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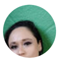
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FUNCTIONAL DIOECY IN *ECHINOCEREUS*: ONTOGENETIC PATTERNS, PROGRAMMED CELL DEATH, AND EVOLUTIONARY SIGNIFICANCE

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Premise of research. Dioecy has evolved numerous times among unrelated Cactaceae taxa. Changes in ontogenetic patterns determine final floral morphology and breeding system. It has not been established whether these changes share a common pattern in the cactus family, which would allow inferences to be made in the evolutionary process that underlies the development of unisexual flowers. *Echinocereus* has related hermaphroditic and dioecious taxa, which makes it a useful system to determine how transitions from hermaphroditic to unisexual flowers occur.

Methodology. Sexual systems in five *Echinocereus* taxa were determined using controlled pollinations and field observations of floral morphology and fruit-set patterns. Floral and seed development were analyzed using histological observations and scanning electron microscopy; a TUNEL assay was used as an indicator of DNA fragmentation to corroborate programmed cell death during floral and seed development.

Pivotal results. Changes in ontogenetic patterns result in unisexual flower development in four species of *Echinocereus* section *Triglochidiati* (Cactaceae). Populations of *E. coccineus*, *E. polyacanthus*, *E. pacificus*, and *E. mombergerianus* are functionally dioecious, whereas *E. triglochidiatus* is hermaphroditic. At anthesis, the male (female-sterile) flowers of dioecious species exhibit a fully developed gynoecium with ovules where, after fertilization, seeds are aborted at early stages of postzygotic development. Flowers on female (male-sterile) plants do not form pollen grains in any of the dioecious species. Programmed cell death mediates both seed and pollen abortion. We propose that heterochrony controls male sterility by accelerating the process of programmed cell death in tapetal cells during anther development in female flowers.

Conclusions. Dioecious taxa of *Echinocereus* share the ontogenetic pattern of male and female sterility. We provide groundwork for future research on developmental pathways of unisexual flowers in closely related taxa in Cactaceae, as well as the embryological framework to elucidate the molecular/genetic mechanisms involved in the development of unisexuality.

Keywords: Cactaceae, dioecy, heterochrony, late female sterility, male sterility, seed abortion.

Online enhancements: appendix tables and figures.

Introduction

In addition to its high number of species (ca. 126 genera and 1900 species [Anderson 2001] or ca. 124 genera and 1438 species [Hunt et al. 2006]), the Cactaceae family is one of the most morphologically diverse plant groups and includes taxa with arborescent, shrub-like, epiphytic, and climbing growth habits, the stems of which are cylindrical, globose, or flattened (Guzmán et al. 2003). Its floral diversity is observed in variations in whorl size, color, and shape and in its breeding systems. This variation and the ecological importance of this family make it an interesting model to study the evolution of floral and morphological diversity (Wolf and Martínez del Rio 2003; Casas et al. 2006; Mandujano et al. 2010; Hernández-Hernández et al. 2011; Grosse-Veldmann et al. 2016).

Hermaphroditism is the predominant breeding system in the family, seeing that more than 98% of its species produce flowers with both a functional gynoecium and androecium. However, unisexual flowers have been described in eight genera in the Pereskioideae, Opuntioideae, and Cactoideae subfamilies (Orozco-Arroyo et al. 2012). Among species that produce unisexual flowers, most are dioecious, in which male or female flowers are separated in different individuals, or subdioecious, which pro-

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duce male, female, and hermaphroditic morphs, although the latter are usually present in low numbers in populations (Leuenberger 1986; Fleming et al. 1994; Strittmatter et al. 2002, 2006; Baker 2006; del Castillo and Argueta 2009; Orozco-Arroyo et al. 2012). Dioecious systems represent an extreme form of sexual separation that ensures outcrossing, although additional evolutionary forces have also been implicated (e.g., resource allocation [Charlesworth and Charlesworth 1981] or pollination system [Bawa and Beach 1981]). Given the importance of dioecious systems in the promotion and maintenance of genetic diversity (Barret 2002; Charlesworth 2006), it is essential to comprehend the proximate causes of how these systems develop in the Cactaceae family. The first step is to analyze the ontogenetic mechanisms that result in the production of male-sterile or female-sterile flowers, thus gaining insight into the development of breeding systems. The appearance of unisexuality in three Cactaceae subfamilies (Pereskioideae, Opuntioideae, and Cactoideae) suggests that unisexuality may be convergent in Cactaceae, as it is in other lineages of angiosperms (Lankester 1870; Renner 2014). However, in closely related taxa, unisexuality could represent a homologous trait. In some species of these subfamilies, unisexual flowers start their development as bisexual. Nevertheless, it has not been determined whether the ontogenetic pattern leading to the abortion of a sexual organ in a particular flower morph is shared or whether it differs among Cactaceae taxa and is thus the outcome of either a parallel or convergent evolutionary trend (Gould 2002; Hall 2007; Wake et al. 2011). If the trend is parallel, candidate genes may be identified and their expression analyzed throughout development in order to understand whether these genes have been recruited repeatedly (Harrison et al. 2005; Scotland 2011; Wake et al. 2011). If the trend is convergent, it may be possible to try to understand the potential selective forces that have led to similarities in the evolution of unisexual flowers (Renner and Ricklefs 1995; Ainsworth 2000; Scobell and Schultz 2005; Stehlik et al. 2008; Spigler and Ashman 2011). In order to make predictions concerning the origin and evolution of dioecy in Cactaceae, members of a dioecious clade with clearly hermaphroditic ancestors must be included in the analysis to establish the sequence of the ontogenetic and physiological events that determine the morphological (sexual) variants upon which natural selection acts.

Echinocereus Engelm. (Cactoideae, Cactaceae) has approximately 65 species divided into eight sections (Sánchez et al. 2018). In section *Triglochidiati*, five species, namely, *E. coccineus*, *E. polyacanthus*, *E. yavapaiensis*, *E. mombergerianus*, and *E. pacificus*, produce unisexual flowers and are also known to be polyploids (Ferguson 1989; Rebman 2003; Baker 2006), yet the nature of their reproductive systems was determined superficially and is based solely on their external floral morphology (Blum et al. 1998; Rebman and Roberts 2012). In all five of these species, flowers have a fully developed gynoecium at anthesis, but field observations indicate that fruits are not developed in the male plants of four species, suggesting they are cryptically dioecious, as described by Hoffman (1992) for some populations of *E. coccineus*. *Echinocereus coccineus*, *E. polyacanthus*, *E. yavapaiensis*, *E. mombergerianus*, and *E. pacificus* are closely related species occurring in northeastern Mexico and in the southeastern United States. Depending on the author, *E. mombergerianus* and *E. pacificus* may be considered as subspecies of *E. polyacanthus* (Ferguson 1989; Sánchez et al. 2014, 2018). In this study, we consider each taxon as a separate species. Flowers appear bisexual in all dioecious taxa in *Echinocereus*, but little is known about the physiological and ontogenetic mechanisms involved in the establishment of unisexuality, which could also help corroborate phylogenetic relationships in this section.

The purpose of this study was therefore to describe and compare anther, ovule, fruit, and seed development; mature morphology of androecium and gynoecium; sexual system; and number of seeds per fruit in male and female flowers of dioecious species of *Echinocereus* section *Triglochidiati*. Emphasis was put on the identification and description of the critical stages that give rise to functionally unisexual flowers and the occurrence of programmed cell death (PCD) as the mechanism involved in the abortion of sexual whorls. We propose that the use of this group of dioecious species and the related hermaphroditic species will enable clarification of aspects of the sexual system based on mature floral morphology, pollination treatments, seed set, and changes in ontogenetic patterns.

Material and Methods

Studied Species, Sites, and Sexual Systems

We included four of the five dioecious species (*Echinocereus coccineus* Engelm., *E. polyacanthus* Engelm., *E. pacificus* (Engelm.) Britton & Rose, and *E. mombergerianus* G. Frank) and one hermaphroditic species (*E. triglochidiatus* Engelm.) as a reference for hermaphroditic floral development, since it is closely related to the dioecious species studied (Sánchez et al. 2014, 2018). *Echinocereus yavapaiensis* was not included because the flowering season was missed during fieldwork, and its origin is likely hybrid since it is a hexaploid taxon. Plants in populations were sexed in spring of 2009–2010 and 2014–2016 using several criteria, including anther morphology (collapsed or turgent), absence/presence of pollen, and mature fruits with seeds. Presence of gynoecium as a sexing criterion was ruled out because all flowers presented an apparently normal gynoecium. Plants with flowers that had both pollen grains and mature fruits with seeds were considered hermaphroditic. In each species, floral buds, flowers, immature fruits, and mature fruits were collected from each sexual morph (from at least 10 male, 10 female, and 10 hermaphroditic plants), fixed in FAA (37% formaldehyde, 96% ethanol, acetic acid, distilled water, 10 : 50 : 5 : 35 v/v), and stored in 70% ethanol for developmental work. One population of each species was studied, either from the middle of the species range or from the endemic population (*E. pacificus* and *E. mombergerianus*). A population of *E. coccineus* was studied in Samalayuca, Chihuahua, Mexico (*D. Sánchez 48*, MEXU), and collections were made using 19 female and 18 male individuals. The *E. polyacanthus* population was located in El Salto, Durango, Mexico (*D. Sánchez 12, 13*, MEXU), and 15 female and 19 male individuals were used. The population of *E. triglochidiatus* was found in the Jicarilla Mountains in Lincoln County, Carrizozo, New Mexico (*M.A. Baker 16191*, ASU), where material was collected from 10 individuals. The endemic *E. pacificus* was studied in San Carlos, Ensenada, Baja California, Mexico (*S. Arias 1828*, MEXU); samples were taken from 33 male and 20 female individuals in this population. The species *E. mombergerianus* is endemic to San Pedro Mártir, Baja California (*S. Arias 1806, 1807*, MEXU), where 33 male and 18 female individuals were sampled.

Mating System

Pollination treatments were performed in the field and in live collections at the Jardín Botánico of the Universidad Nacional Autónoma de México (*E. coccineus*) and at the Desert Botanical Garden (DBG) in Phoenix, Arizona (*E. triglochidiatus*). In dioecious species, 10 male plants and 10 female (male-sterile) plants were used for cross-pollination. Eight hermaphroditic plants were used for cross-pollination treatments, five male and five hermaphroditic plants were used for self-pollination, and three female plants were subjected to an apomixis treatment (freshly opened flowers were covered with fine mesh bags), using at least two flowers per plant. Fruits were collected 6–8 wk later. We compared the number of seeds to assess the allocation of resources to seed production in females among species and between females and the hermaphroditic species. The number of seeds per fruit was counted, and differences among morphs and treatments were determined by Kruskal-Wallis (K-W) and Dunn's multiple comparisons tests, using GraphPad Prism, version 6.00, for Windows (GraphPad Software, La Jolla, CA; http://www.graphpad.com). Since male flowers presented gynoecia, we wanted to determine their functionality by evaluating pollen tube growth through styles. To this end, we performed self- and cross-controlled pollinations in all studied species, sampling five plants from each floral morph (male, female, and hermaphroditic), using two flowers from each, per pollination treatment. Pollination treatments in males and hermaphroditic plants were carried out using either cross-pollen or self-pollen in emasculated flowers. Pollination of female plants was performed using only cross-pollen. All the flowers were covered with fine mesh bags. Five flowers from each treatment were harvested 72 h after pollination (determined by pilot experiments). Pistils were fixed in FAA and softened in a 10% $NaHSO_3$ solution, using a steam autoclave for 5 min; stained with decolorized 1% aniline blue diluted in 0.1 N K_3PO_4 for 12 h; mounted; squashed on glass slides; and examined for pollen tube growth using an epifluorescence microscope (Olympus Provis AX70). Observations were made at three locations, namely, the stigma surface, median region, and basal region of the style. The rest of the pollinated flowers continued development to await fruit and seed production.

