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Gynoecium structure and pollen tube pathway in the cactus family with emphasis on tribe Trichocereeae (Cactaceae: Cactoideae)

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The structural details of the gynoecium are key to understanding the reproductive systems and successful diversification of flowering plants. However, the gynoecium morpho-anatomy in South American species of Cactaceae that evolved in the Andean region remains largely unknown. Here we selected 18 species, most of them of evolutionarily related genera of Cactaceae tribe Trichocereeae, to conduct a detailed comparative study. Observations were made using light, fluorescence, differential interference contrast and scanning electron microscopy. Most of the characters of the ovary and ovule were typical of the family, except for the nucellar beak in *Echinopsis aurea* and *E. haematantha*, here reported for the first time in cacti. We found evidence suggesting that the stigmatic surface covered with multiseriate trichomes, the semi-closed style type and the pollen tube transmitting tract are conserved characters among species of Trichocereeae; this finding may be explained by a phylogenetic conservatism of the investigated genera of the tribe. We integrated the available information about structural and histological characters of the gynoecium in the family, taking into account the current phylogenetic context of the examined genera. Our results reinforce the significance of floral anatomical traits for the systematics of Cactaceae.

ADDITIONAL KEYWORDS: circinotropous ovule – funicular obturator – nucellar beak – pollen tube – semi-closed style – transmitting tissue.

INTRODUCTION

The angiosperm carpel is a complex structural and functional unit that provides ovules/seeds with complete protection from external environmental factors; thus, it has considerable adaptive significance for reproductive success in flowering plants (Endress, 1982, 2015). Specialized carpellary tissues, such as the stigma and pollen tube transmitting tissue (PTTT), play an active role in pollen tube growth during the progamic phase, a key event for the completion of the sexual plant reproduction process (Herrero & Hormaza, 1996). The PTTT supports the heterotrophic pollen tube growth towards the female gametophyte and offers ample opportunity for pollen competition and selection (Endress, 1982, 1994, 2015; Erbar, 2003; Lora, Hormaza & Herrero, 2016).

Cactaceae comprises *c*. 1850 species in 130 genera, which are grouped in subfamilies Cactoideae, Maihuenioideae, Opuntioideae and Pereskioideae (Nyffeler & Eggli, 2010). Cacti are a lineage that is highly specialized to arid environments (Barthlott & Hunt, 1993; Anderson, 2001). Flowers of cacti are morphologically diverse, exhibiting several traits related to the adaptation to dry environments and different pollination modes (Cota Sánchez, 1993). An example of an adaptive character is the syncarpous gynoecium with an inferior ovary completely enclosed by the end of a modified shoot, the pericarpel (Tiagi, 1955; Boke, 1980; Ross, 1982; Mauseth, 2006; Rosas-Reinhold *et al.*, 2021). Bracts and areoles usually cover

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the pericarpel, which protects developing ovules and seeds and prevents them from desiccation (Gibson & Nobel, 1986; Nyffeler, 2002). Some convergent floral features, such as pollination syndromes, have been used in morphology-based classifications, which do not reveal phylogenetic relationships (Schlumpberger & Raguso, 2008; Schlumpberger & Renner, 2012).

The number of carpels in cacti, as indicated by the number of dorsal bundles in the styles (Boke, 1964; Tiagi, 1955, 1961; Volgin, 1988) and by the number of stigmas, ranges from three to > 20 arranged in one series (Barthlott & Hunt, 1993). Syncarpy takes place by congenital fusion, forming a unilocular cavity, a single style and free stigmas (Boke, 1963; Ross, 1982). Records in the literature indicate that stigma traits and style type are not uniform in Cactaceae (Boke, 1963, 1966, 1968; Heslop-Harrison & Shivanna, 1977; Strittmatter, Negrón-Ortiz & Hickey, 2002; Fuentes-Pérez, 2004; Fuentes-Pérez, Terrazas & Arias, 2009; Almeida, Sartori-Paoli & Souza, 2010; Villalpando-Martínez *et al.*, 2020). Despite available evidence for variation in these features, detailed structural aspects of the gynoecium have only been studied in a few genera.

As with most floral traits, the significance of the gynoecium characters in the classification of Cactaceae is still poorly understood, partly due to a lack of sufficient information and comparative studies. Cactoideae are the largest monophyletic subfamily of Cactaceae, with *c.* 1530 species (Nyffeler & Eggli, 2010). However, only 11 species of this subfamily have studied with special attention on flower anatomy and histology (Fuentes-Pérez, 2004; Fernández *et al.*, 2020; Villalpando-Martínez *et al.*, 2020). Thus, we examined the gynoecium structure in 18 species of Cactoideae, most belonging to the tribe Trichocereeae in the traditional sense, but also including species of tribes Notocacteae and Browningieae (Hunt, 2006), with the aim to explore the diversity in characters and to evaluate its putative relevance to the systematics. A second aim of this survey is to investigate the pollen tube pathway by assessing the association between the gynoecium structure and the pollen tube routes.

MATERIAL AND METHODS

Pre-anthetic buds and open flowers were collected from plants growing in the wild and fixed in formalin/acetic acid/70° alcohol (FAA) for anatomical and scanning electron microscopy (SEM) examination. Voucher specimens were deposited in the herbarium of the Instituto de Botánica del Nordeste (CTES), Argentina. The list of species with voucher specimens and the two tribal classifications consulted in this study are presented in Table 1. It should be noted that, according to Schlumpberger & Renner (2012), *Cleistocactus* Lem., *Denmoza* Britton & Rose, *Echinopsis* Zucc., *Gymnocalycium* Pfeiff. ex Mittler and *Harrisia* Britton are included in the *Echinopsis s.l.* clade.

Permanent microscope slides of the gynoecium were prepared by processing the fixed material by dehydration through an ethanol series with a pre-impregnation rinsing with tertiary butyl alcohol (Gonzalez & Cristóbal, 1997). Infiltration in paraffin Histoplast (Biopack, Buenos Aires, Argentina) was performed according to Johansen (1940). The pieces of the fertile floral whorl were sectioned transversely (10–12 µm thickness) with a rotary microtome. The sections were stained with astra blue-safranin (Luque, Sousa & Kraus, 1996), and then mounted with synthetic Canada balsam (Biopur, Buenos Aires, Argentina). Morphological and anatomical observations were performed under a Leica MZ6 stereomicroscope and a Leica DM LB2 compound microscope (Leica, Wetzlar, Germany), respectively, both equipped with a digital camera. For SEM observations, the fixed material was dehydrated through a series of increasing ethanol concentrations. The material was then critical point-dried with solvent-substituted liquid carbon dioxide and coated with a thin layer of gold palladium. SEM micrographs were obtained with a JEOL 5800 LV scanning electron microscope (JEOL USA, Peabody, MA, USA) operating at 20 kV.

The structure of mature ovules in *Echinopsis aurea* Britton & Rose and *Echinopsis haematantha* (Speg.) D.R.Hunt was analysed following the pistil clarification technique of Young, Sherwood & Bashaw (1979), with the treatment times adapted to the characteristics and nature of the material. To eliminate the cellular content, starting from material previously fixed in FAA, the ovules were dissected from the ovaries and placed in Eppendorf tubes with 70% alcohol for 30 min. Then they were dehydrated and clarified with 85% ethanol (30 min); 100% ethanol (30 min); ethanol:methyl salicylate 1:1 (15 min); ethanol: methyl salicylate 1:3 (15 min) and 100% methyl salicylate (15 min). The preparations were mounted in a drop of 100% methyl salicylate and observed under a Leica DM2500 microscope equipped with a differential interference contrast (DIC) device and a Leica EC3 digital camera.

