EVIDENCE OF GYNO DioECY IN KALLSTROEMIA GRANDIFLORA (ZYGOPHYLLACEAE): MICROSPOROGENESIS IN HERMAPHRODITE AND FEMALE PLANTS AND LACK OF REPRODUCTIVE COMPENSATION

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Understanding mechanisms involved in the maintenance of gynodioecy in natural populations requires evidence concerning the phenotypic expression and reproductive consequences of male sterility. We have recently discovered male sterility in populations of Kallstroemia grandiflora from northwestern Mexico. Here we describe the development of anther and pollen grains in hermaphrodite and female plants and explore whether females experience any compensatory advantage over hermaphrodites. The major difference in anther development between morphs was the early disintegration of tapetal cells in anthers of pistillate flowers. Abnormalities in developing pollen grains in pistillate flowers were seen in the microspore mother cells, microspore tetrads, and young and mature pollen grains. Biomass allocation to flowers, petals, and stamens were larger in hermaphrodites, while both sexes had the same number of ovules per flower. Hermaphrodite flowers were self-compatible. Pollination experiments revealed evidence of pollinator limitation for female plants but not for hermaphrodites. No evidence of reproductive compensation was detected, as flower and fruit production were similar for both sexes in two populations of K. grandiflora. We suggest alternative mechanisms that could maintain male sterility in natural populations of K. grandiflora.

Keywords: male sterility, pollen development, pollen limitation, self-compatibility, Sonoran Desert.

Introduction

Male sterility in plants is the result of the suppression of the male function in hermaphrodite flowers. The phenotypic expression may range from indehiscent anthers that contain viable pollen to the absence of male reproductive organs in flowers (Kaul 1988). Male sterility may be an inherited trait that arises spontaneously, or it may be induced by environmental stress (Kaul 1988). Embryological studies of sporogenous male sterility in plants have detected that pollen abortion usually occurs during or after the tetrad stage and is often associated with problems in the tapetum layer (Raghavan 1997). Male sterility has arisen independently in at least 71 angiosperm families, as spontaneous mutants in natural populations or as products of inter- or intraspecific crosses among agricultural plants (Laser and Lersten 1972; Dem’ya-nova 1985; Kaul 1988). The incidence of male steriles in crosses of agricultural species has led some authors to suggest that it is the result of incompatibility between nuclear and cytoplasmic genes (Hanson and Conde 1985; Frank and Barr 2003). Studies on the inheritance of male sterility in wild and agricultural plants have demonstrated that the transmission of this trait is nucleocytoplasmic, where cytoplasmic genes induce sterility while nuclear genes act as restorers of the male function (Charlesworth and Laporte 1998; Budar et al. 2003).

Male sterility has played an important role in the evolution of plant breeding systems such as gynodioecy and dioecy (Charlesworth and Charlesworth 1978; Maurice et al. 1993; Schultz 1994). The origin, spread, and maintenance of male sterility in gynodioecious populations have been problems of great interest to plant population biologists. Male sterility may occur with very low frequency (6%; Williams and Fender 1998), or it may reach high frequency (99%; Manicacci et al. 1998) in gynodioecious populations, depending on the mechanism driving the evolutionary dynamics. Theoretical models have identified a variety of mechanisms that could be involved in the maintenance and evolutionary dynamics of male sterility (Frank 1989; Gouyon et al. 1991; Jacobs and Wade 2003). As models have identified parameters such as the compensatory advantage of females or the restoration cost as critical, empirical studies have focused on the estimation of those variables in natural populations. Female plants usually have a compensatory advantage over hermaphrodites, producing more (smaller) flowers and greater fruit set, especially among species with many ovules per flower (Shykoff et al. 2003). However, although the fecundity advantage may be a sufficient condition for maintenance, it is not a necessary condition, as compensation has not been detected in species with high frequency of male steriles (Alonso and Herrera 2003).
2001). In those plants, other mechanisms might be involved in the maintenance of male sterility. The first steps toward understanding the maintenance of gynodioecy in wild populations usually require empirical evidence on the phenotypic expression and reproductive consequences of male sterility. Thus, the discovery of male sterility in new taxa often raises questions about how it is expressed and whether females achieve a fitness advantage over hermaphrodites.

We have recently discovered male sterility in populations of *Kallstroemia grandiflora* Torr ex Gray (Zygophyllaceae) from northwestern Mexico. As male sterility has not been previously described in this family (Dem’yanova 1985; Kaul 1988), we start by describing the development of anthers and pollen grains in hermaphrodite and pistillate flowers of this species. In addition, we explore whether female plants experience any compensatory advantage over hermaphrodites by comparing several reproductive traits. Thus, here we use a combination of embryological and ecological evidence to explore the developmental basis and the mechanism that could maintain male sterility in *K. grandiflora*.

