were dissected from the pistils and soaked in $NaHCO_3$ (1%) for 1 h, then washed thoroughly in distilled water, and softened by autoclaving in Na_2SO_3 (1%) for 20 min. The softened styles were soaked for 30 min in acetone (100%), then were squashed and stained with lacmoid blue (Rosas and Pimienta, 1986). Pollen tube growth was recorded at the base of the style 72 h after pollination using a bright field optic with a Zeiss microscope.

Ovule Penetration and Fruit and Seed Set

Ovules were removed from ovaries collected 72 h after pollination, and placed in $NaHCO_3$ (1%) for 1 h. Afterward, the ovules were washed with distilled water and mounted on slides in aniline blue (0.005 %) in 0.15 M phosphate buffer (pH 9.1). Ovules were observed under UV light using a fluorescence microscope (Zeiss) equipped for epi-illumination. In order to score pollen tube penetration of ovules (Polito and Pimienta, 1982) we calculated the number of penetrated ovules.

Fruit were collected before fruit ripening (80 days after pollination) at which time fruit set was also determined. This early harvest was done, to avoid fruit losses by predators, or by people from rural villages who commonly gather fruits from *O. streptacantha* wild populations. Seed set was calculated as the percentage of ovules fertilized relative to number of ovules per flower.

Statistical Analysis

To homogenize variances prior to ANOVA, values for pollen grains on the stigmata, pollen tubes in the style, and pollen tubes penetrating ovules were ln-transformed, seed set were arcsine-square-root transformed. One-way ANOVAs were used to test for significant differences between treatments. Means were compared with the Student-Newman Keuls Method Test ($P<0.05$). (Zar, 1984). Analyses were conduced with Sigma Stat statistical software, version 2.0.

Insect Activity

Insects were collected using paper bags between 10 to 18 h while they foraged flowers of *O. streptacantha*. To do so, we walked a 50 m transect every 2 hours during two days at the peak of blooming. Insects were scored and identified in the entomology laboratory at the Universidad de Guadalajara.

Chromosome Number

Elongating secondary root tip cells were placed in cold 8-hydroxyquinoline (0.002 M) for 2 h. Then, the root tips were hydrolyzed with hydrochloric acid (1N) for 10 min at 60°C and transferred to Feulgen

reagent for 7 min at 60°C. Slides were prepared using the squash technique. In metaphase, five cells from each of three plants of each population were observed (García-Velázquez, 1990).

RESULTS AND DISCUSSION

Different pollination treatments, including control (open pollinated), achieved statistically indistinguishable numbers of pollen grains per mm^2 of stigma surface at 12 h after pollination ($F_{3,56}$ = 0.396, P = 0.7559) (Table 1). However, the numbers of pollen tubes at the base of the style 72 h after pollination varied significantly among treatments ($F_{3,56}$ = 62.4, P < 0.0001). Pollinations between populations (*ERB* and *GB* treatments) resulted in fewer pollen tubes reaching the base of style (94 and 15 tubes on average, respectively) than control (209 tubes) or within-population hand pollinations (*ERW* treatment; 196 tubes) (Table 1).

Table 1. Number of Pollen Grains 24 h after Pollination at the Stigmatic Surface, Frequency of Pollen Tubes at the Base of the Style, and Approaching the Ovules at 72 h Under Different Pollination Treatments

Pollination treatment	Number of pollen grains on stigmatic lobules 24 h after pollination (mm^2)	Number of pollen tubes at base of style after 72 h	Number of pollen tubes arriving at ovules after 72 h	Fruit set (%)	Seed set (%)
Control (natural pollination)	8 [a*]	209 [a]	98 [a]	93	53 [a]
Cross-pollination within population in El Rayo (ERW)	8 [a]	196 [a]	81 [a]	60	58 [a]
Cross-pollination between El Rayo and Laguna de Guadalupe (ERB)	8 [a]	94 [b]	33 [b]	53	32 [b]
Cross-pollination between populations in Laguna de Guadalupe and El Rayo (GB)	8 [a]	15 [c]	34 [b]	13	21 [b]

*Average with same letters means no significant differences (Student-Newman Keuls, α=0.05; N=15 flowers per treatment).