To elucidate the pollen tube pathway, pollinated flowers of one representative per genus were analysed. The pistils, previously fixed in FAA, were dehydrated and embedded in paraffin. Cross sections (10 µm thick) of ovaries, styles and stigmas were made with a rotary microtome. They were deparaffinized and stained with 0.05% aniline blue. Observations were made with a Leica DM 1000 fluorescence microscope with a callose filter, equipped with a Canon EOS Rebel TDi digital camera.

Table 1. Species studied, voucher specimens and authors of two tribal classifications based on morphology and molecular phylogenetics, respectively. CTES: herbarium, Instituto de Botanica del Nordeste, Argentina

Species	Voucher specimens	Hunt (2006)	Nyffeler & Eggli (2010)
Cleistocactus baumannii (Lem.) Lem.	Argentina. Salta, *González V.V. et al. 5* (CTES)	Trichocereeae	Cereeae
Cleistocactus hyalacanthus (K.Schum.) Rol.-Goss.	Argentina. Salta, *González, V.V. et al. s.n.* (CTES)	Trichocereeae	Cereeae
Cleistocactus smaragdiflorus (F.A.C.Weber) Britton & Rose	Argentina. Salta, *González, V.V. et al. 4* (CTES)	Trichocereeae	Cereeae
Denmoza rhodacantha (Salm-Dyck) Britton & Rose	Argentina. Salta, *González, V.V. et al. 17* (CTES)	Trichocereeae	Cereeae
Echinopsis ancistrophora Speg.	Argentina. Salta, González, V.V. *et al.* 20 (CTES)	Trichocereeae	Cereeae
Echinopsis atacamensis (Phil.) H.Friedrich & G.D.Rowley	Argentina. Salta, *González, V.V. et al. 14* (CTES)	Trichocereeae	Cereeae
Echinopsis aurea Britton & Rose	Argentina. Salta, *González, V.V. et al. 11* (CTES)	Trichocereeae	Cereeae
Echinopsis haematantha (Speg.) D.R.Hunt	Argentina. Salta, *González, V.V. et al. 16* (CTES)	Trichocereeae	Cereeae
Echinopsis schickendantzii F.A.C.Weber	Argentina. Salta, *González, V.V. et al. 18* (CTES)	Trichocereeae	Cereeae
Echinopsis terscheckii (Parm. ex Pfeiff.) Friedrich & G.D.Rowley	Argentina. Salta, *González, V.V. et al. 10* (CTES)	Trichocereeae	Cereeae
Echinopsis thionantha (Speg.) D.R.Hunt	Argentina. Salta, *González, V.V. et al. 15* (CTES)	Trichocereeae	Cereeae
Echinopsis tubiflora (Pfeiff.) Zucc. ex A.Dietr.	Argentina. Salta, *González, V.V. et al. 9* (CTES)	Trichocereeae	Cereeae
Gymnocalycium saglionis (F.Cels) Britton & Rose	Argentina. Salta, *González, V.V. et al. 3* (CTES)	Trichocereeae	Cereeae
Gymnocalycium schickendantzii (F.A.C.Weber) Britton & Rose	Argentina. Salta, *González, V.V. et al. 6* (CTES)	Trichocereeae	Cereeae
Gymnocalycium spegazzinii Britton & Rose	Argentina. Salta, *González, V.V. et al. 13* (CTES)	Trichocereeae	Cereeae
Harrisia pomanensis (F.A.C.Weber ex K.Schum.) Britton & Rose	Argentina. Salta, *González, V.V. et al. 8* (CTES)	Trichocereeae	Cereeae
Parodia microsperma (F.A.C.Weber) Speg.	Argentina. Salta, *González, V.V. et al. 7* (CTES)	Notocacteae	Notocacteae
Stetsonia coryne (Salm-Dyck) Britton & Rose	Argentina. Salta, *González, V.V. et al. 19* (CTES)	Browningieae	Cereeae

RESULTS

Pericarpel, ovary and ovules

In all species, the gynoecium displays congenital fusion of a variable number of carpels, resulting in a synascidiate region with a unilocular ovary (Fig. 1A, G). The pericarpel surrounding the ovarian tissue shows homogeneous characteristics among species. The epidermis is glabrous; the underlying parenchyma has abundant mucilaginous cavities and vascular bundles arranged in rings, among which the recurrent bundles stand out (Fig. 2A–C). The ovary wall consists of a few layers of cells that are smaller than the cells of the pericarpel parenchyma. Furthermore, the tissue contains starch grains, showing a Maltese cross and large intercellular spaces (Fig. 2D, E).

In all species the ovules are attached to the ovary through a modified axile placenta (Fig. 1G). Small ridges of a limited extent radiating from the ovarian roof and represent the limits of each carpel (Fig. 1F). In the locule, two rows of ovules develop in the placentae belonging to the same carpel, between adjacent ridges (Fig. 1G). Ovules are bitegmic and crassinucellate; a gap is frequently formed between both integuments at