**Material and Methods**

**Studied Species**

The Zygophyllaceae is a relatively small family composed of 25 genera and 250 species, distributed mostly in the arid tropics and subtropics (Porter 1969; Sheahan and Chase 1996). The family is currently positioned in the Eurosid I clade (APG II 2003). *Kallstroemia* is a large genus with 17 taxa distributed in the New World that belong to the tribuloid (Tribulidae) clade (Porter 1969; Sheahan and Chase 2000). The distribution of *Kallstroemia grandiflora* ranges from the Sonoran and Chihuahuan Deserts of North America to the tropical dry forest of the state of Guerrero in Mexico (Porter 1969). We have detected male sterility in the Sonoran Desert, where *K. grandiflora* is a summer annual that flowers from July to October, depending on water availability (Dimitt 2000).

The flowers last 1 d and are solitary and pentamous, with five petals, five sepals, and two whorls of five stamens. The gynoecium has 10 locules and a single ovule per locule. Pollen release and stigma receptivity are simultaneous, and apparently flowers are self-compatible (Porter 1969). Throughout its range, flowers are visited by more than 40 species of insects, mainly bees and wasps (Cazier and Linsley 1974, 1975; Osorio-Beristain et al. 1997). From the behavior of flower visitors, at least 10 species could be considered effective pollinators (Cazier and Linsley 1974, 1975).

**Study Area**

The frequency of females has been estimated using line transects and sampling of at least 200 plants per population. Female plants are easily detected by their reduced stamens and white anthers. Although some forms of partial male sterility have been detected (i.e., pistillate and perfect flowers in the same plant), their frequency was low (<1%). The frequency of male sterility among populations ranges from 0% to 44% throughout the Sonoran Desert (F. Molina-Freaner et al., unpublished data). We selected two populations in Sonora to study the development of pollen grains and explore whether compensatory advantages occur in female plants. One population (La Colorada) is located east of Hermosillo along Highway 16 (28°45.68’N, 110°28.64’W), and the other one (San Juanero) is located west of Hermosillo (28°58.53’N, 111°13.11’W). The frequency of females at La Colorada was 20%, while at San Juanero the frequency was 32% during 2001. Both populations were large, i.e., thousands of plants, and are located within the Plains of Sonora vegetational subdivision of the Sonoran Desert (Shreve 1964).

**Embryology**

During August 2001, a random sample of 20 hermaphrodite and 20 female plants were transplanted from each population into a shadehouse located at the Instituto de Ecología in Hermosillo. Plants were grown in 10-cm pots containing soil from each site, were watered every day, and were fertilized with Osmocote (NPK) every 15 d. After an acclimation period in the shadehouse, buds at several stages of development, as well as open flowers, were collected from female and hermaphrodite plants, fixed in FAA (63% ethanol, 5% glacial acetic acid, 5% formaldehyde, and 27% distilled water), and dehydrated in a graded ethanol series. We collected 200 samples from both morphs, and 50 random samples were processed and analyzed per morph (five plants per morph, two samples per stage, five stages). Two-thirds of the samples were embedded in LR-W (London resin white), cut at 1–2 μm, and stained with 1% toluidine blue, while the rest of the samples were embedded in Paraplast, sectioned at 9 μm, and stained with safranin and fast green.

Forty samples were processed for scanning electron microscopy (SEM; model JSM-5310LV, JEOL, Peabody, MA). These samples were dehydrated in absolute ethyl alcohol and critical-point dried, glued with silver paste to SEM stubs, blown with air to remove dust, coated with a layer of gold, and viewed at 15 kV.

**Flower Allocation**

We used a sample of 10 hermaphrodite and 10 female plants transplanted to the shadehouse from La Colorada to measure flower allocation. After the plants acclimated to the shadehouse, three recently opened flowers were collected from each plant early in the morning and immediately weighed to determine their fresh mass to the closest milligram. Flowers were then carefully dissected and dried, and the dry mass of petals, sepals, stamens, and pistil were obtained. In addition, we estimated flower diameter and the number of ovules per ovary, using flowers from the same plants. We explored whether females and hermaphrodites differed in flower allocation through a nested ANOVA (gender, plants nested within gender) on log-transformed data, using JMP software, version 3.1 (SAS Institute 1997).