Furthermore, percent of pollen tubes arriving at the ovules 72 h after pollination varied significantly among treatments ($F_{3,56}$ = 7.34, P = 0.0003), with *ERB* and *GB* treatments being similar (33 and 34 ovules penetrated per fruit, respectively) and well below control (98) or the *ERW* treatment (81) (Table 1).

Finally, fruit and seed set varied significantly among treatments. Fruit set was 1.75 times lower in the *ERB* treatment, 7 times lower in the *GB* treatment, and 1.55 times lower in the *ERW* treatment than in control (Table 1). Seed set was statistically different ($F_{3, 29}$ = 3.68, P = 0.0381). Seed sets were 1.65 and

2.52 times lower in the *ERB* and *GB* treatments than in the control treatment, which were statistically indistinguishable from one another (Table 1). Control and *ERW* were statistically indistinguishable.

Pollinations between isolated populations of *O. streptacantha* revealed partial crossing barriers as indicated by low numbers of pollen tubes reaching the base of the style and the ovules, and reductions in fruit and seed set, compared with crosses within a population or open pollination. Similar partial crossing barriers have been documented in *Dalechampia scandens* (Pélabon *et al.,* 2005), *Erythronium grandiflorum*, *Delphinium nelsonii, Ipomopsis aggregata* and *Phlox drummondii*, with local pollen performing better than pollen from distant plants (Levin, 1989; Waser and Price, 1989, 1991, 1993; Cruzan, 1990; Baker and Shore, 1995; Fischer and Matthies, 1997). In some cases, the strength of partial crossing barriers appears to vary with the distance between parents in a way that suggests the existence an optimal out-crossing distance (Waser and Price, 1989, 1991). Reductions in numbers of pollen tubes reaching and penetrating ovules, as we have documented for *O. streptacantha*, resembles some forms of interspecific incompatibility that contributes to the isolation of allopatric populations and sympatric species (De Netancourt, 1977, Waser 1993, Waser et al. 2000).

Furthermore, partial prezygotic crossing barriers in *O. streptacantha* were asymmetrical, with their strength depending on the source population of plants used as female parents. This asymmetry appears to be similar to, although less extreme than, the "unidirectional incompatibility" or "unilateral incompatibility" observed in some crosses between closely-related species. For example, the cross between *Lycopersicon esculentum* and *L. pennellii* is successful, but the reciprocal cross fails, therefore the cross can be successfully made in one direction but not the other because in interspecific crosses *L.esculentum*×*L. pennellii* are similar to the self-pollination of *L. esculentum* or *L. pennellii* in that the pollen tubes traveled the length of the style and successfully set seed, but in crosses between *L. pennellii* and *L. esculentum*, the pollen tube entered the transmitting tissue of the style and stopped growth approximately 2–3 mm into the style (Liedl *et al*. 1996).

Flowers of *O. streptacantha* were visited by a variety of bees, including honey bees [*Apis mellifera* (Apidae)] and solitary bees [*Diadasia rinconis* (Apidae), *Lithurge* sp. (Megachilidae), *Agapostemon texanus* (Halictidae), and *Perdita bicolor* (Andrenidae)]. However, virtually all visits were by two species, *Apis mellifera* (68% of all visits) and *Diadasia rinconis* (31%). These species used the *Opuntia* stigma for landing, at which time the pollen attached to the insects was deposited on the stigmatic surface.

The observations on foraging behavior of insects that visit *O. streptacantha* flower, revealed that *Apis mellifera* will travel up to 13 km to obtain pollen and nectar from wild *Opuntia* populations (Roubik, 1992). In contrast, *Diadasia rinconis* the main pollinator in *Opuntia* (Grant and Hurd, 1979; Grant *et al.*, 1979; Mandujano *et al.,* 1996) usually travel distances not greater than superior to 30 m from their nests. (García-Sánchez, 1984). The populations of *O. streptacantha* under study were separated by a distance greater than 30 km, and thus it is likely that few insect pollinators flew between them. This condition would surely reduce gene flow between populations of *Opuntia* under study.