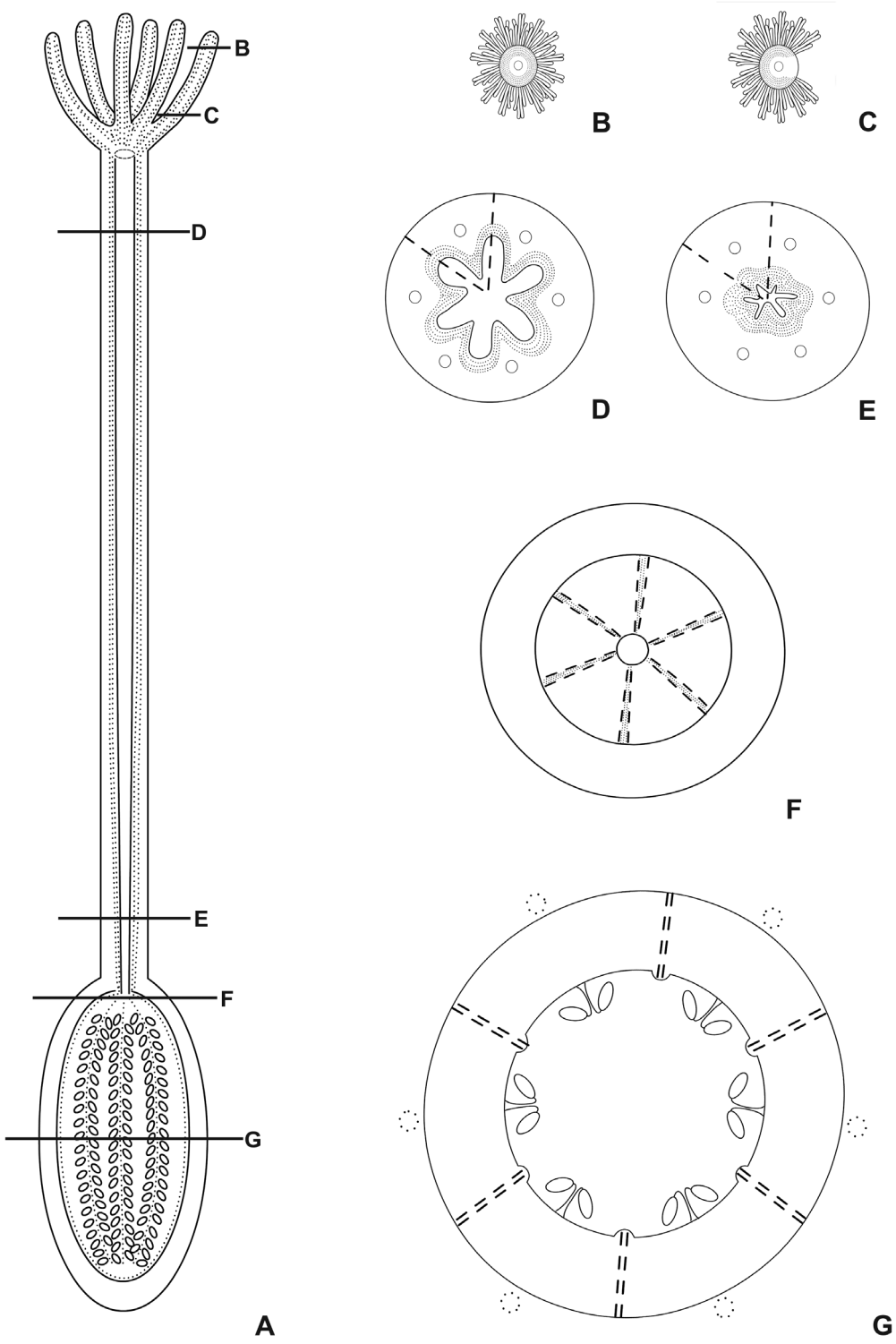

Figure 1. Schematic diagram of the syncarpous gynoecium structure in cacti. A, longitudinal section. B–G, series of cross sections. B, distal section of stigma lobe. C, proximal section of the stigma lobe (the ventral side is on the left of the diagram). D, upper portion of the style. E, basal portion of the style. F, upper portion of ovary. G, middle portion of ovary. Interpretations of the arrangement of dorsal bundles and the limits of the carpels at the level of the ovary were taken from Boke (1980). Note: dotted lines = pollen tube transmitting tissue (PTTT); dashed lines = distal end of the stylar canal; thin lines circles = vascular bundles; ellipses = ovules; broken lines indicate hypothetical carpel limits.

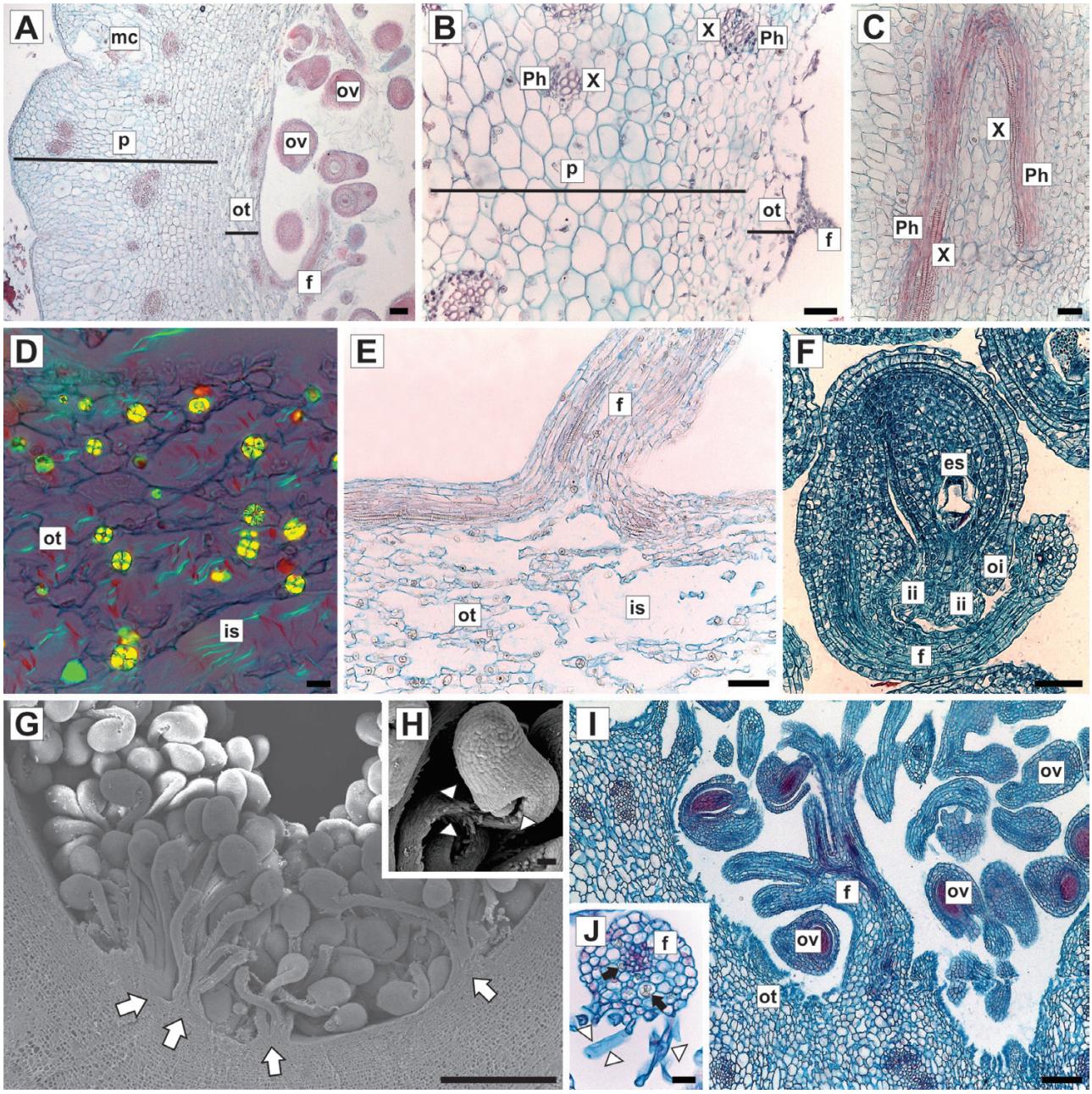

Figure 2. Pericarpel, ovary and ovules in species of Trichocereeae. A, C–E, *Cleistocactus baumannii.* A, cross section of ovary portion. B, *C. hyalacanthus,* detail of ovary wall; note the inverted position of xylem and phloem in vascular bundles close to the locule. C, detail of a recurrent vascular bundle in longitudinal section. D, detail of starch grains showing a Maltese cross in ovarian tissue, under polarized light. E, detail of ovary wall and base of a funiculus. F–H, *Denmoza rhodacantha.* F, longitudinal section of circinotropous ovule. G, section of ovary under SEM; note branching of funiculi (white arrows). H, detail of circinotropous ovule; note funiculus ventral epidermis with unicelular trichomes (white arrow heads). I, J, *Echinopsis schickendantzii.* I, cross section of ovary portion; note branching of funiculus. J, cross section of funiculus; note starch grains (black arrows) and ventral epidermal trichomes (white arrow heads). Abbreviations: es = embryo sac; f = funiculus; ii = inner integument; is = intercellular space; mc = mucilage cavity; oi = outer integument; ot = ovarian tissue; ov = ovule; p = pericarpel; Ph = phloem; X = xylem. Scales: A, I = 100 µm; B, C, F, H = 50 µm; D = 10 µm; E = 30 µm; G = 1 mm; J = 20 µm.

the chalazal region. Ovule curvature is circinotropous, a large funiculus developed longitudinally causing the ovule to rotate in a spiral on its own axis (Fig. 2F–H). A single vascular bundle runs through each funiculus and ends in the chalaza. The ventral epidermis of the funiculus presents unicellular trichomes (Fig. 2G, H, J). Ovules are connected by their funiculi, with a variable number arising from a common funicular base (Fig. 2G, I). Starch grains are observed within the parenchyma cells of the funiculus (Fig. 2J). The micropyle is formed by the inner integument and faces the obturator, the trichomatic surface of the funiculus (Fig. 2F, H). The inner integument is two cell layers thick, but is three to four cell layers in the micropylar region.