Developmental and Histological Analyses

To perform a light microscopy analysis of floral buds, flowers, fruits, and seed development, fixed material was dehydrated in an ascending ethanol series and embedded in either LR White resin (Electron Microscopy Sciences) or Paraplast (Sigma-Aldrich). Eight stages were established during bud and flower development based on the days elapsed from the detection of small floral buds up to anthesis (table A1, pt. A; tables A1, A2 are available online). For seed development, the stages used ranged from pollination to the mature fruit (table A1, pt. B). Five samples were used at each stage. LR White–embedded material was sectioned at 1–2 μm, using an RM2164 Leica ultramicrotome, and stained with 0.05% toluidine blue. Paraplast-embedded material was sectioned at 5–7 μm, using a rotary microtome, and stained with safranin and fast green. Histochemical seed analysis was conducted on Paraplast-embedded seed sections, using periodic acid–Schiff's reagent to identify nonsoluble polysaccharides and starch granules and Lugol (IKI) solution (2.5% iodine, I_2, 5% potassium iodine) to discern starch granules. Proteins were identified by staining sections with naphtol blue black (O'Brien and McCully 1981; Ruzin 1999; Márquez et al. 2016). Photomicrographs were taken using an Olympus Provis AX70 light microscope equipped with a digital camera. For scanning electron microscopy analysis, two samples of each stage fixed in FAA were dehydrated in a graded series to 100% ethanol and critical-point dried with CO_2. Samples were mounted on metallic stubs, sputter-coated with gold, viewed, and photographed using a Jeol JSM-5310LV scanning electron microscope.

Programmed Cell Death during Flower and Seed Development

In order to detect DNA fragmentation during flower and seed development, as a normal process or causative factor of the loss of function, a TUNEL assay (Roche) was performed. In this assay, the enzyme terminal deoxynucleotidyl transferase (TdT) identifies fragments of DNA at free 3′-OH− groups and catalyzes the addition of fluorescein 12-dUTP for subsequent detection. Ovaries, anthers, and developing fruits from each stage were fixed with 4% formaldehyde diluted in phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 $7H_2O$, 2 mM KH_2PO_4 H_2O), pH 7.3, dehydrated, and embedded in Paraplast. Semithin sections were mounted on Superfrost glass slides (Fisher). The TUNEL assay was performed using a PCD kit (Roche Applied Science, Mannheim) following the manufacturer's instructions, with minor modifications, as reported previously (Flores-Rentería et al. 2013). Finally, slides were mounted with 4′,6-diamidino-2-phenylindole (DAPI) diluted in fluorescent mounting medium (Dako). Positive and negative controls were used to exclude background labeling; the former was performed on sections after pretreatment with DNase I, whereas the terminal transferase enzyme was omitted in the latter. Tissue sections were visualized using an Olympus FV1000 confocal microscope.

Results

Field Observations and Sexual System Determination

Examination of flowers from individual plants at anthesis revealed that populations of *Echinocereus coccineus*, *E. polyacanthus*, *E. mombergerianus*, and *E. pacificus* contain two floral morphs—functional female (male-sterile) and functional male (female-sterile) flowers. Hereafter, these will be referred to as female and male, respectively. Female flowers exhibited whitish shriveled anthers positioned under stigmatic lobes (fig. 1*A*, 1*I*), with no pollen grains (fig. 1*B*, 1*J*). At anthesis, the gynoecium was fully developed, presenting a bright green stigma with long and divergent stigmatic lobes (fig. 1*A*, 1*I*) and an ovary filled with mature curved ovules with long funiculi (fig. 1*C*, 1*K*). Ovule viability was evidenced by the formation of seeds and fruits following cross-pollination experiments, as well as in field observations (fig. 1*D*, 1*L*). In contrast, the male morph produced flowers with turgescent pink-purple anthers (fig. 1*E*, 1*M*, 1*N*)

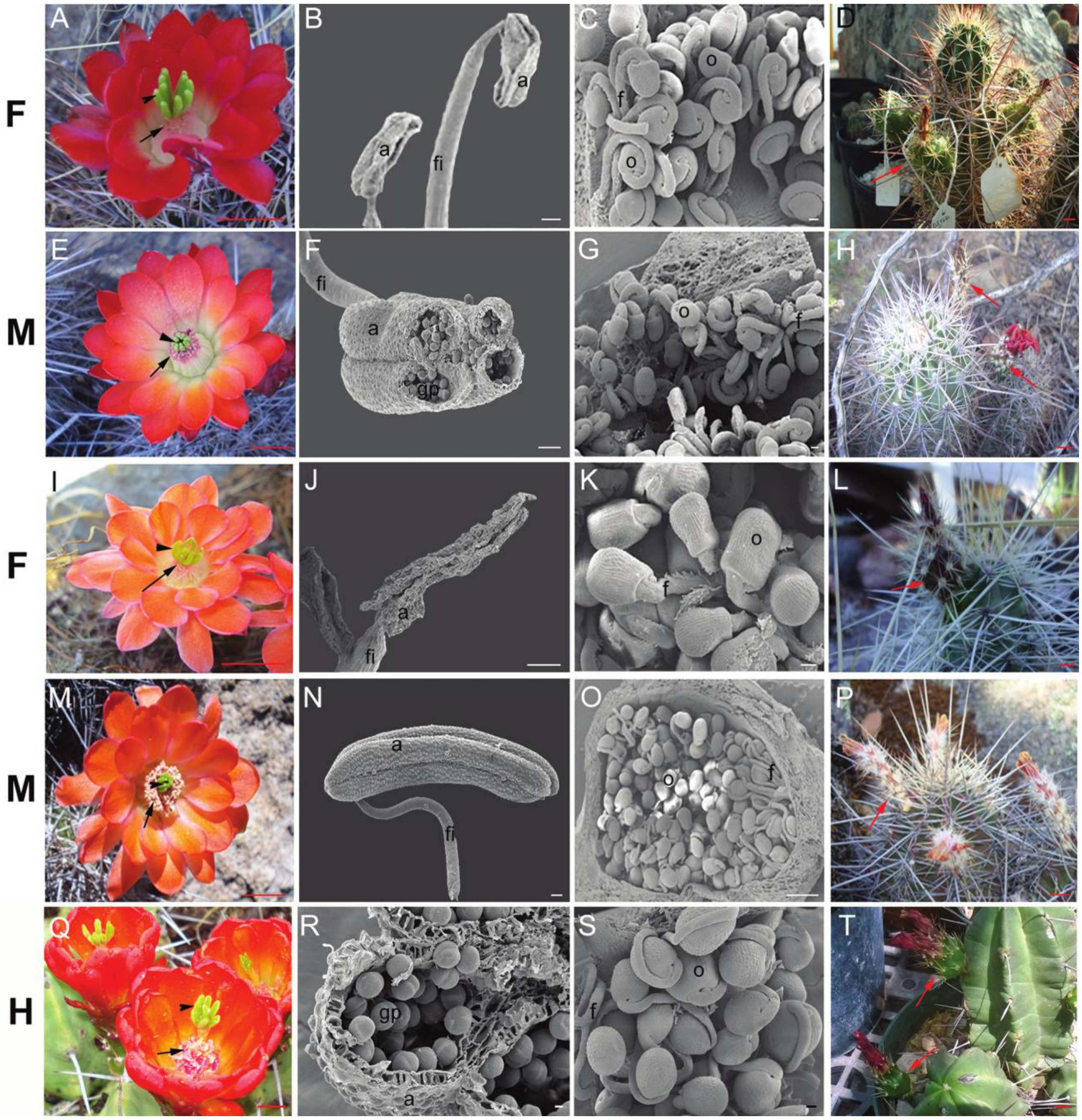

Fig. 1 Floral morphology of *Echinocereus*. *Echinocereus coccineus* (*A*–*H*), *E. polyacanthus* (*I*–*P*), and *E. triglochidiatus* (*Q*–*T*). *A, I*, Female flowers showing green stigma (arrowhead) and reduced yellow anthers without pollen (arrow). *B, J*, Close-up view of collapsed anthers lacking pollen. *C*, *G*, *K*, *O*, *S*, Ovaries from female, male, and hermaphroditic flowers with multiple curved ovules attached to placenta by long funiculi. *D*, *L*, *T*, Developing fruits (arrows) from cross-pollinated female and hermaphroditic flowers. *E*, *M*, Male flowers showing closed stigma (arrowhead) and purple anthers (arrow). *F*, *N*, *R*, Close-up view of anthers. Numerous pollen grains are observed inside microsporangia (*F*, *R*). *H, P*, Abortive fruits (arrows) in pollinated male flowers. *Q*, Hermaphroditic flowers showing open stigma (arrowhead) and purple anthers (arrow). a = anther; f = funicle; fi = filament; gp = pollen grains; o = ovule. Scale bars = 1 cm (*A*, *D*, *E*, *H*, *I*, *L*, *M*, *P*, *Q*, *T*), 100 μm (*B*, *C*, *F*, *G*, *J*, *K*, *N*, *S*), 1 mm (*O*), 10 μm (*R*).

that contained numerous pollen grains (fig. 1*F*, 1*N*) and were positioned at the same level as the stigmatic lobes. The ovary in the male morph contained numerous mature and well-developed ovules (fig. 1*G*, 1*O*), yet only remnants of undeveloped fruits were observed in any of the populations visited (fig. 1*H*, 1*P*) or any of the individuals used in cross-pollination treatments. Flowers in the male morph wilted 5–7 d after pollination. These observations confirm that the sexual system in *E. coccineus*, *E. polyacanthus*, *E. mombergerianus*, and *E. pacificus* is functionally dioecious. In contrast, the hermaphroditic *E. triglochidiatus* is both male fertile and female fertile (fig. 1*Q*–*T*). Its anthers produced pollen grains (fig. 1*R*), and its ovary, filled with numerous ovules (fig. 1*S*), produced fruit and seeds after fertilization (fig. 1*T*).

Pollen Grain Germination and Pollen Tube Growth along the Gynoecium

Cross-pollinations in male, female, and hermaphroditic flowers of all studied species indicated that gynoecia in the three morphs were functional. Although the stigmatic lobes of the male morph do not fully expand, the stigma is functional in both males and females of dioecious species, as evidenced by the germination of pollen grains (fig. 2*A*, 2*B*). Numerous pollen tubes were traced growing along the style in both floral morphs (fig. 2*C*, 2*D*). In both males and females of the four species, the pollen tubes entered the ovary, surrounded the ovules, penetrated the micropyle (fig. 2*E*, 2*F*), and carried out fertilization. This suggests that the loss of female function in male flowers is not prezygotic, but rather postzygotic, and results from an early abortion of seeds and fruits. Cross-pollination treatments in hermaphroditic flowers showed pollen grain germination in stigma and pollen tube development through style and ovary (not shown).

Self-pollination treatment showed that the male morph of dioecious species is self-compatible and that the stigma accepts and promotes pollen grain hydration and germination (fig. A1*A*; figs. A1, A2 are available online). Also, pollen tubes were observed growing through the style and reaching the ovules (fig. A1*C*, A1*E*). In contrast, the self-pollination treatment in hermaphroditic *E. triglochidiatus* showed that it is self-incompatible (fig. A1*B*, A1*D*, A1*F*), since no pollen tubes were observed beyond the stigma and no seeds were formed.