**Pollination Treatments**

We explored whether hermaphrodites are self-compatible and whether plants experience pollinator limitation by performing pollination treatments at La Colorada. Four flowers...
from each of 10 hermaphrodite and 10 female plants were used for each pollination treatment. Flowers were bagged the day before flowering and subjected to manual outcrossing (MOC), manual self-pollination (MSP; hermaphrodites only), and a control open pollination treatment (CON) once they opened. For outcrossing, the stigmas were rubbed with a fresh anther from a random hermaphrodite in the population. Stigmas of hermaphrodites were rubbed with an anther of the same flower for the self-pollination treatment. Flowers were bagged after the treatment to prevent access to pollinators and predators. Flowers in the control treatment were available to any visitor. Fruit set was scored one week later, mature fruits were collected 15 d after the pollination treatments, and the number of seeds per fruit was counted. We explored whether females and hermaphrodites differ in response to the same pollination treatments (MOC, CON) and whether significant differences were detected between pollination treatments in each gender. Fruit set (arc sine-transformed) and number of seeds (log-transformed) were analyzed through a split-plot ANOVA model using JMP software, version 3.1 (SAS Institute 1997).

Reproductive Compensation

We explored whether female plants experience any compensatory advantage by measuring lifetime flower and fruit production in both populations during 2001. At the beginning of the flowering season (early August), we tagged a random sample of 20 female and 20 hermaphrodite plants in each population. Each week, we counted the number of open flowers and the number of developing or mature fruits on each plant. We calculated overall flower and fruit production by averaging weekly data for individuals of each gender in each population. We explored whether females and hermaphrodites differ in flower and fruit production (both log-transformed) through a nested ANOVA (population, plants nested within gender), using JMP software, version 3.1 (SAS Institute 1997). At La Colorada we estimated the mean number of seeds and seed mass per fruit, and at the end of the season we harvested a sample of 20 female and 20 hermaphrodite plants in which we estimated fruit production by counting the number of fruit scars from each plant.

Results

Embryology

The stamens of perfect flowers are larger than stamens of pistillate flowers (fig. 1A, 1B). Early in anther development, the four microsporangia are formed. The anther wall consists of two different layers: epidermis and two or three parietal strata surrounding the sporogenous cells (fig. 1C, 1D). At this stage of anther development, we do not detect any major difference between anthers of perfect and pistillate flowers.

At the microspore mother cells developmental stage, the middle layers are more evident in anthers of perfect flowers (fig. 2A). The other layers of the anther wall look quite similar between sexes (fig. 2A, 2B). However, the microspore mother cells in developing anthers of perfect flowers are round and occupy all the locular space (fig. 2A). In contrast, microspore mother cells in pistillate flowers are half-moon-shaped and adhere to the tapetal cells, leaving an empty area in the locular space (fig. 2B). Meiosis takes place in anthers of both morphs, and it is possible to observe tetrads of haploid microspores with an incipient exine enclosed in a callose wall (fig. 2C, 2D). However, even though we analyzed the same number of samples of both sexes, microspore tetrads in pistillate flowers were rarely observed. In addition, microspore tetrads of perfect flowers show round microspores (fig. 2C), contrasting with microspores of pistillate flowers that show the half-moon shape observed in a previous stage (fig. 2D).

Once the microspores are released from the tetrads, major differences appear. While the anther wall of hermaphrodite flowers maintains tapetal cells, these cells disappear in pistillate flower anthers of the same stage (fig. 3A, 3B). In addition, the anthers of perfect flowers show round-shaped pollen grains (fig. 3A), whereas ca. 30% of the locules of anthers from pistillate flowers usually possess a few elongated, empty pollen grains, because the nucleus and cytoplasm have degenerated (fig 3B). At this stage pollen grains of hermaphrodite plants show a regular exine pattern that contrasts with the abnormal exine deposition in pollen grains of female plants (fig. 3A, 3B).

In hermaphrodite flowers during anther dehiscence, the middle wall layers and tapetum disappear. The only layers of the anther wall remaining are the epidermis and the enlarged endothecial cells, and it is also possible to observe the stomium region (fig. 3C). In contrast, in pistillate flowers, the anther undergoes a striking cell death program, and the septum region is still present (fig. 3D). However, in some pistillate flowers, anther breakage takes place at the stomium region within the epidermal layer (fig. 4A). Mature pollen grains from hermaphrodite flowers reach equatorial diameters of 61.9 ± 1.72 μm (mean ± 1 SD here and hereafter), with the exine fully developed (fig. 4B), while in pistillate flowers, pollen grains are smaller (23.1 ± 2.45 μm), with an abnormal exine (fig. 4C).