First record of ovules with nucellar beak in Cactaceae

In *Echinopsis aurea* and *E. haematantha*, the nucellus apex grows out of the micropyle, forming a nucellar beak at anthesis (Fig. 3A–D). In both species, the embryo sac begins to form normally within the body of the ovule, but as the nucellar tissue divides just before flower anthesis, the embryo sac is displaced towards the micropylar region until it is expelled from the integuments. The dimensions of the nucellar beak in mature ovules differ between species. In *E. haematantha*, this structure is notably larger and the entire female gametophyte protrudes out of the micropyle (Fig. 3C, D). In *E. aurea*, nucellus growth may cause the enlarged and laterally compressed appearance of the female gametophyte.

Style and stigma

Styles of *Cleistocactus*, *Denmoza rhodacantha* (Salm-Dyck) Britton & Rose, *Echinopsis*, *Gymnocalycium*, *Harrisia pomanensis* (F.A.C.Weber ex K.Schum.) Britton & Rose and *Stetsonia coryne* (Salm-Dyck) Britton & Rose are the symplicate regions of the gynoecium, with a semi-closed structure, i.e. a central stylar canal consisting of continuous inner surface and the underlying PTTT several cell layers thick (Fig. 1A, D, E). The lumen of the canal is large in the upper portion of the style and is considerably reduced in the basal portion, but the open condition is never lost (Fig. 1D, E). *Parodia microsperma* (F.A.C.Weber) Speg. is the exception to this pattern, because the style is semi-closed in its apical portion and is completely occluded by PTTT in the basal portion (Fig. 4A–G).

In transverse section, the semi-closed style consists of: (1) outer epidermis; (2) cortical parenchyma, where the dorsal vascular tissue is located; (3) PTTT and (4) inner epidermis surrounding the stylar canal (Fig. 4A–F). The outer epidermis of the style is covered with a relatively thick cuticle. This epidermis consists of quadrangular cells in species of *Cleistocactus*, *Denmoza*, *Echinopsis* and *Harrisia* and *Stetsonia coryne* (Fig. 4A, B), and of papillose cells in *Gymnocalycium* (Fig. 4D, E). The cortical parenchyma is composed of isodiametric cells of different dimensions; it includes the dorsal vascular bundles arranged in a ring and numerous mucilaginous cavities (Fig. 4A–G). In all the species analysed, the number of vascular bundles that innervate the style is equal to the number present in stigmatic lobes. Before pollination, the PTTT appears quite compact compared to the surrounding parenchyma (Fig. 4C, D). However, the transmitting tissue generally becomes lax after pollination (Figs 4A, 5E, G, H). It consists of round cells of small diameter, with dense cytoplasm and a large nucleus that occupies most of the cell (Fig. 4A–G). The inner epidermis of the style is trichomatic in *Cleistocactus*, *D. rhodacantha*, *Echinopsis ancistrophora* Speg., *Echinopsis terscheckii* (Parm. ex Pfeiff.) Friedrich & G.D.Rowley, *Echinopsis thionantha* (Speg.) D.R.Hunt, *Gymnocalycium, H. pomanensis* and *S. coryne* (Fig. 4B–D, F), whereas it is glabrous in *Echinopsis atacamensis* (Phil.) H.Friedrich & G.D.Rowley, *E. aurea*, *E. haematantha* and *Echinopsis tubiflora* (Pfeiff.) Zucc. ex A.Dietr. (Fig. 4A). *Gymnocalycium saglionis* (F.Cels) Britton & Rose has compact multiseriate multicellular trichomes filling the lumen of the canal, giving the appearance of a solid style (Fig. 4D, F). In *S. coryne*, the inner epidermis consists of uniseriate trichomes with tannins (Fig. 4C).

The stigmatic lobes correspond to the asymplicate region of the gynoecium (Figs 1A–C, 4H, I). The number of stigmatic lobes varies among species and shows some intraspecific variation (Table 2). In all the analysed species, in cross section, the stigmatic lobes are structurally organized in: (1) outer trichomatic epidermis covered with a thin cuticle; (2) PTTT forming a ring; and (3) vascularized parenchyma (Fig. 4I–K). In species of *Cleistocactus*, *Denmoza*, *Echinopsis*, *Harrisia* and *Gymnocalycium* and *S. coryne*, stigmatic lobes are almost completely covered with multiseriate trichomes (Fig. 4H–K). In *S. coryne*, these trichomes have phenolic compounds (Fig. 4K), since they stained positively with ferric chloride (data not shown). In *P. microsperma*, papillae are both unicellular and multicellular multiseriate. In all species, the sub-stigmatic PTTT has the same characteristics as those observed in the style (Fig. 4I–K). The underlying parenchyma is a variable number cell layers thick and has mucilaginous cavities. Each stigmatic lobe has a single vascular bundle, which consists predominantly of xylem elements (Fig. 4L). On the pollinated stigma surface of *E. tubiflora*, a small amount of secretion was observed under SEM (Fig. 5A).