Embryology

Androecium development. Carpel and stamen were initiated in floral buds in both morphs of the dioecious species and hermaphroditic *E. triglochidiatus* (fig. 3*A*, 3*J*). In male, female, and hermaphroditic flowers, the secondary parietal layer had divided by stages 1 and 2, forming the middle layer and tapetum as occurs in monocotyledonous anther wall development (fig. 3*B*). By stage 3, the anther wall consisted of the epidermis, endothecium, middle layer, and a secretory binucleate tapetum, and microspore mother cells were surrounded by callose inside the locule (fig. 3*C*). At stage 4, the microspore mother cells underwent simultaneous meiosis (fig. 3*D*). By stage 5, tetrahedral tetrads enclosed in callose were observed in the microsporangium. At this point, the anther wall consisted of the epidermis, endothecium, and secretory tapetum, and the cells in the middle layer began to collapse (fig. 3*E*). During stage 6, the microsporangium showed free uninucleate microspores, abundant connective tissue, and a persistent septum between locules (fig. 3*F*). At stage 7, the tapetum started to disintegrate, the anther wall was comprised of a thin epidermis and an endothecium with fibrous thickenings, and microspores became rounded and underwent mitosis, forming bicellular pollen grains (fig. 3*G*). The pollen grains have starch granules (fig. 3*H*). Finally, by stage 8, mature tricellular and tricolpate pollen grains with a thick exine were observed (fig. 3*I*).

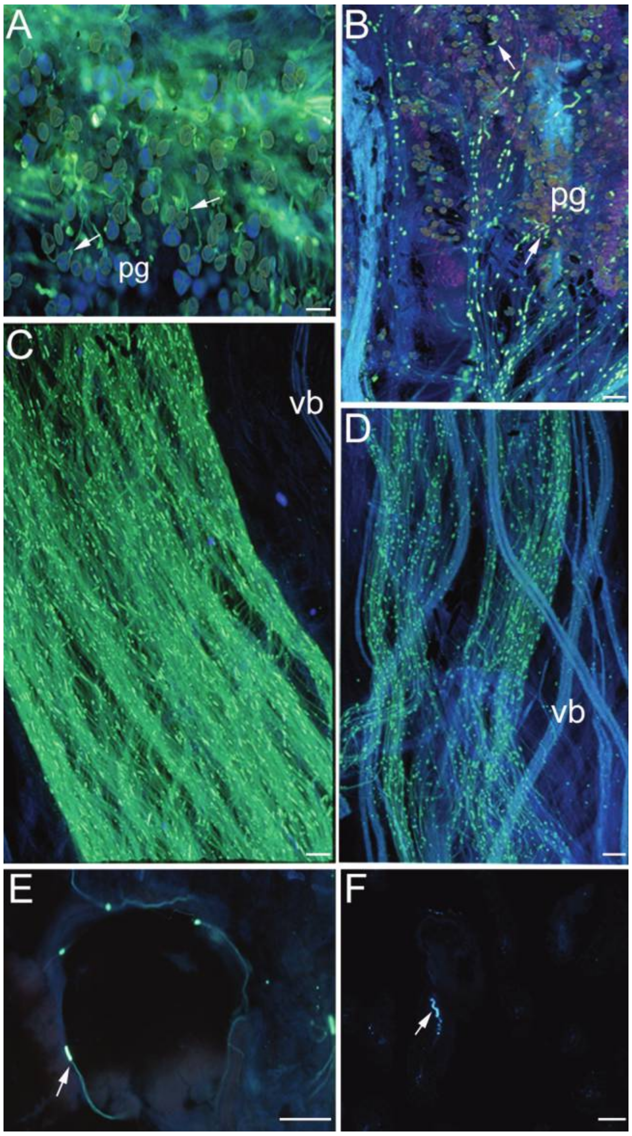

Fig. 2 Trajectory and growth of pollen tubes through style. Gynoecia of female flower (*A*, *C*, *E*) and male flower (*B*, *D*, *F*). *Echinocereus coccineus* (*B*, *D*), *E. polyacanthus* (*A*, *C*, *E*), and *E. mombergerianus* (*F*). *A*, *B*, Germinated pollen grains on the stigma; arrows indicate pollen tubes. *C*, *D*, Pollen tubes grow through the transmitting tissue along the style. *E*, *F*, Pollen tubes reaching ovules; arrows indicate the pollen tubes surrounding the ovule. pg = pollen grains; vb = vascular bundles. Scale bars = 100 μm (*A*), 200 μm (*B*–*F*).

In female flowers, despite initiation of anther development (fig. 3*J*), the anthers were wilted and empty at anthesis as a result of a premeiotic abortion of microspore mother cells. The early stages (St1–3) of anther development proceeded as in the male flowers, and the anther wall consisted of the epidermis, endothecium, middle layer, and secretory tapetum; in the latter, however, cells were uninucleate (data not shown). By the time the microspore mother cells began to differentiate, the tapetum remained uninucleate and started to disintegrate (fig. 3*K*, 3*L*). The microspore mother cells (St3–4) were not enclosed in callose

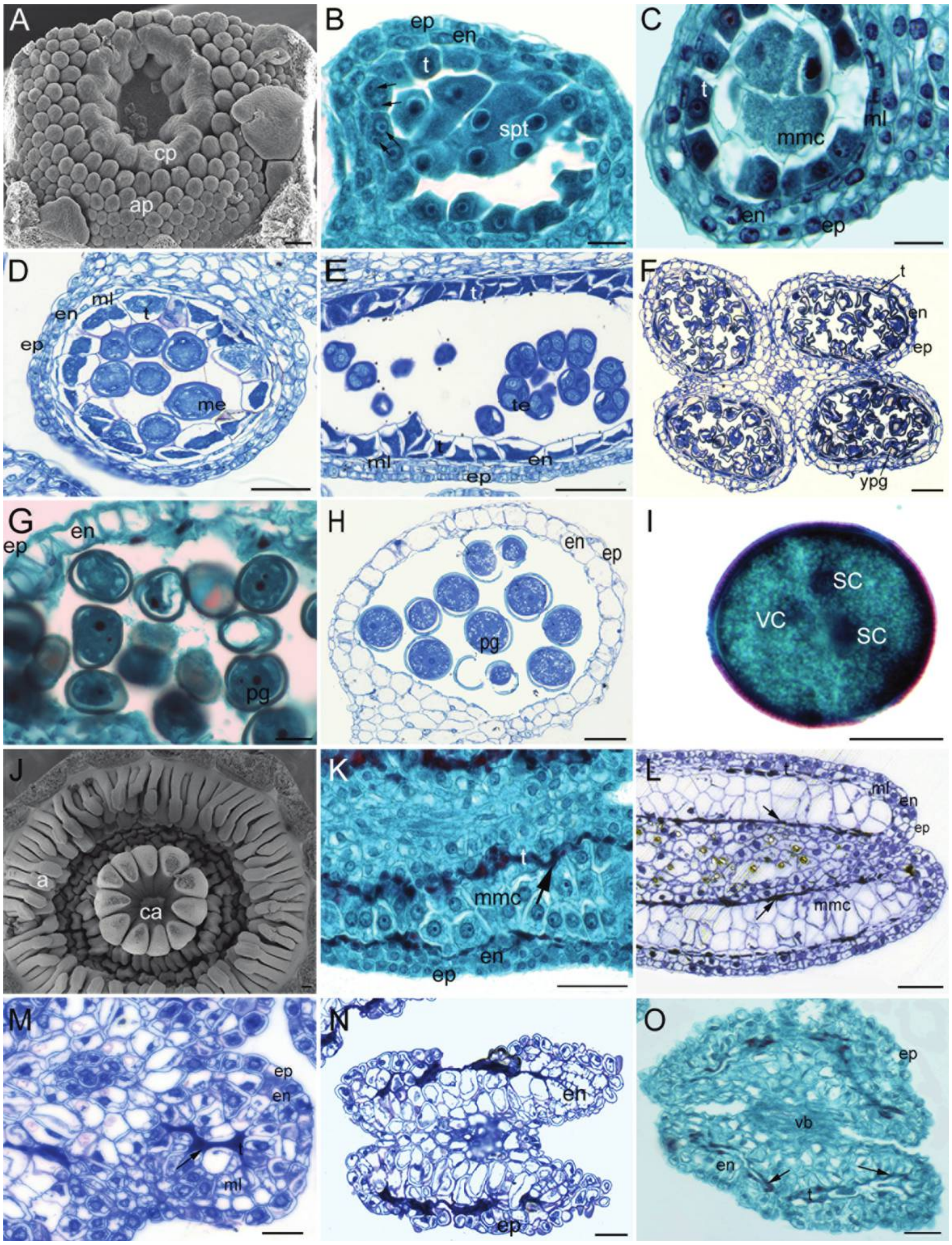

Fig. 3 Androecium development. Male flowers (*A–I*), female flowers (*J–O*). *Echinocereus coccineus* (*A*, *G*, *K*), *E. polyacanthus* (*B*, *J*, *L*), *E. mombergerianus* (*C*, *F*, *I*, *M*, *N*), *E. triglochidiatus* (*D*, *E*, *H*), and *E. pacificus* (*O*). *A*, Floral bud in stage 1, showing carpels and stamen primordia; some tepals are covering them. *B*, Young anther at sporogenous tissue stage. Anther wall development is monocotyledonous type (arrows). *C*, The anther wall has four differentiated strata at microspore mother cell stage. *D*, Microsporangium showing meiocytes; the anther wall has four differentiated strata. *E*, Anther at tetrad stage consisting of the epidermis, endothecium, middle layer, and secretory tapetum. *F*, Anther at young pollen grain stage; anther wall with epidermis and endothecium. The four microsporangia are attached by connective tissue. *G*, Microsporangium during tricellular pollen grain stage; the wall is formed by persistent epidermis and endothecium with cellular thickenings. *H*, Microsporangium showing pollen grains with starch granules. *I*, Tricellular and tricolpate pollen grain. *J*, Floral bud showing androecium

and degenerated prior to meiosis (fig. 3*K*), thus preventing the formation of pollen grains. At later stages, only wall remnants of the microspore mother cells were observed (fig. 3*M*). The cell layers comprising the anther wall gradually collapsed, and connective tissue became scarce (fig. 3*N*). At maturity, only the epidermis and a slightly thickened endothecium were distinguishable. The apex of the filament collapsed at the juncture with the anther (fig. 1*B*, 1*J*), unlike the male flower (fig. 1*F*, 1*N*). By anthesis, the anthers were completely empty, shriveled, and indehiscent (figs. 1*B*, 1*J*, 3*J*).

Gynoecium development. Gynoecium development followed a similar pattern in both floral morphs in the four dioecious taxa and the hermaphroditic species. In all species, the stigma formed between six and 10 stigmatic lobes (fig. 4*A*, 4*B*, 4*E*) covered by unicellular papillae filled with tannins (fig. 4*B*, 4*F*). Adjacent to the papillae, various layers of transmitting tissue, consisting of small cells with dense cytoplasm, were observed. Each stigma lobe contained a central vascular bundle surrounded by parenchyma (fig. 4*F*). The style was semisolid, with a stylar canal formed by elongated and papillate cells from the inner epidermis, filled with tannins. Next to the epidermis, several layers of transmitting tissue were observed forming a ring around the stylar canal (fig. 4*C*, 4*G*). Concentric vascular bundles were closed and amphicribral and surrounded by parenchymatic tissue, which was abundant in the style. Many large mucilage cells were observed between the ring of vascular bundles and the outer tanniniferous epidermis (fig. 4*C*, 4*G*). In all studied species, the ovary was filled with ovules encased by a long curved funicle with papillae at its ventral epidermis (figs. 4*D*, 4*H*, A2*A*–A2*D*). Cross sections of the ovary at early developmental stages showed that ovule primordia originated from parietal placenta and were distinguished by nucellar parenchymatic cells surrounded by protodermis (fig. 4*I*). Each ovule primordium began to curve by stages 3 and 4, and the division of a single hypodermal archesporial cell produced the primary parietal cell and megaspore mother cell. The primordia of the outer and inner integuments were also observed at this stage. The integuments were two cell layers thick, although the outer integument, positioned closer to the funicle, was not as differentiated or elongated due to the curvature of the ovule (fig. 4*J*). Further mitotic divisions of the parietal cell added more layers to the initial nucellus, giving rise to a crassinucellate ovule. By stages 5 and 6, ovules were campilotropous, bitegmic, and endostomous (fig. 4*K*). The embryo sac was the *Polygonum* type, surrounded by eight to 10 layers of parenchymatic nucellar tissue and by the two bilayered integuments that became multilayered near the micropyle (figs. 4*L*, A2*E*–A2*L*). At anthesis, the ovules could be seen arranged in clusters. Gynoecium development was similar in all morphs and studied species (fig. A2*M*, A2*N*).