Pollination Treatments

Hermaphrodite plants were self-compatible (fig. 6). No significant differences were detected in fruit set between female and hermaphrodite plants (F = 1.08, df = 1, P = 0.30). However, significant differences were detected between pollination treatments (gender; F = 8.06, df = 2, P = 0.0014).
Fig. 1  Flowers of hermaphrodite and female plants and microsporangia of perfect and pistillate flowers. A, Hermaphrodite flower showing the normal stamens at dehiscence; bar = 1000 µm. B, Pistillate flower showing the reduced stamens; bar = 1000 µm. C, Cross section of a young hermaphrodite microsporangium (light field); bar = 10 µm. D, Cross section of a young pistillate microsporangium (phase contrast); bar = 10 µm. 

\( a = \) anther; \( f = \) filament; \( p = \) pistil; \( e = \) epidermis; \( ps = \) parietal stratus; \( sc = \) sporogenous cells.
Fig. 2 Microsporangia and tetrads of hermaphrodite and pistillate flowers. A, Cross section of a hermaphrodite microsporangium, showing normal microspore mother cells; bar = 10 μm. B, Cross section of a pistillate microsporangium, showing degenerating microspore mother cells and the locular space; bar = 10 μm. C, Tetrad with callose wall and normal microspores from hermaphrodite anther; bar = 10 μm. D, Tetrad with the half-moon-shaped microspores and the callose wall from a pistillate anther; bar = 10 μm. e = epidermis; en = endothecium; ml = middle layer; t = tapetum; mmc = microspore mother cell; ls = locular space; mi = microspore; cw = callose wall.
Fig. 3 Anther sections. A, Longitudinal section of hermaphrodite anther at the young microspore stage, showing the tapetal cells and the microspores with a thick wall cell; bar = 10 μm. B, Cross section of a pistillate anther at the young microspore stage, lacking tapetum but having empty microspores with abnormal exine deposition; bar = 10 μm. C, Hermaphrodite anther at dehiscence, showing the stomium and mature pollen grains; bar = 10 μm. D, Pistillate mature anther, showing a collapsed locule and degenerated anther wall with some abnormal pollen grains; bar = 10 μm. mi = microspore; e = epidermis; en = endothecium; t = tapetum; st = stomium; mpg = mature pollen grain; apg = abnormal pollen grain; s = septum; cl = collapsed locule; ct = connective tissue.
Contrast tests showed significant differences only in females ($t = 4.01$, df = 1, $P < 0.0003$), indicating pollinator limitation. No significant differences were detected in number of seeds for pollination treatments between sexes ($F = 0.25$, df = 1, $P = 0.61$) or among pollination treatments (gender; $F = 0.38$, df = 2, $P = 0.68$).

**Reproductive Compensation**

We did not detect significant differences in lifetime flower ($F = 1.49$, df = 2, $P = 0.23$) and fruit production ($F = 1.65$, df = 2, $P = 0.19$) between sexes during 2001. We did, however, detect significant differences in fruit production between populations ($F = 48.6$, df = 1, $P < 0.001$; fig. 7). At La Colorado, no significant differences were detected in number of seeds per fruit (for hermaphrodites, 9.7 ± 0.9, females, 9.4 ± 0.8; $t = -1.16$, df = 48, $P > 0.25$), seed mass (for hermaphrodites, 0.057 ± 0.008 g, for females, 0.053 ± 0.007 g; $t = -1.49$, df = 48, $P > 0.142$), or total number of fruits per plant, estimated as the number of fruit scars (for hermaphrodites, 80.9 ± 109; for females, 76.5 ± 108; $t = -0.11$, df = 32, $P > 0.90$).

**Discussion**

As far as we know, this is the first report of male sterility in the Zygophyllaceae. Most members of this family have hermaphroditic flowers, and only Neoluederitzia has unisexual flowers (Porter 1969; Sheahan and Chase 1996). The embryological study of *Kallstroemia grandiflora* showed abnormalities in the anthers of pistillate flowers in the microspore mother cells, tetrad formation, and the young and mature pollen grain stages. In particular, the early degeneration found in the tapetal cells of pistillate flowers in *Kallstroemia* has some resemblance to the pattern of cytoplasmic male sterility of some other species. Our data revealed no evidence of reproductive compensation in female plants. The apparent lack of compensation and the retention of reduced stamens with low quantities of nonfunctional pollen in anthers of pistillate flowers could be interpreted as evidence of a relatively recent evolutionary origin of gynodioecy in *Kallstroemia*.