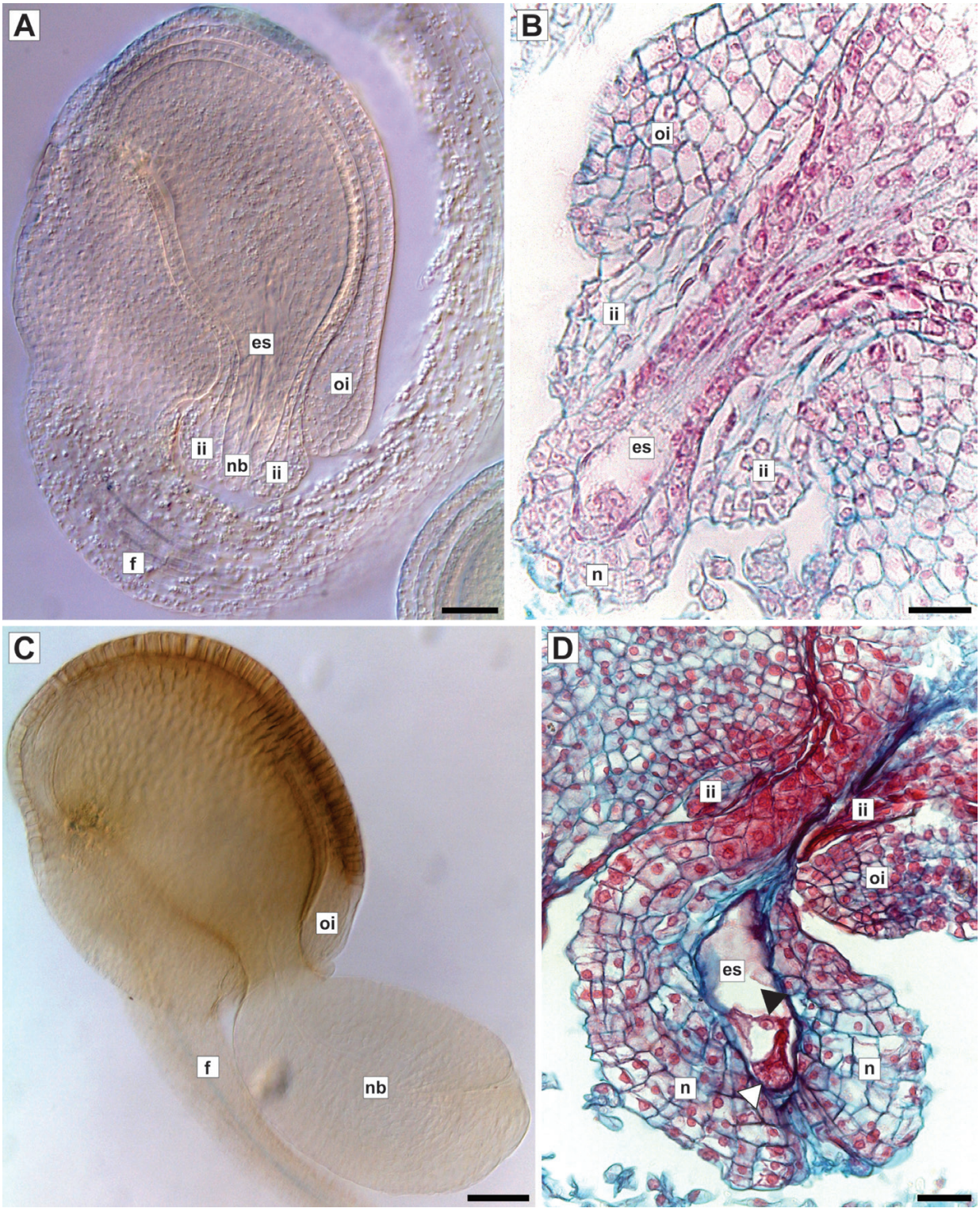

Figure 3. Mature ovules with developed nucellar beak. A, B, *Echinopsis aurea*. A, general view of circinotropous ovule under DIC microscopy. B, detail of nucellar beak. C, D, *E. haematantha*. C, general view under DIC microscopy. D, nucellar beak detail; note the embryo sac with a central cell (black arrowhead) and a synergid cell (white arrowhead). Abbreviations: es = embryo sac; f = funiculus; ii = inner integument; n = nucellus; nb = nucellar beak; oi = outer integument. Scales: A = 50 µm; B = 20 µm; C = 100 µm; D = 30 µm.

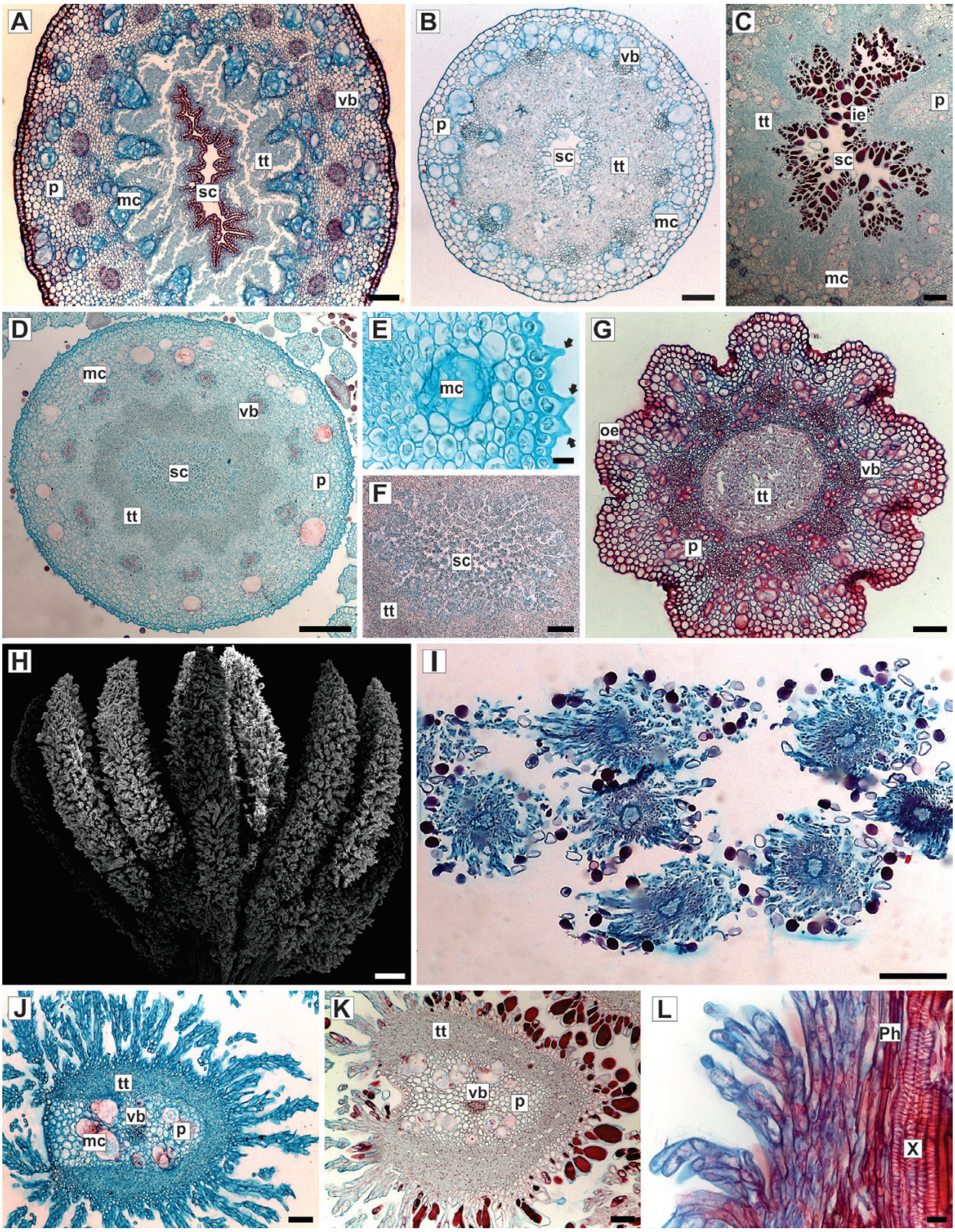

Figure 4. Style and stigma structure in species of Trichocereeae. A–G, cross sections of styles. A, distal symplicate portion of style in *Echinopsis atacamensis*. B, *E. thionantha.* C, *Stetsonia coryne.* D–F, *Gymnocalycium saglionis.* D, distal symplicate portion of style; note trichomes of inner epidermis filling the lumen of the stylar canal, giving the appearance of a solid style

Pollen tube pathway

The pollen grains adhere to the trichomes of the receptive surface and germinate (Fig. 5B). Once germination starts, the pollen tubes penetrate the cuticle and the cells of these trichomes, which may appear curved, retain the pollen units (Fig. 5C). Pollen tubes continue growing through the sub-stigmatic PTTT and then through the intercellular matrix of the stylar PTTT (Fig. 5D–F). The PTTT of the style forms an internal compitum, where the pollen tubes from one carpel can pass to another carpel. Pollen tubes do not enter the lumen of the stylar canal. After the tubes grow down through the style, the PTTT becomes lax and large intercellular spaces are generated (Fig. 5G–I). Once the pollen tube enters the ovary cavity, it continues its journey through the papillose ventral surface of the funiculus until it reaches the micropyle and finally fertilizes the ovule (Fig. 5J–L).