Fruit and Seed Development

Ovule and seed development was characterized in both male and female floral morphs in all four dioecious taxa and in the hermaphroditic species. At anthesis, the ovary (St0; table A1, pt. B) contained many ovules (fig. 1*C*, 1*G*, 1*K*, 1*O*, 1*S*), each with a well-developed embryo sac (figs. 4*L*, A2*E*–A2*L*, A2*N*). The ovules at this stage were turgescent and surrounded by the funicle, with papillae on the abaxial surface (figs. 4*D*, 5*H*). At subsequent stages, differences were observed in the color and anatomy of seeds formed in male flowers, in comparison with hermaphroditic and female flowers.

Fruit and seed development in females and the hermaphroditic species. In the early stages (St1; table A1, pt. B), the pericarpel was green and the ovules were turgescent (fig. 5*A*) and beige, turning brown as seed maturation progressed. At this point, zygotes could be observed in the seeds, and exotesta cells accumulated tannins (fig. 5*D*, 5*H*). Later (St2; table A1, pt. B), the pericarpel widened, and the seeds acquired a brown color. Images obtained with a scanning electron microscope showed that the seed coat was already formed and the external walls of the exotesta were inflated, except in the area surrounding the micropyle, where cells were smaller and less inflated (fig. 5*B*), tannin deposits were observed in the endotegmen, and the developing embryos were surrounded by endosperm and perisperm (figs. 5*E*, 5*F*, A2*O*). Once the fruit was formed (St3; table A1, pt. B), the pericarpel acquired a reddish color, the funiculi comprised the whitish pulp, and the seeds were brownish black. The cells of the exotesta were globose, except for those surrounding the hilum-micropylar zone (fig. 5*C*). At this time, embryos were at the cotyledonary stage and slightly curved with an elongated wide hypocotyl, a short radicle, and two wide cotyledons; the apical meristem was at the epicotyl. The perisperm had been almost entirely consumed, and only one or two endosperm cell layers were persistent, with abundant tannins in the seed coat (fig. 5*G*). In female flowers that were covered to avoid pollination (i.e., apomixis treatment), the ovules and funiculi collapsed 8 d after anthesis and did not develop fruits or apomictic seeds. Normal fruit development in the female and the hermaphroditic species was completed in approximately 2 mo.

Seed number and micromorphology. The number of seeds formed per fruit in female and hermaphrodite individuals was compared. Since no seeds were formed in the self-pollination and apomixis treatments, the data comparison was conducted using only the seeds obtained from the cross-pollination treatment. The average number of seeds produced by cross-pollinated females ranged from 185 ± 49 (mean ± SE) in *E. mombergerianus* ($n = 5$ fruits) to 545 ± 69 in *E. coccineus* ($n = 7$). In hermaphroditic *E. triglochidiatus*, 287 ± 75 ($n = 21$) seeds per

with multiple stamens that have already differentiated into filaments and anthers. In the gynoecium, the carpel apex is distinguished by developing stigma; the tepals have been removed. *K*, Stage that should correspond with microspore tetrads, but most of the microspore mother cells do not undergo meiosis; the tapetum is collapsed (arrow), and the rest of the layers of the anther wall are undifferentiated. *L*, Anther showing collapsed tapetum with signs of degradation (arrows). *M*, Microspore mother cells and microsporangium inner layers are collapsed (arrow). *N*, Hypertrophied connective tissue; the microspore mother cells and various strata of the anther wall are collapsed. *O*, At anthesis, the microspore mother cells and various strata of the anther wall are collapsed. Arrows show the inner layers collapsed. a = anther; ap = anther primordia; ca = carpel; cp = carpel primordium; en = endothecium; ep = epidermis; me = meiocytes; ml = middle layer; mmc = microspore mother cell; pg = pollen grain; sc = spermatic cell; spt = sporogenous tissue; t = tapetum; te = tetrad; vc = vegetative cell; vb = vascular bundle; ypg = young pollen grains. Scale bars = 100 μm (*A*, *D*, *J*, *L*–*O*), 25 μm (*B*), 10 μm (*C*, *I*), 40 μm (*E*), 80 μm (*F*), 50 μm (*G*, *H*, *K*).

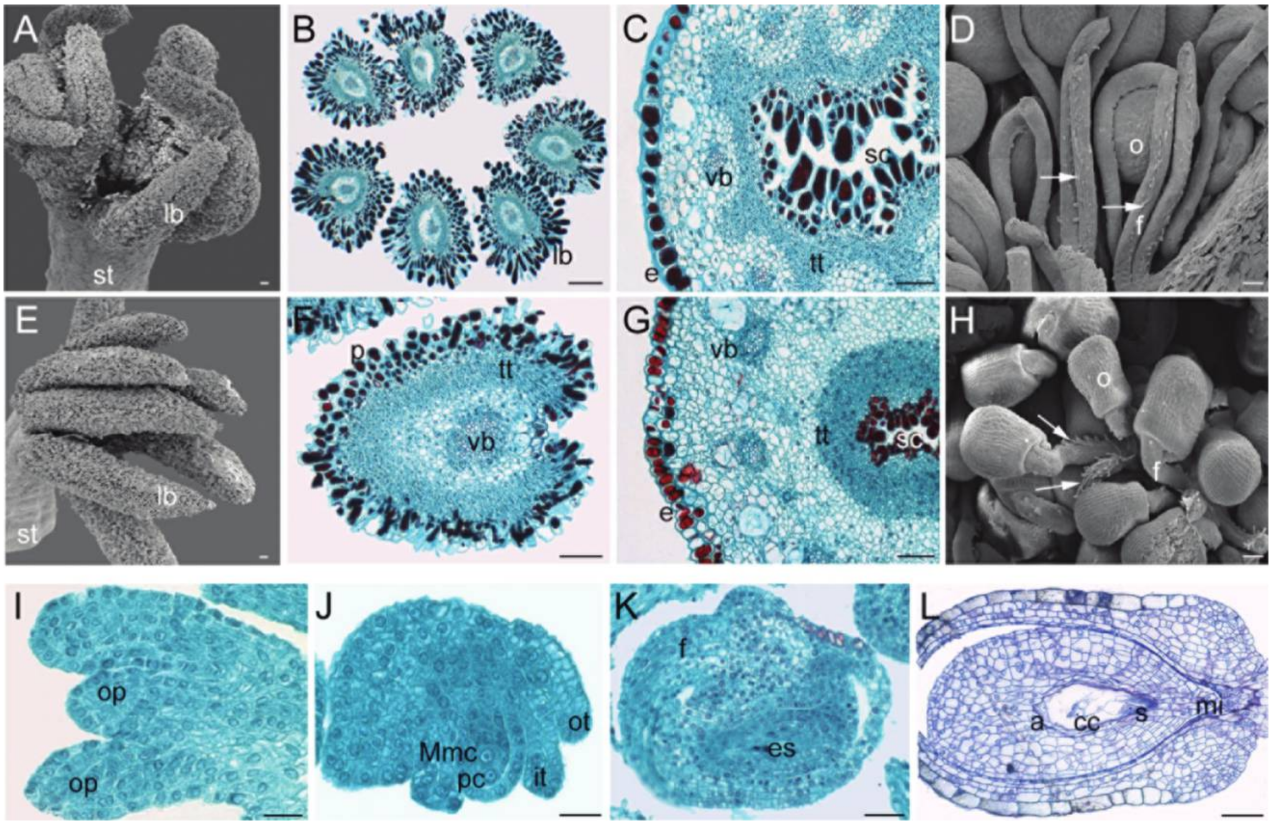

Fig. 4 Morphology and development of gynoecium. Male flowers (*A–D*), female flowers (*E–H*). *Echinocereus polyacanyhus (A–F, H, I)* and *E. coccineus (G, J–L)*. *A*, *E*, Panoramic view of stigma. *B*, Cross section of the stigma. *C*, Cross section of style, showing in the center the tanniniferous cells of stylar canal, followed by the transmitting tissue. *D*, Ovules in male flower; the funicular trichomes are smaller than those observed in female flowers (*H*). *F*, Transverse section of a stigmatic lobe showing the central vascular bundle, transmitting tissue, and stigmatic papillae. *G*, Style cross section at basal position; the stylar canal seems more closed; the transmitting tissue presents more cellular layers than those observed in male flowers (*C*). *I*, Three ovule primordia. *J*, Young ovule after archesporial cell mitosis showing the megaspore mother cell and parietal cell; the outer and inner integuments are bilayered. *K*, Ovule during embryo sac development showing an integumentary space. *L*, Mature ovule showing some cells of the embryo sac. a = antipodes; cc = central cell; e = outer epidermis; es = embryo sac; f = funicle; it = inner integument; lb = stigmatic lobe; mi = micropyle; Mmc = megaspore mother cell; o = ovule; op = ovule primordium; ot = outer integument; p = stigmatic papilla; pc = parietal cell; s = synergid; sc = stylar canal; st = style; tt = transmitting tissue; vb = vascular bundle. Scale bars = 100 μm (*A*, *C–H*, *L*), 400 μm (*B*), 25 μm (*I*, *J*), 50 μm (*K*).

fruit were produced (table A2). Significant differences in seed numbers were found (K-W, 17.29; $P < 0.05$). Although fruits from the species with unisexual flowers (*E. coccineus*, *E. polyacanthus*, and *E. pacificus*) generally averaged more seeds than the hermaphrodite *E. triglochidiatus*, the statistical Dunn's multiple comparison test showed that only *E. pacificus* and *E. triglochidiatus* differed significantly. Fruit and seed production in *E. mombergerianus* was very low after both natural pollination and cross-pollination. In general, considerable variation was found in the seed number and fruit size across all studied species, since smaller fruits bore more seeds compared with larger fruits (fig. 6*A*). External and internal seed micromorphology and histochemical composition were analyzed. Following Barthlott and Hunt (2000), mature seeds produced in females and hermaphroditic species are ovoid and asymmetric, present a shiny black surface, and are surrounded by fleshy whitish funiculi (fig. 6*A*). The outer periclinal walls of the exotesta presented a high convex relief. Cells were isodiametric and polygonal in dorsal and ventral zones but small and low convex near the hilum-micropylar area (fig. 6*B*, 6*C*). The hilum-micropylar region was conjunct, basal, and ovoid (fig. 6*D*). The seed coat was crustaceous, and the testa and tegmen were bilayered. The exotesta was formed by large sclerenchymatic cells, whereas the endotesta cells were flattened and parenchymatic. Cells of the exotegmen were collapsed, and the endotegmen contained tannins (fig. 6*E*–6*H*). Histochemical staining showed that the tegmen was rich in polysaccharides (fig. 6*I*), the perisperm was rich in both proteins (fig. 6*J*) and starch granules (fig. 6*K*), and the embryo was rich in proteins (fig. 6*J*) and that the cuticle remained attached to the endosperm once the perisperm had been consumed (fig. 6*L*).