The chromosome number was 88 for all individuals of *O. streptacantha* examined. Our observations coincide with recent reports of chromosome numbers obtained from *O. streptacantha* collected in other arid zones of Mexico and near the study site that reported the occurrence of octoploids plants (Pimienta-Barrios and Muñoz-Urias, 1995; Palomino and Heras, 2001). Because the two populations under study showed similar chromosome number (2n=88), we discard sympatric instantaneous speciation by changes in chromosome numbers in the populations under study (Futuyma, 1997).

CONCLUSION

We conclude that isolation of wild populations of *O. streptacantha* have reduced pollen transfer by insects between fragmented populations in this study. This isolation seems attributable to the development of prezygotic sexual incompatibility mechanisms that affect both pollen-grain germination and pollen-tube growth and, ultimately, a low percentage of ovules that develop into seeds.

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Figure 1. Mature plant of *Opuntia streptacantha* growing in an alluvial soil in a semiarid environment in the locality of El Rayo Pinos, Zacatecas

Figure 2. Morphology of mature cladode of *Opuntia streptacantha*

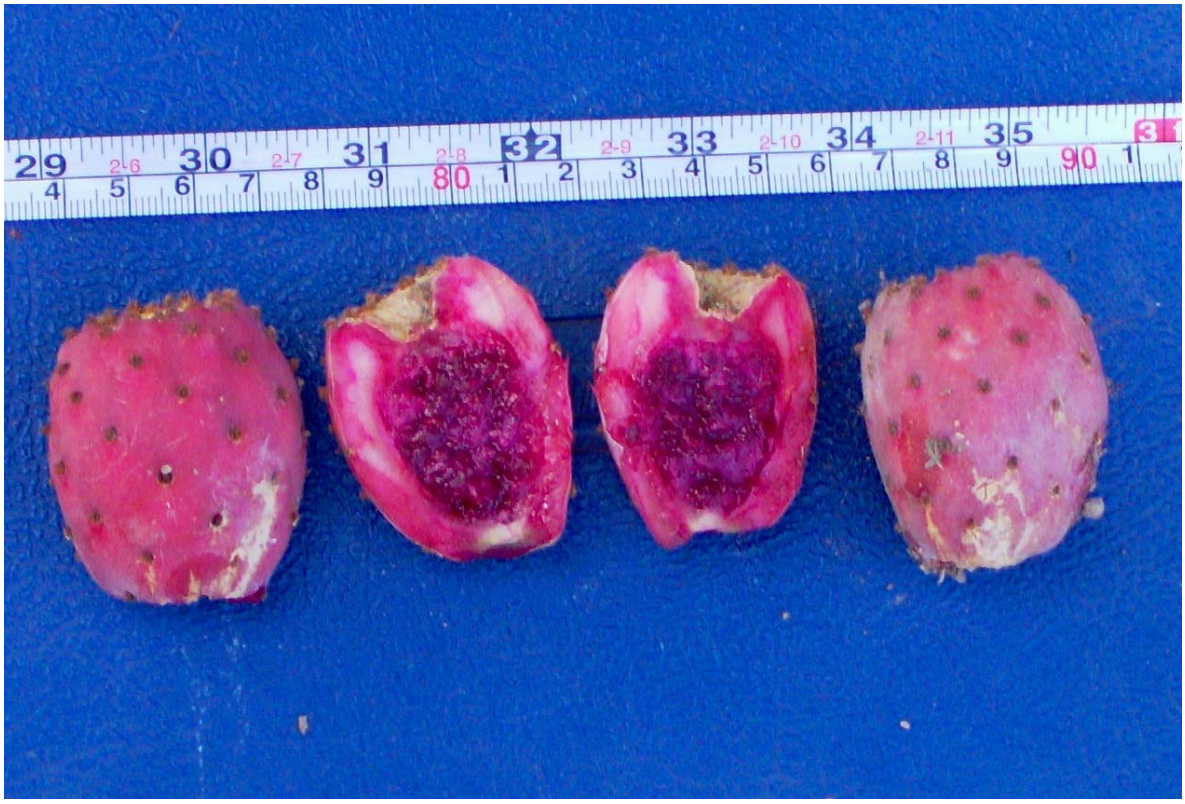

Figure 3. Mature fruit of *Opuntia streptacantha*

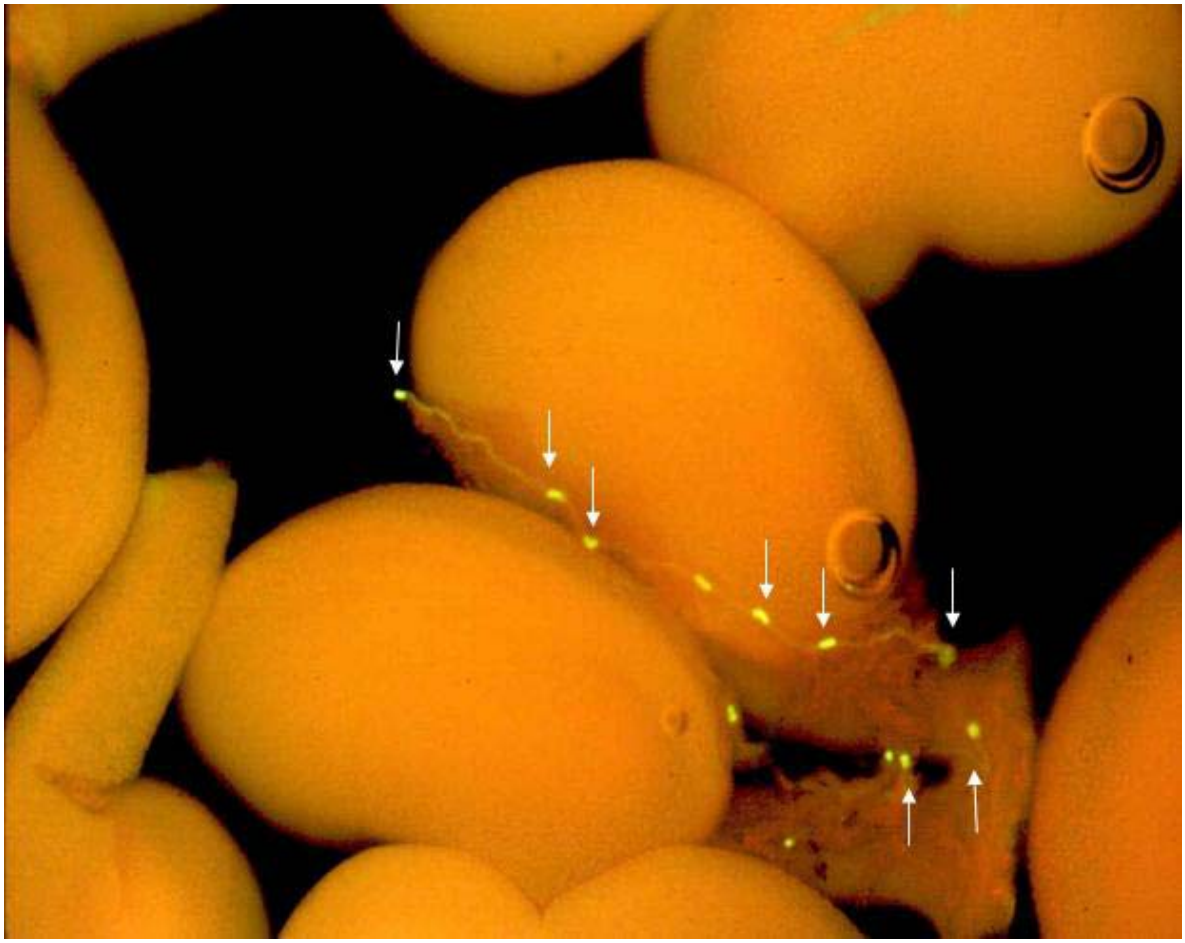

Figure 4. Aniline-blue-induced fluorescence showing the course of pollen tubes in the locule and approaching the micropile on the ovules. The trace of pollen tubes is distinguished by the fluorescence emitted by callose plugs (indicated by arrows) along the pollen tube. (25X)

Figure 5. *Apis mellifera* visiting *Opuntia streptacantha* flowers in El Rayo, Zacatecas, population