DISCUSSION

Pericarpel, ovary and ovules

This is the first analysis of the gynoecium structure in 18 species belonging to Cactaceae subfamily Cactoideae. The studied species and other cacti share carpels that are congenitally connate throughout most of their length and free in the distal portion, forming the stigmatic lobes (Boke, 1964; Ross, 1982; Villalpando-Martínez *et al.*, 2020). In Cactaceae, the ovary is inferior, with exceptions in some species of *Pereskia* Mill. (Pereskioideae, Boke, 1980). Inferior ovaries are characteristic of highly specialized groups, such as orchids and sunflowers (Gibson & Nobel, 1986). According to Endress (2011), an inferior ovary characterizes some monocots (Dioscoreales, Orchidaceae and Zingiberales) and some core eudicots (the Cucurbitales-Fagales clade, Santalales, Cornales, Rubiaceae and campanulids). Minor occurrences are found in some early-diverging angiosperms [Nymphaeaceae, Eupomatiaceae, Gomortegaceae, Hernandiaceae, Aristolochiaceae and some Alismatales (monocots)] (Endress, 2011). It is also found in ancient angiosperm lineages, including *Hedyosmum* Sw. (Sokoloff, 2016). However, in cacti, the inferior ovary has evolved in a different way from that of other angiosperms (Boke, 1964; Volgin, 1988). In the species analysed here, the stem tissue encloses the ovary, forming the pericarpel. Such a result can be supported by the histological characteristics of the epidermis, cortical parenchyma and vascular anatomy of the outer region of the ovary, which show structural similarities to previous records in shoots (Terrazas & Mauseth, 2002; Mauseth, 2006; Fuentes-Pérez *et al.*, 2009). The recurrent vascular bundles, with inverted xylem and phloem orientation, prove that the ovary is included in axial tissue (Boke, 1980; Volgin, 1988). However, Volgin (1988) reported evolutionary trends towards the reduction of the recurrent vascular bundle system in Cactaceae. Thus, in the evolutionary process it is possible for the vascular system of flowers with an inferior, axial ovary not to preserve the vestiges of the axial nature of the ovary wall (Volgin, 1988).

Ovules have modified axile placentation in all the taxa studied. The term ‘parietal placentation’ is widespread in previous studies on representatives of all the subfamilies of Cactaceae (Tiagi, 1955, 1961; Boke, 1963, 1964, 1966, 1968, 1980; Nuñez-Mariel *et al.*, 2001; Fuentes-Pérez, 2004; Fuentes-Pérez *et al.*, 2009; Almeida *et al.*, 2010; Villalpando-Martínez *et al.*, 2020). In the conventional parietal placentation, the median placenta of two different carpels is contacted, each one disposed on the respective carpel margin. In the modified placentation of cacti, we observed that the two rows of placentas are confined to the same carpel, suggesting carpel margins are involute. This re-interpretation of the placentation in Cactaceae was well studied by Boke (1964, 1980).

The number of ovules per ovary is certainly variable, although in the family there appears to be a trend towards an increase of ovule number. For example, in Pereskioideae (*Pereskia*) the number of ovules is < 100 (Boke, 1963, 1964), whereas in Cactoideae, in *Echinopsis*, it is > 1000 (V. V. González, pers. obs.) and in *Hylocereus* (A.Berger) Britton & Rose it varies among species from 2000 to 7200 (Nerd & Mizrahi, 1997; Tel-Zur *et al.*, 2005; Cisneros, Benega García & Tel-Zur, 2011). The circinotropous ovule type is typical in Cactaceae (Johri, Ambegaokar & Srivastava, 1992; Strittmatter *et al.*, 2002; Almeida *et al.*, 2010; this study). According to Johri *et al.* (1992), in Aristolochiaceae, Cactaceae and Plumbaginaceae,

type. E, detail of outer epidermis with papillose cells. F, detail of inner epidermis with trichomes lining the stylar canal. G, basal portion of style in *Parodia microsperma*. H, I, *Cleistocactus smaragdiflorus*. H, asymplicate region consisting of stigmatic lobes, under SEM. I, cross section of stigmatic lobes. J, detail of a stigmatic lobe in *E. terscheckii*. K, detail of a stigmatic lobe in *S. coryne*. L, longitudinal section of portion of stigmatic lobe in *E. atacamensis*. Abbreviations: ie = inner epidermis; mc = mucilage cavity; oe = outer epidermis; p = parenchyma; Ph = phloem; sc = stylar canal; tt = transmitting tissue; vb = vascular bundle; X = xylem. Scales: A, C, H, I = 200 µm; B, F, G, J, K = 100 µm; L = 20 µm; D = 300 µm; E = 30 µm.