Fruit and seed abortion in the male morph. Fruits abort at early developmental stages in male individuals. The pericarpel was initially (St1, table A1, pt. B) a bright green color. Ovules appeared beige and turgescent (fig. 5*H*). After fertilization, zygotes were observed (figs. 5*K*, A2*P*–A2*R*). As in the fe-

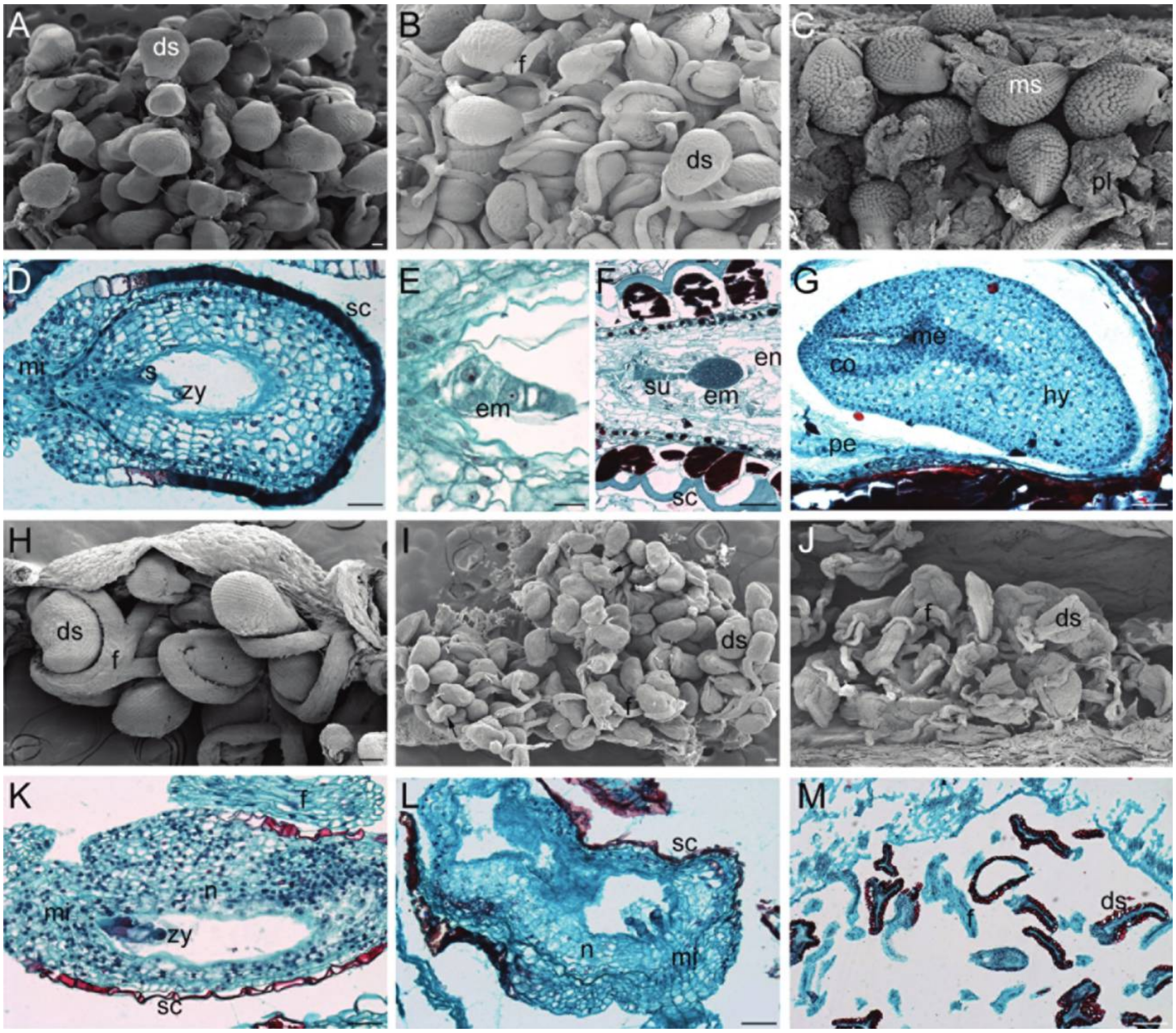

Fig. 5 Seed development in female (*A–G*) and abortion of seeds in male (*H–M*) individuals. *Echinocereus pacificus (A, C, G–J, L), E. mombergerianus (B, D, K, M),* and *E. coccineus (E, F). A, H*, Panoramic view of fruit in stage 1; young seeds and funiculi are swollen. *B*, Stage 2 fruit; ovules have begun to form seeds, and the formation of blisters on the seed coat is observed. *C*, Fruit with almost mature seeds. *D*, Longitudinal section of young seed at stage 1; a zygote can be observed. *E*, Seed in stage 2; a developing embryo is observed. *F*, A globular embryo stage. *G*, At stage 3, the seed coat has been formed and cotyledonary embryo can be observed. *H*, Panoramic view of the fruit in stage 1; the young seeds and the funiculi at this stage begin to collapse. *I, J*, During stage 3 in the male morph, both funiculi and seeds are observed to be flattened and collapsed. *K*, Longitudinal section of seed in stage 1 showing the zygote. *L*, Seed in stage 2; the nucellus, seed coat, and zygote begin to collapse. *M*, Cross section of fruit at stage 3, with collapsed seeds, in which only the exotestal epidermis persists. co = cotyledon; ds = developing seed; em = embryo; en = endosperm; f = funicle; hy = hypocotyl; me = meristem; mi = micropyle; ms = mature seed; n = nucellus; pe = perisperm; pl = placenta; s = sinergyd; sc = seed coat; su = suspensor; zy = zygote. Scale bars = 100 μm (*A–C*, *H–J*), 40 μm (*D*, *K*, *L*), 20 μm (*E*), 50 μm (*F*), 80 μm (*G*), 200 μm (*M*).

male counterpart, seeds turned brown as maturation progressed. The pericarpel widened (St2) and reached a greenish-yellow color, seeds became darker, and some seeds began to collapse (fig 5*I*). Zygotes could still be observed in these seeds, though they degenerated approximately 12 d after anthesis, whereas females produced fleshy fruits with a whitish pulp and black seeds (fig. 6*A*). This led to the conclusion that female function is regulated postzygotically by fruit and seed abortion. By maturity (St3), the pericarpel was yellow and entirely collapsed. Seeds were dark brown and collapsed (fig. 5*J*); histological sections showed that they were formed only by remnants of the seed coat, which exhibited tannins and a collapsed funicle (fig. 5*L*, 5*M*).

Programmed Cell Death

Programmed cell death during floral development. DNA fragmentation as a sign of PCD by a TUNEL-positive signal was observed in various androecium and gynoecium tissue

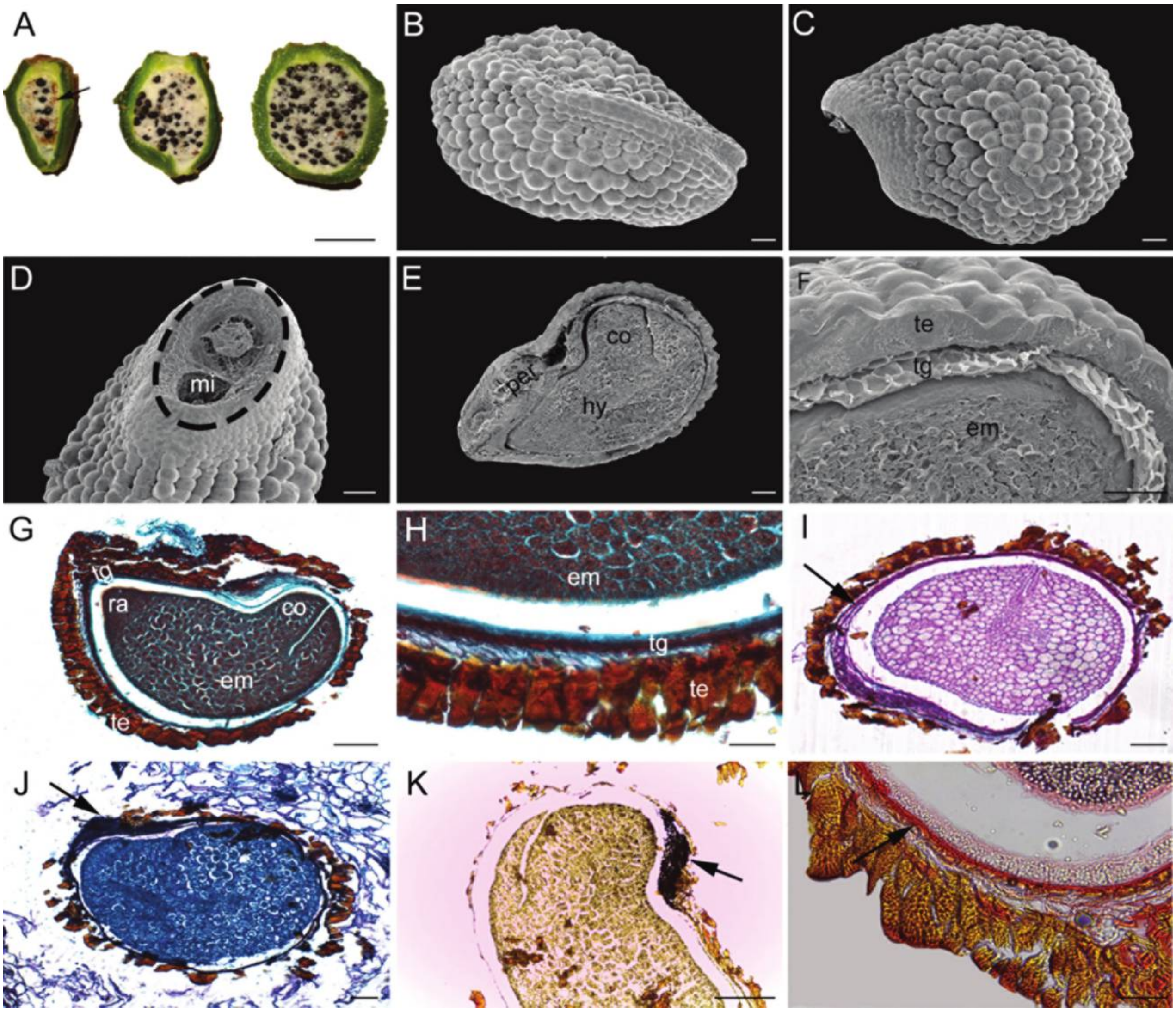

Fig. 6 Morphology and composition of the seeds. *Echinocereus triglochidiatus* (*A*), *E. pacificus* (*B*, *E–J*), and *E. mombergerianus* (*C*, *D*, *K*, *L*). *A*, Mature fruits, showing different numbers of seeds; the arrow indicates unfertilized ovules. *B*, Apical view of the seed. *C*, Lateral view of the seed. *D*, Hilum-micropylar region (dashed line). *E*, Longitudinal section of the seed; the mature embryo and the perisperm are observed. *F*, Close-up of the seed coat and part of the embryo; cells of the testa and tegmen are observed. *G*, Histological longitudinal section of mature seed; the seed coat and embryo are observed. *H*, Layers of the seed coat. *I*, Result of the Schiff reagent test, showing in pink purple the polysaccharides of the perisperm (arrow) and the cellulose of the cell walls. *J*, Result of the black naphthol blue test, in dark blue the perisperm proteins (arrow) and in the embryo cells are evident. *K*, Lugol test result, makes evident starch deposits of the perisperm in black (arrow) and in embryo cells. *L*, Result of the test of red O; the deposits of oils of the tegmen are shown in red (arrow). co = cotyledon; em = embryo; hy = hypocotyl; mi = micropyle; per = perisperm; ra = radicle; te = testa; tg = tegmen. Scale bars = 1 cm (*A*), 100 μm (*B–F*, *H*, *K*, *L*), 200 μm (*G*, *I*, *J*).