The embryological study showed at least four different developmental stages in which abnormalities were detected in anthers of pistillate flowers. The first abnormalities were found early in development, at the premeiotic stage in the microspore mother cells, indicating that the effects of male sterility are expressed early in the development of pollen.
plants of hermaphrodites and females may outperform the outcrossed progeny of female plants, as has been reported for the great majority of gynodioecious species (Delph 1996; Shykoff et al. 2003). In Kallstroemia, hermaphrodite flowers were 1.6 times larger than pistillate flowers, which is slightly larger than the reported average of 1.3 (Delph 1996). Nevertheless, we did not find any evidence of reproductive compensation (fig. 7), although clear differences were detected in resource allocation between sexes. Embryological evidence indicates that some pistillate flowers invest some resources in the partial pollen production and could be at least partially responsible for the lack of reproductive compensation found in Kallstroemia. Female plants in Kallstroemia allocate fewer resources to flowers and floral organs, which is a prerequisite for fecundity compensation. Hermaphrodite flowers were larger than pistillate flowers, as has been reported for the great majority of gynodioecious species (Delph 1996; Shykoff et al. 2003). In Kallstroemia, hermaphrodite flowers were 1.6 times larger than pistillate flowers, which is slightly larger than the reported average of 1.3 (Delph 1996). Nevertheless, we did not find any evidence of reproductive compensation (fig. 7), although clear differences were detected in resource allocation between sexes. Embryological evidence indicates that some pistillate flowers invest some resources in the partial production of pollen. Reproductive compensation requires the reallocation of resources not used in pollen in order to produce more and/or better seeds in female plants. Thus, it is possible that reallocation in females might be prevented by partial pollen production and could be at least partially responsible for the lack of reproductive compensation found in Kallstroemia.

Given the invariant number of ovules per flower in Kallstroemia, compensation effects could be expected in seed mass or total seed production (Shykoff et al. 2003). However, our data showed similar values of seed mass and seed production between sexes. It is possible that the progeny of females may outperform the outcrossed progeny of
hermaphrodites even if seed mass of female plants is either equal to or smaller than that of hermaphrodites (Delph et al. 1999). Thus, future studies should address the relative performance of seeds from female and hermaphrodite plants in order to explore the role of postgermination compensation. Unfortunately, we have been unable to germinate seeds of *Kallstroemia* to explore this issue.

Our data revealed evidence of pollinator limitation in females but not in hermaphrodites. However, given that no differences were detected in overall seed production between sexes, pollinator limitation in females may occur during brief periods of time. Because the available evidence indicates that flowers attract a large number of insect visitors (Cazier and Linsley 1974, 1975; Osorio-Beristain et al. 1997), spatiotemporal variation in pollinator availability could affect the pollination success of female plants. Future studies of pollinator activity and pollen deposition in morphs under contrasting frequencies are required to explore the role of pollinators in the dynamics of male sterility in *K. grandiflora*.

As in other members of Zygophyllaceae, *K. grandiflora* is self-compatible (Porter 1969; Simpson et al. 1977; Debandi et al. 2002). However, it is unclear whether hermaphrodites are capable of autonomous self-pollination. It is also unclear whether inbreeding depression is expressed in the progeny of selfed hermaphrodites. Thus, future studies should explore the ability of hermaphrodites to self in the absence of pollinators, the actual levels of outcrossing rates, and the possible role of inbreeding depression in the maintenance of male sterility.

Currently, we do not know what mechanism maintains male sterility in *K. grandiflora*. Several processes could be involved. It is possible that compensation occurs after germination either because seeds from females are better provisioned or through inbreeding effects in hermaphrodites. In addition, once females are established in populations, it is likely that seed dormancy and the persistent seed bank could be involved in delaying their elimination independently of any possible compensation. Desert annuals usually posses a persistent seed bank that buffers changes to rapidly growing individuals of the population (Venable and Lawlor 1980; Moriuchi et al. 2000). Because populations of *K. grandiflora* can be maintained even after several years without new seed input, their seeds have remarkable longevity under desert conditions (Cazier and Linsley 1975; E. Cuevas García, personal observation). Therefore, female plants could persist in populations for several years without exhibiting major compensatory advantages. Finally, if male sterility is nucleocytoplasmic, gynodioecy in *K. grandiflora* could be maintained.
by the dynamics imposed by the interaction between cytoplasmic and nuclear restorer alleles (Frank 1989).

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