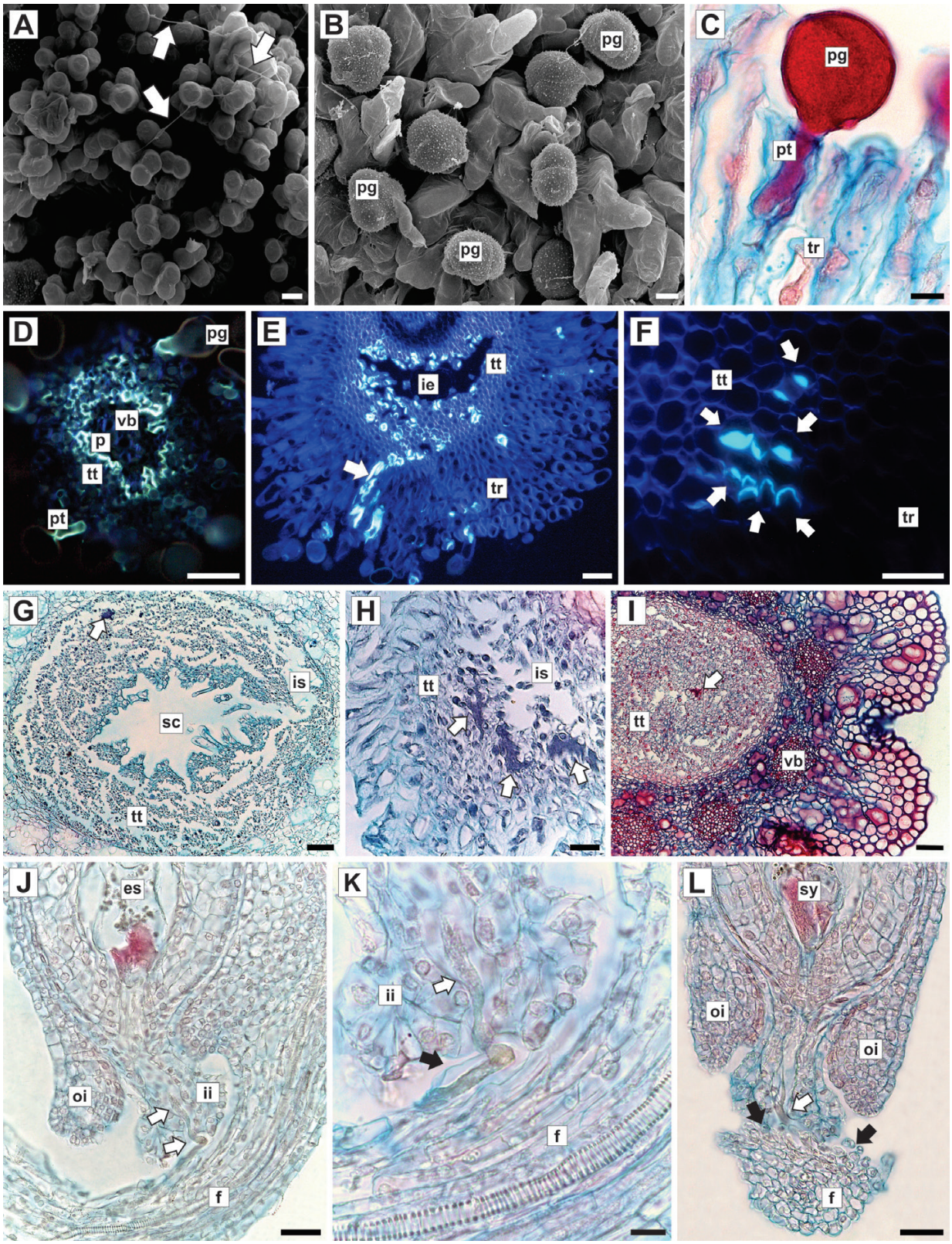

Figure 5. Pollen tube pathway in species of Trichocereeae. A, B, SEM of portion of stigmatic lobes. A, *Echinopsis tubiflora*; note a small amount of secretion (white arrows). B, *E. terscheckii*, portion of stigmatic lobe with pollen grains attached to trichomes of the receptive surface. C, E, F, *Gymnocalycium spegazzinii.* C, pollen tube entering the stigmatic trichome. D,

the circinotropous condition derives from anatropous ovules. In these ovules, the axis of the funiculus exhibits uneven growth, which causes the ovule to curve until the micropyle is upwards. However, in several other Cactaceae there are records of curved and elongated funiculus in amphitropous ovules (Cisneros *et al.*, 2011) and campylotropous ovules (Elizondo-Elizondo *et al.*, 1994; Fuentes-Pérez *et al.*, 2009; Villalpando-Martínez *et al.*, 2020). The branched funiculus is a common feature of all the cacti studied; consequently, two or more ovules are borne on a single funiculus base (Tiagi, 1955; Johri *et al.*, 1992; this study). According to Almeida *et al.* (2010), the ventral surface of the funiculus in contact with the micropyle and covered with unicellular trichomes has a potential role as an obturator. Here we confirm this assumption, since this ovular structure helps in directing the growth of the pollen tube towards the micropyle. According to Shamrov's classification (1998), this corresponds to a funicular obturator.

The crassinucellate condition, the presence of two integuments, and the micropyle formed by the inner integument are constant ovule features in Cactaceae (Boke, 1964; Johri *et al.*, 1992; Strittmatter *et al.*, 2002; Fuentes-Pérez, 2004; Fuentes-Pérez *et al.*, 2009; Almeida *et al.*, 2010; Cisneros *et al.*, 2011; present study). The small gap between the inner and outer integuments in the chalazal region reported here also appears to be frequent in ovules of cacti (Johri *et al.*, 1992; Cisneros *et al.*, 2011). In *Hylocereus*, this cavity is subsequently occupied by the embryo at the early cotyledonary stage (Cisneros *et al.*, 2011).

The presence of the nucellar beak in ovules of Cactaceae reported here is a novelty for the family. The nucellar beak is an extension of the nucellus at the micropylar end that protrudes partially into the placenta or the transmitting tissue of the ovary (Lizarazu & Pozner, 2014). It is a rare characteristic in ovules, which serves to bridge the gap between the placenta and the micropyle (Johri, 1984; Endress, 1994; Bhojwani, Bhatnagar & Dantu, 2015). The occurrence of a nucellar beak has been reported in other families of eudicots, such as Cucurbitaceae, Euphorbiaceae, Malphigiaceae, Polygonaceae, Rhamnaceae and Lythraceae (as Trapaceae) (Bouman, 1984; Rangan & Rangaswamy, 1999; Lizarazu & Pozner, 2014; Bhojwani *et al.*, 2015). In Cactaceae, this feature of the ovule is probably more labile than other features. *Echinopsis aurea* and *E. haematantha* belong to two separate lineages (Schlumpberger & Renner, 2012). In this context, the occurrence of nucellar beaks may be viewed as a case of parallel evolution in *Echinopsis*.

Table 2. Number of vascular bundles in the style and stigmatic lobes in species of Trichocereeae

Species	Number of vascular bundles in style and stigmatic lobes
Cleistocactus baumannii	7–8
C. hyalacanthus	10
C. smaragdiflorus	10
Denmoza rhodacantha	11–12
Echinopsis ancistrophora	8-9
E. atacamensis	16 or 19
E. aurea	13
E. haematantha	12
E. schickendantzii	18
E. terscheckii	18 or 21
E. thionantha	8
E. tubiflora	10–13
Gymnocalycium saglionis	11
G. schickendantzii	13–15
G. spegazinii	12–15
Harrisia pomanensis	12, 13 or 15

Style

In transverse sections of the style, histological characteristics of the cortical parenchyma, PTTT and vascular tissue are similar among species of Cactaceae. Most of the differences in the style lie

cross section of stigma lobe under fluorescence microscopy in *Harrisia pomanensis*. E, portion of stigmatic lobe; note the fluorescence of growing pollen tube, callose outlining, within a stigmatic trichome (white arrow) and intercellular space in transmission tissue. F, detail of pollen tube growing in transmission tissue cells under fluorescence microscopy (white arrows). G, H, *Denmoza rhodacantha.* G, cross section of basal portion of style. H, detail of transmission tissue; note growing pollen tubes (white arrows) and large intercellular spaces. I, *Parodia microsperma,* cross section of basal portion of style; note lax tissue and growing pollen tubes (white arrow). J–L, longitudinal sections of ovule in *Cleistocactus hyalacanthus.* J, lateral view; note the pollen tube entering the ovule through the micropyle (white arrows). K, detail of a growing pollen tube; note the trichome on the ventral side of the funiculus (black arrow) and the pollen tube growing between the internal integuments (white arrow). L, front view of ovule; note the proximity between the ventral face of the funiculus with trichomes (black arrows) and the micropile with a pollen tube entering the ovule (white arrow). Abbreviations: is = intercellular space; p = parenchyma; pg = pollen grain; pt = pollen tube; sc = stylar canal; sy = synergids; tr = trichome; tt = transmitting tissue; vb = vascular bundle. Scales: A, B, H = 20 µm; C, K = 10 µm; D = 100 µm; E, F = 50 µm; G, I = 50 µm; J, L = 30 µm.

in the shape of the outer epidermis cells and in the morphology of the epidermal cells lining the canal (e.g. trichomatic or non-trichomatic, absence or presence of tannins) (Strittmatter *et al.*, 2002; Fuentes-Pérez, 2004; Fuentes-Pérez *et al.*, 2009; Almeida *et al.*, 2010; Villalpando-Martínez *et al.*, 2020; this study). Among the previously mentioned characters of the style, the papillose outer epidermis was the unique trait that allowed the differentiation of *Gymnocalycium* from *Cleistocactus*, *Denmoza*, *Echinopsis* and *Harrisia*, all of which are currently assembled in the *Echinopsis s.l.* clade (Schlumpberger & Renner, 2012). In Cactaceae, previous integrative studies combining stem anatomy and molecular characters have demonstrated the importance of including epidermal traits from surface and cross sections to provide data that allow us to recognize relationships between genera (Martínez-Quezada *et al.*, 2020, and references therein). The evidence provided here can support the importance of epidermal traits in systematic studies of cacti.