in all studied species, though at different developmental stages, as part of normal development or associated with the abortion of a sexual whorl in dioecious species. Regarding androecium development in male and hermaphroditic morphs, no signs of PCD were found during the early stages of anther development. At the free microspore stage, the first TUNEL-positive signals were observed in the tapetum, coinciding with the normal degeneration pattern observed during anther development (fig. 7*A*). Finally, when the anther neared anthesis, PCD was detected in epidermal cells of the stomium, which allows dehiscence (fig. 7*B*). In contrast, the female morph exhibited TUNEL-positive signals early in anther formation. This process started when cells in the sporogenous tissue differentiated to produce the microspore mother cell; thus, DNA fragmentation was detected in cells of the secondary parietal layer, as well as in sporogenous tissue, tapetal cells, and microspore mother cells (fig. 7*C*). By anthesis, TUNEL-positive signals were widespread throughout different anther layers (fig. 7*D*). These results confirm PCD in the development of unisexuality. This process started earlier in different anther wall layers and in the microspore mother cell in premeiotic

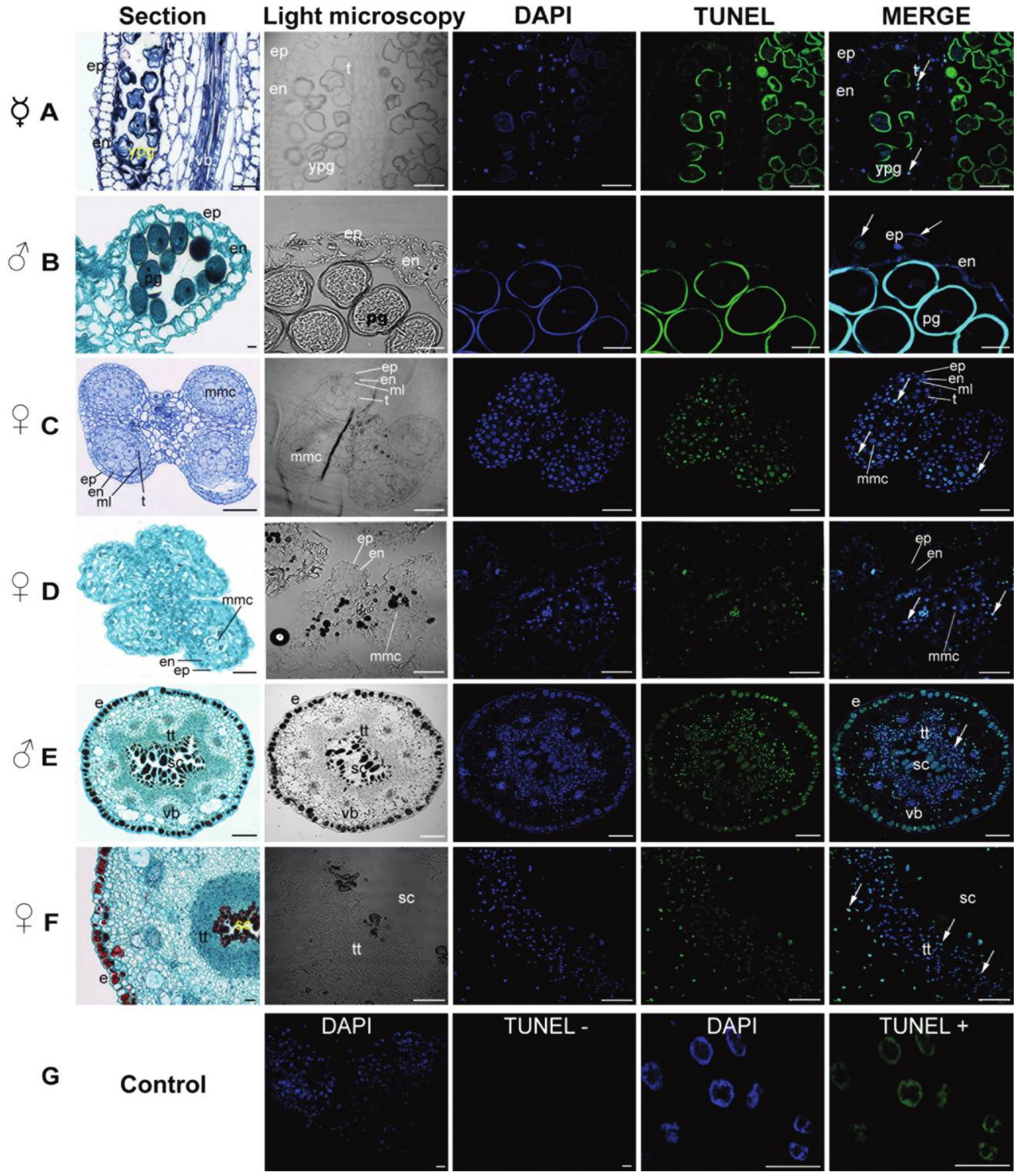

Fig. 7 Programmed cell death during floral development. *Echinocereus triglochidiatus* (*A*), *E. polyacanthus (B, C, E)*, and *E. coccineus (D, F)*. The stages are shown by *A*–*G*. The staining of DAPI (blue fluorescence) corresponds with the nuclei. Green fluorescence corresponds with nuclei showing evidence of programmed cell death (PCD) result of the TUNEL assay observed by confocal microscope. Arrows (*A*–*G*) indicate locations with evidence of PCD. *A*, Cross section of anthers of male flowers in stage 6; evidence of programmed cell death is seen in tapetal cells. *B*, Microsporangium of male flowers at stage 7; PCD in the epidermis and remnants of the tapetum; fluorescent nuclei are observed with TUNEL staining. *C*, Cross section of female flower anthers at stage 3; in the tapetum and microspore mother cell, evidence of PCD is observed. *D*, Aborted anther of a female flower at stage 6; all nuclei show evidence of PCD. *E*, Cross section of male flower styles in stage 7. PCD is observed in transmitting tissue cells. Autofluorescence is seen in the vascular bundles and tanniniferous big cells. *F*, Cross section of female flower style at stage 8; in the transmitting tissue cells, TUNEL-positive nuclei are observed. *G*, Positive and negative control. e = outer epidermis; en = endothecium; ep = epidermis; ml = middle layer; mmc = microspore mother cell; pg = pollen grain; sc = stylar canal; t = tapetum; tt = transmitting tissue; vb = vascular bundle; ypg = young pollen grains. Scale bars = 80 μm (*A*), 100 μm (*B*, *C*, *F*), 50 μm (*D*), 200 μm (*E*).

female flowers, whereas in male and hermaphrodite flowers it occurred after meiosis and was limited to the tapetum and middle layer.

With regard to gynoecium development, no TUNEL-positive signals were detected in male, female, or hermaphroditic flowers during stigma and style development. Near anthesis, PCD was observed in transmitting tissue cells adjacent to the papillae from the stylar canal, as well as in parenchymatic cells surrounding the vascular bundles; this was more intense in male flowers (fig. 7*E*) than in female flowers (fig. 7*F*). Both the positive (added DNase) and negative (no TdT) controls of the TUNEL assay corroborated the PCD (fig. 7*G*).

Programmed cell death during fruit and seed development. No TUNEL-positive signals were detected during the first stages of seed and fruit development in female and hermaphroditic flowers (fig. 8*A*), although at later stages DNA fragmentation was evidenced in cells that would normally form the seed coat during seed development (fig. 8*D*). In ovaries from the apomixis treatment, PCD was detected in the nucellus of the collapsed ovules and throughout the funicle 8 d after anthesis (fig. 8*B*, 8*C*). In fruits with developing seeds (St2), TUNEL-positive signals were observed in unfertilized ovules or in selectively aborted seeds, which were completely collapsed and exhibited a thick autofluorescent epidermis, indicating tannin deposition in the incipient seed coat (fig. 8*E*). Both the positive (added DNase) and negative (no TdT) controls of the TUNEL assay corroborated the PCD (fig. 8*F*).

In contrast with female and hermaphroditic flowers, where PCD is a normal process in late seed development, TUNEL-positive signals were detected earlier in male flowers both in young fruits and developing seeds. During stage 1, DNA fragmentation was not apparent in any of the seeds (fig. 9*A*), though some funiculi gave a positive signal (fig. 9*B*). By stage 2, both apparently normal seeds initiating seed coat formation and those with abnormal development showed evidence of DNA fragmentation in cells from the nucellus, funicle, and fruit wall (fig. 9*C*, 9*D*). A TUNEL-positive signal spread throughout the fruit wall (St3, fig. 9*D*). In cells with a collapsed and autofluorescent exotesta, the DAPI signal was diffuse as a consequence of cellular degeneration (fig. 9*E*). Both the positive (added DNase) and negative (no TdT) controls of the TUNEL assay corroborated the PCD (fig. 9*F*). These results demonstrate that PCD operates similarly in males of all four dioecious species and results in the postzygotic abortion of seeds.

Discussion

Floral Dimorphism and Sexual System

Detailed examination of floral development in the four dioecious taxa identified differences between the male and female morphs and confirmed the sexual system. Comparison with the floral development of hermaphroditic *Echinocereus triglochidiatus* allowed us to infer the developmental divergences that occur in unisexual flowers in *Echinocereus*. Our results demonstrate that *E. pacificus*, *E. mombergerianus*, *E. polyacanthus*, and *E. coccineus* are functionally dioecious. As reported for other functionally dioecious populations of *E. coccineus* in New Mexico (Hoffman 1992) and Arizona and New Mexico (Scobell 2008), floral dimorphism is subtle in Mexican populations. In females, anthers are produced but do not contain pollen, whereas in males, failure of female function does not occur until postfertilization.

Although the sex ratio in the four studied dioecious populations was not statistically corroborated, it showed a tendency of 1 : 1 (male : female), which coincides with observations made by Hoffman (1992) for *E. coccineus* and *E. yavapaiensis* populations in Arizona (Baker 2006), whereas subdioecious populations of *Consolea spinosissima* (Strittmatter et al. 2002) and *Pachycereus pringlei* (Fleming et al. 1998) are reported to have a greater incidence of male individuals.

Embryology

This is the first embryological study reported for the genus *Echinocereus*. Normal androecium development occurs in male flowers of *E. coccineus*, *E. polyacanthus*, *E. pacificus*, and *E. mombergerianus*, as well as in hermaphroditic flowers of *E. triglochidiatus*. As in other Cactaceae, the anther wall is monocotyledonous, meiosis in the microspore mother cell is simultaneous, and the tapetum is secretory (Flores-Rentería et al. 2013; Jiménez-Durán et al. 2014; Sánchez and Vázquez-Santana 2018). Gynoecium development in female, male, and hermaphrodite morphs is similar to what has been described by Johri et al. (1992) and that described for *Pereskia lychnidiflora* (Jiménez-Durán et al. 2014) and *Mammillaria dioica* (Sánchez and Vázquez-Santana 2018), where the megaspore mother cell forms a linear tetrad. The embryo sac is the *Polygonum* type with ephemeral antipodes, and the ovule is campilotropous, crassinucellate, and bitegmic. This shows that embryological characteristics are highly conserved in different Cactaceae subfamilies.

Male Sterility

To reveal the ontogenetic mechanisms underlying male sterility, comparative studies of floral morphology and development have been carried out in Cactaceae, as well as in other species with functionally unisexual individuals. Our results show that the androecium is reduced and sterile in female flowers of the four dioecious taxa. Similar androecium morphology has been reported in several other unisexual Cactaceae, including *E. yavapaiensis* (Baker 2006), *Opuntia stenopetala* (Orozco-Arroyo et al. 2012), *Opuntia robusta* (del Castillo and González-Espinosa 1988; Hernández-Cruz 2008), *Consolea spinosissima* (Strittmatter et al. 2002), *Pachycereus pringlei* (Fleming et al. 1994), and *M. dioica* (Sánchez and Vázquez-Santana 2018). In the female flowers of the four dioecious species studied here, anther development is arrested prior to meiosis, and microspore mother cells cease development as a result of tapetum degeneration. However, there is some variation among species in the timing of anther abortion in the female flowers of the studied taxa, either at the sporogenous tissue stage or later during anther development. Abortion of anther development at the microspore mother cell stage has been observed in other cacti, such as *O. stenopetala* (Flores-Rentería et al. 2013), the *Consolea* unisexual group (Strittmatter et al. 2002, 2006), and *E. yavapaiensis* (Baker 2006).

The tapetum plays an important role in nutrient transportation and sustains microspore development (Papini et al. 1999; Márquez 2013). A correct synchronization of PCD in the tapetum

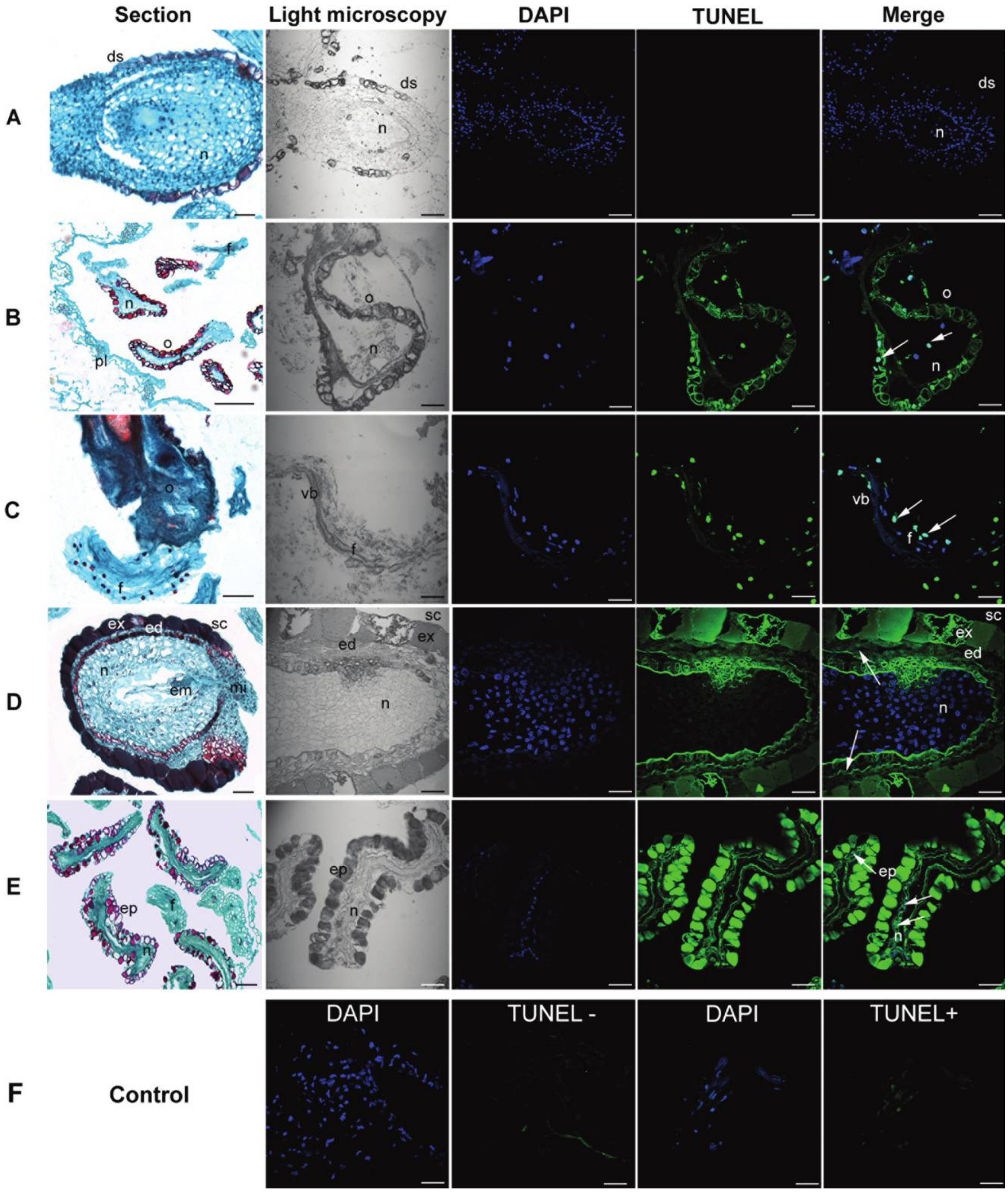

Fig. 8 Results of the TUNEL assay in seed development of female flowers. *Echinocereus mombergerianus (A, D–F)* and *E. pacificus (B, C)*. Arrows in *A–F* indicate sites with evidence of programmed cell death (PCD). *A*, During stage 1, the TUNEL assay is negative. *B, C*, Ovules and funiculi from an unpollinated flower; the TUNEL test was positive. *D*, Developing seeds at stage 2 show evidence of PCD in the cells of the seed coat (arrows). *E*, Abortive seeds in stage 2 show evidence of PCD in the cells of the seed coat (arrows). *F*, Positive and negative control. ds = developing seed; ed = endotesta; em = embryo; ep = epidermis; ex = exotesta; f = funicle; mi = micropyle; n = nucellus; o = ovule; pl = placenta; sc = seed coat; vb = vascular bundle. Scale bars in left column = 50 μm (*A, E*), 200 μm (*B*), 40 μm (*C, D*). Scale bars in fluorescence columns = 80 μm (*A*), 40 μm (*B–F*).

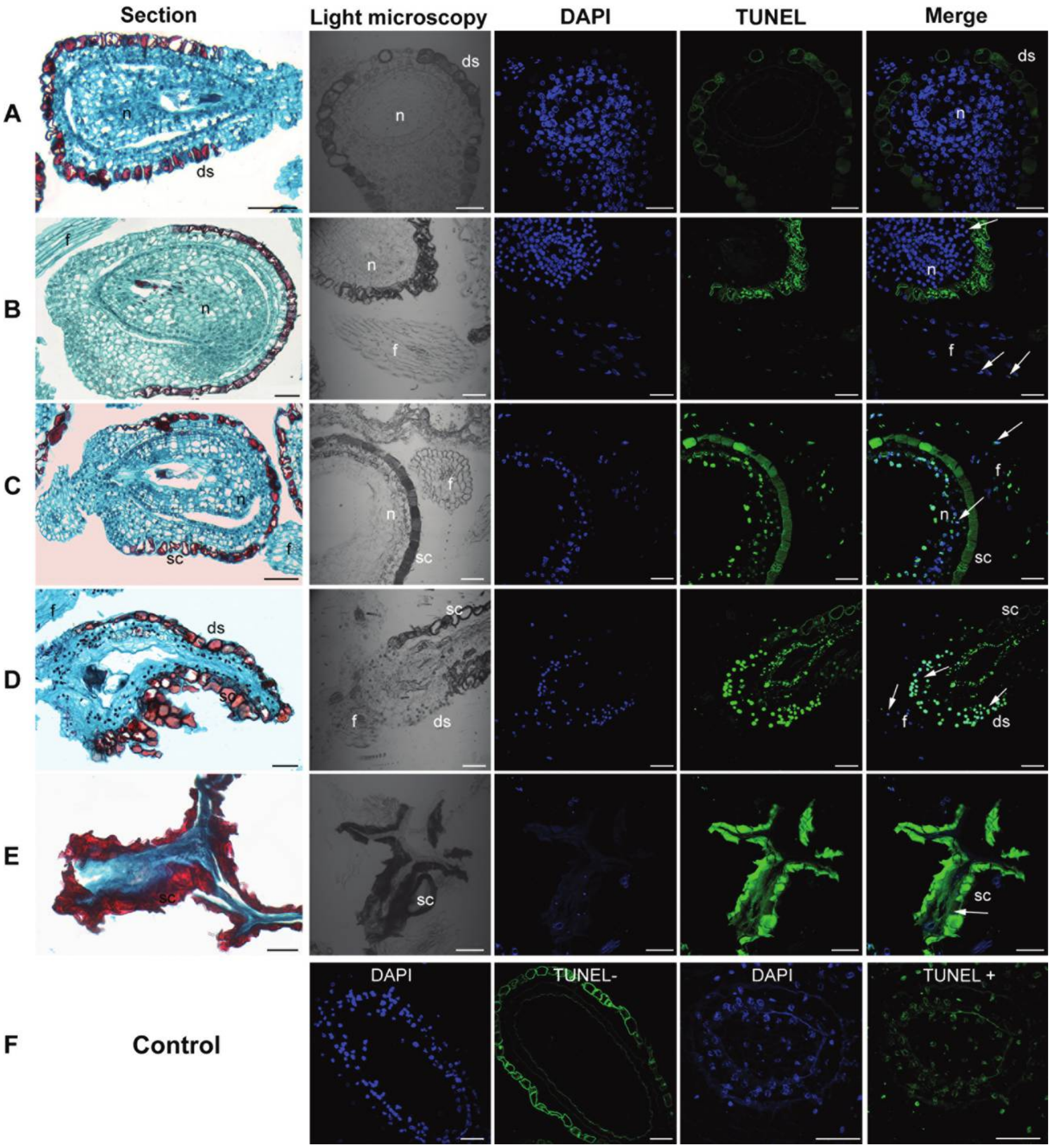

Fig. 9 Results of the TUNEL assay in seed development of male flowers. *Echinocereus mombergerianus* (*A*–C) and *E. pacificus* (*D*, *E*). Arrows in *A*–*F* indicate sites with evidence of programmed cell death (PCD). *A*, In stage 1 the developing seed is negative for TUNEL. *B*, Nuclei with fragmented DNA are observed in the funicle. *C*, Developing seed in stage 2; evidence of PCD is observed in the ovary wall and funicle and in developing seeds. *D*, At stage 2 the developing seed is completely collapsed, with PCD evidence in the funicle and nucellus. Autofluorescence is observed in epidermis cells. *E*, Seed completely aborted at stage 3. *F*, Positive and negative controls. ds = developing seed; f = funicle; n = nucellus; sc = seed coat. Scale bar in left column = 100 μm (*A, C*), 40 μm (*B, D*), 25 μm (*E*). Scale bars in fluorescence columns = 40 μm.

is necessary for normal microsporogenesis to occur (Kawanabe 2006). According to Papini et al. (1999), PCD is a characteristic of the secretory tapetum, from which PCD expands radially to the rest of the cell layers in the anther wall, allowing anther dehiscence (Varnier et al. 2005). In hermaphroditic and male flowers, the tapetal cells undergo PCD during the microspore and pollen grain stage in normal anther wall development (Papini et al. 1999; Varnier et al. 2005; Kawanabe 2006; Li et al. 2006; Parish and Li 2010). Alterations in the spatial and temporal expression of PCD in the anther wall, especially in the tapetum, cause male sterility in many angiosperm species. In the female flowers of the four dioecious studied species, PCD occurs prematurely in the tapetum, which has also been described in *O. stenopetala* (Flores-Rentería et al. 2013), several species in *Consolea* (Strittmatter et al. 2006), and male-sterile mutant lines of *Allium cepa* (Holford et al. 1991), *Arabidopsis thaliana* (Kawanabe 2006), *Capsicum annuum* (Luo et al. 2006), *Helianthus annuus* (Balk and Leaver 2001), and *Oryza sativa* (Ku et al. 2003). The premature expression of PCD in tapetal cells during microsporogenesis is the primary factor leading to male sterility in *Echinocereus* and other species with unisexual flowers in Cactaceae, except *M. dioica* (Sánchez and Vázquez-Santana 2018).

No Evidence of Female Sterility during Floral Development

Thus far, no single ontogenetic pattern leading to female sterility has been found in unisexual Cactaceae, since male flowers exhibit diverse morphological configurations. Male flowers in *E. coccineus*, *E. polyacanthus*, *E. pacificus*, and *E. mombergerianus* have a fully developed gynoecium (with stigma, style, and numerous apparently normal ovules) yet do not produce fruits, confirming reports by Hoffman (1992) on populations of *E. coccineus* in the southwestern United States. This differs from other species of cacti considered to be functionally cryptic, in which male flowers also retain an apparent fully developed gynoecium yet by anthesis have papery ovules (*C. spinosissima*; Strittmatter et al. 2002) or present underdeveloped funicle and nucellus (*Pachycereus pringlei*; Fleming et al. 1994) and thus cannot be fertilized. By contrast, in *O. stenopetala* the male floral morph lacks a stigma, has a reduced style, and exhibits an ovary with varying degrees of underdevelopment, which may produce few or no ovules or, in extreme cases, lack an ovary cavity (Orozco-Arroyo et al. 2012). In *O. robusta*, most male flowers also exhibit reduced ovaries with no ovules, although occasionally some may be found at the base of the ovary but do not reach maturity by anthesis (Hernández-Cruz 2008). Even though seeds were not formed in the male flowers of any of the studied *Echinocereus* species, no differences in ovule ontogeny were detected between these and the female or hermaphroditic flowers that do produce seeds. In other dioecious species, such as *C. spinosissima*, ovule development ceases at preanthesis, as the result of the disintegration of the nucellus (Strittmatter et al. 2002). Our results show that in *Echinocereus*, ovule development in the male flower is intact despite the lack of seed set. Fruit abortion in the male morph starts at approximately 12 d postanthesis, when seeds become a dark brown color and finally collapse, whereas females produce fleshy fruits with a whitish pulp and black seeds. The presence of a fully developed gynoecium in the dioecious species of *Echinocereus* is atypical, since the rest of the species with female sterility in this family either abort early or suppress the development of the ovules, stigma, and style.

Stigma and style function. Growth of pollen tubes along the style in male flowers suggests that the gynoecium remains functional. No differences were observed in the stylar canal ontogeny or in the transmitting tissue of the three floral morphs. Generally, in angiosperms with hollow styles, pollen tubes grow through the inner epidermis or along a stylar canal filled by nutritive secretions (Raghavan 1997). In the studied species of *Echinocereus*, as well as in *C. spinosissima*, the style is semisolid (Strittmatter et al. 2002). The stylar canal lacks secretions, and pollen tubes grow through the transmitting tissue below the inner epidermis. In *Echinocereus* male flowers, pollen tubes were observed growing throughout the style, reaching the ovary, and fertilizing ovules. Pollen tube growth through the gynoecium of male flowers evidences stigma and style functionality, as occurs in pollinated male flowers of *C. spinosissima* (Strittmatter et al. 2002) or other angiosperms such as *Mussaenda pubescens* (Li et al. 2010), which do not set fruits either. Gynoecium functionality is still maintained in these species, indicating that, morphologically, dioecy has not been fully established or its origin is recent. This incomplete loss of female function might give rise to subdioecious populations, since some male individuals could occasionally produce fruits (Delph 2003; Delph and Wolf 2005). Interestingly, pollen tube growth in the stigma and style of male flowers that underwent the self-pollination treatment is consistent with Scobell's (2008) work on self-compatibility in *E. coccineus*. To the contrary, the lack of pollen tube growth observed beyond the style of hermaphroditic flowers of *E. triglochidiatus* showed that it is self-incompatible, as has been observed in other cacti (Boyle 1997; Boyle and Idnurm 2001).

Evidence of PCD was detected in different tissue of the gynoecium. Near anthesis, DNA fragmentation can be seen in the transmitting tissue in both male and female flowers and constitutes a normal part of gynoecium development. This has been shown to occur normally prior to pollination, when cellular degeneration and the concomitant liberation of cell material allow pollen grain germination in the stigma and pollen tube growth along the style (Wu and Cheung 2000).

Fruit Development and Seed Number

Seeds from the *Echinocereus* genus were morphologically characterized using scanning electron microscopy (Barthlott and Hunt 2000). In the mature seed, the embryo is curved, the hypocotyl is larger than the cotyledons, and there is little perisperm. These characteristics can be seen in the seeds of the five taxa described herein, despite variation in seed size. The fruit in all the studied species is fleshy and contains many seeds. During development, the pericarpel widens and the pulp develops, arising mainly from the funiculi and the inner epidermis of the ovary. The pulp is acidic and promotes seed dispersal, since it is consumed by small mammals, such as rabbits and rodents, and even by ants (Lozano and Reid 1982).

Preliminary treatments show that the number of seeds per fruit for each taxon is highly variable, indicating that it may depend on pollinator efficiency or the amount of resources available to the plant for fruit development (Scobell 2008). Furthermore, seed size is larger in fruits with fewer seeds, though further experiments are needed to test this. Despite the small sample size, two trends are noticeable. The female plants of some species in this study produced more seeds than the hermaphroditic plants (table A2), so additional research is needed to determine whether resource allocation is involved (Ainsworth 2000). On the other hand, *E. mombergerianus* showed few fruits with few seeds (table A2), perhaps as a consequence of a scarcity of pollinators (R. Hernández and D. Sánchez, personal observations) or a result of endogamy, considering the species is microendemic with a low number of individuals. Further studies on the reproductive biology and population genetics should be addressed.

Seed Abortion by Programmed Cell Death Is the Evidence of Female Sterility

Fruit and seed abortion has been described in different species and occurs as a result of many different factors, such as limited resources and exposure to environmental stress (Sutherland 1986; Diggle 1997; Rosati et al. 2012). Selective abortion of both seeds and fruits also occurs in the female and hermaphrodite flowers of the studied taxa. In well-developed fruits, collapsed ovules, showing signs of PCD, could be seen next to mature seeds, indicating that those ovules were not fertilized or perhaps not enough resources were available for all the fertilized ovules to mature. Also, it may be that some of the fruits were selectively aborted, even if the ovules had been fertilized, since only three or four developing fruits and a great number of undeveloped remnant fruits were observed in individual plants. The fruiting season in these taxa occurs in late winter and spring, during the driest and hottest months of the year (Pimienta-Barrios and del Castillo 2002). This has also been reported in *Olea europea* (olive), in which the apparent competition between flowers and adverse environmental factors limits the number of fruits that can be produced (Rosati et al. 2012). In contrast to the extrinsic features, fruit abortion in male flowers is genetically determined, since no fruit development was observed in any of the male individuals, either in plants marked and observed later in the field or in plants collected and maintained in academic collections (Jardín Botánico, Universidad Nacional Autónoma de México). Seed abortion occurs acropetally, similar to the leaky-male flowers described in the *Consolea* genus (Strittmatter et al. 2008). Abortion of developing seeds could be the result of changes in the hormonal gradients that guide the PCD process (Ruan et al. 2012). Evidence of PCD was seen in the tissues that nurture the seeds, funicle, and nucellus, and thus seeds do not complete their development.

The Role of Heterochrony in the Establishment of Unisexuality

The evolution of unisexuality in *Echinocereus* could be clarified by determining the differences in ontogenetic timing and patterns observed in the sexual whorls of *E. coccineus*, *E. polyacanthus*, *E. pacificus*, and *E. mombergerianus* dimorphic flowers, when compared with hermaphroditic flowers of *E. triglochidiatus*. In dioecious species, female flowers begin their development as bisexual; stalling and atrophy of the androecium occur at later stages, resulting in flowers with a functional gynoecium that retains vestiges of the androecium. These are type I unisexual flowers, or unisexual by abortion, which, according to Mitchell and Diggle (2005), will eventually lose organ vestiges through a process of heterochrony. Heterochrony is considered a primary cause of ontogenetic modification in evolution and can be observed at cellular, tissue, or individual levels (Gould 1977; Li and Johnston 2000). Thus, the alteration of the sequence of events leading to PCD in the tapetal cells driving male sterility can be considered a heterochronic process. Heterochrony has also been reported as the mechanism underlying female loss of function during flower development in *Consolea* (Strittmatter et al. 2008); however, in dioecious *Echinocereus*, ovules were not aborted in the male flower during its development but rather after fertilization. Although PCD is observed during normal development of the seed, this happens in very specific tissue and not in all the development seed, as occurs in the male flowers; thus, there is no change in the timing patterns where PCD was observed. The physiological and/or molecular mechanism leading to seed abortion deserves to be addressed using a transcriptomics tool.

The Origin of Unisexuality in Echinocereus *Section* Triglochidiati

Section *Triglochidiati* represents a monophyletic group within *Echinocereus* (Sánchez et al. 2014, 2018), notwithstanding that the exact number of species and their breeding systems remains to be clarified (Ferguson 1989; Rebman 2003; Baker 2006; Rebman and Roberts 2012; Sánchez et al. 2013, 2014). According to Sánchez et al. (2014, 2018), *E. coccineus* and *E. polyacanthus* (including *E. pacificus* and *E. mombergerianus*) are sister species in section *Triglochidiati*, suggesting a common history regarding the evolution of unisexuality in this group. Dioecy can be assumed to have arisen in the section from a hypothetical hermaphrodite ancestor that lost its capacity to form bisexual flowers. More precisely, the untimely expression of PCD in the tapetum led to male sterility in female flowers, whereas the loss of female function in male flowers arose postfertilization, since ovules appear to develop normally. It is likely that dioecy evolved through gynodioecy, in which the genes responsible for male sterility spread throughout an ancestral hermaphrodite population, resulting in female and hermaphrodite individuals, and later mutations in the gynoecium of hermaphrodite individuals originated male flowers (Ainsworth 2000; Barrett 2002; Delph and Wolf 2005). Sexual systems in Cactaceae have evolved independently within each subfamily, probably via intermediate systems similar to those observed in *M. dioica* (Sánchez and Vázquez-Santana 2018).

Miller and Venable (2000) highlighted the relation between self-compatibility and polyploidy in various species of angiosperms and their closely related diploid and self-incompatible species, including the cacti *Echinocereus* and *Pachycereus*. Polyploidy disrupts gametophytic self-incompatibility, which leads to self-fertilization and possible inbreeding depression (Miller and Venable 2000). This phenomenon has been observed experimentally in the cactus *Hatiora* × *graeseri* (Karle and Boyle 1999). In section *Triglochidiati*, except *E. santaritensis*, all hermaphroditic species are diploid, whereas dimorphic species are either tetraploid or hexaploid (Baker 2006). This study showed that the diploid and hermaphroditic *E. triglochidiatus* is self-incompatible, whereas the tetraploid and dioecious *E. coccineus*, *E. polyacanthus*, *E. pacificus*, and *E. mombergerianus* are self-compatible. Probably, in this dioecious lineage of *Echinocereus*, self-compatibility led to high levels of endogamy, and flower unisexuality was selected to promote outcrossing, as suggested by Miller and Venable (2000) for several angiosperms and by Scobell (2008) for *E. coccineus*.

In conclusion, the abortion of anthers in female flowers and fruits in male individuals is common to the four dioecious species of *Echinocereus* and absent in hermaphroditic *E. triglochidiatus*. Furthermore, this study shows that both male and female sterility are stable in the studied dioecious species of section *Triglochidiati*.

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