Styles may vary structurally according to the three types recorded in angiosperms (open, closed and semi-closed) (Johri, 1984; Gotelli *et al.*, 2017). Open styles are distinguished by one or more canals between the stigma and the ovary, each one surrounded by a secretory epithelium. They often occur in basal angiosperms and monocotyledons. Semi-closed styles are defined by the presence of a PTTT several cell layers thick surrounding the stylar canal (Hanf, 1935; Johri, 1984; Gotelli *et al.*, 2017). The latter type is known for a few angiosperms, as in *Persea americana* Mill. (Sedgley & Buttrose, 1978) and *Mangifera indica* L. (de Wet, Robert & Coetzee, 1990). Finally, closed styles have one or more strands of transmitting tissue and are most common in eudicots.

In Cactaceae, open styles were reported in Opuntioideae (*Opuntia* Mill.; Fuentes-Pérez *et al.*, 2009), Pereskioideae (*Pereskia*; Boke, 1963, 1966, 1968) and tribe Phyllocacteae of Cactoideae [*Escontria chiotilla* (F.A.C.Weber ex K.Schum.) Rose, *Lophocereus* Britton & Rose and *Neobuxbaumia* Backeb.; Fuentes-Pérez, 2004]. However, according to our re-interpretation, the previously mentioned styles may fit the semi-closed type due to the occurrence of a several-layered PTTT beneath the inner epidermis. In styles of Opuntioideae, an unusual structure was reported: the presence of parenchyma between the epidermis of the canal and the PTTT (Fuentes-Pérez *et al.*, 2009), a characteristic that is absent in species of Cactoideae studied to date. A semi-closed style seems to be the most common character in Cactaceae; it has been observed in Opuntiodeae (*Consolea* Lem.; Strittmatter *et al.*, 2002), in Cactoideae tribes Cereeae (*Cereus* Mill. by Hanf, 1935; *Cleistocactus*, *Denmoza*, *Echinopsis*, *Harrisia*, *Gymnocalycium* and *Stetsonia*, this study) and Phyllocacteae [*Polaskia* Backeb. and *Stenocereus* (A.Berger) Riccob. by Fuentes-Pérez, 2004, and *Hylocereus* by Cho & Ding, 2021]. A closed style is reported here in the style base of *Parodia* Speg. (Notocacteae) and was reported in *Myrtillocactus* Console by Fuentes-Pérez (2004) (Phyllocacteae).

Stigma

According to Heslop-Harrison & Shivana (1977), the characteristics of the receptive surface may be constant at the family level or may vary considerably between and sometimes even in families. The comparative analysis based on the species of Cactaceae studied so far indicates structural and histological variations at the stigma level. Most of the species of Cactoideae here examined had multiseriate trichomatic stigmatic surfaces, even though there were differences in the accumulation of tannins and in the stigmatic papillae, which were both unicellular and multiseriate in *Parodia microsperma*. Multiseriate stigmatic trichomes with tannins are found in *Stetsonia coryne* (this study) and *Stenocereus pruinosus* (Otto ex Pfeiff.) Buxb. (Fuentes-Pérez, 2004) of tribes Cereeae and Phyllocacteae, respectively. Stigmatic papillae are uniseriate and with tannin content in Pereskioideae (*Pereskia*, Boke, 1963), Opuntioideae (*Opuntia*, Fuentes-Pérez, 2009) and possibly in genera of Phyllocacteae (Cactoideae) such as *Escontria* Rose, *Lophocereus*, *Myrtillocactus, Neobuxbaumia*, *Pachycereus* (A.Berger) Britton & Rose and *Polaskia* (Fuentes-Pérez, 2004) and *Echinocereus* Engelm. (Villalpando-Martínez *et al.*, 2020).

Heslop-Harrison & Shivanna (1977) reported that the stigma of cacti is of the wet type. However, a dry-type stigma was mentioned, for example, in *Hylocereus polyrhizus* (F.A.C.Weber) Britton & Rose (Cho & Ding, 2021). In this study, no evidence for any copious secretion at the mature stage or cuticle rupture was found to support classification of the stigmas as wet. An additional finding of the present work, which is also observed in other plants possessing a dry stigma, was that the point of adhesion of the pollen grain to a papilla corresponds to the point of entry of the pollen tube into the transmitting tissue. In contrast, in a wet stigma, the presence of an extracellular matrix allows pollen tubes to grow to some extent along the papillae, before penetrating the style (Hiscock *et al.*, 2002). The stigma type is important due to its association with the self-incompatibility systems. Dry stigmas are associated with the occurrence of sporophytic self-incompatibility, whereas wet stigmas are correlated with the occurrence of gametophytic self-incompatibility (Heslop-Harrison & Shivanna, 1977). For example, species of *Schlumbergera* Lem. have wet stigmas (Heslop-Harrison & Shivanna, 1977) and gametophytic self-incompatibility (Boyle,

1997). According to our SEM results, only *Echinopsis tubiflora* revealed patches of exudate on the stigma surface at the mature stage. Thus, since the distinction between wet and dry types of stigma is not always clear, future histochemical and ultrastructural studies may help to interpret the nature of the stigmatic secretions and the characteristics of the cuticle of stigmatic papillae in concomitance with the pollination event.

Intraspecific variation in the number of stigmatic lobes in Cactoideae has been repeatedly reported (Britton & Rose, 1920; Shurly, 1946; Buxbaum, 1953; Villalpando-Martínez *et al.*, 2020; this study). Villalpando-Martínez *et al.* (2020) attributed this variability to fusion events of the stigmatic lobes, which is reinforced by the presence of two vascular bundles. However, in this study the number of vascular bundles in the style was always equal to the number of bundles in the tip of stigmatic lobes, suggesting that differences in quantity of stigmatic lobes may be directly associated with the variable number of carpels per flower rather than with fusion of stigmas. Furthermore, the number of carpels may vary with cultural conditions (Boke, 1964).

Pollen tube pathway

The gynoecium structure, the histological features of the transmitting tissue and the location of chemotropic substances are involved in pollen tube guidance, with all of them affecting the route of the pollen tubes during their growth in the carpels (Heslop-Harrison, 1987; Sage *et al.*, 2009; Prychid *et al.*, 2011; Gotelli *et al.*, 2017). In the species studied here, histological similarity and continuity were observed between the receptive stigmatic surface and the inner epidermal cell layer of the style. This study confirms findings of Cho & Ding (2021), who reported that the epidermis of the canal is not directly involved in pollen tube growth in any case; indeed, we observed that pollen tubes did not grow in the epidermal region of the canal and we did not find secretions produced by this tissue. Two pollen tube pathways were recorded in semi-closed styles of different angiosperms: the intercellular matrix of the transmitting tissue and the secretion in the canal (Gotelli *et al.*, 2017). In the styles of Cactaceae studied to date, pollen tubes grow through a tract multiple cell layers thick. However, these cells lose turgidity and intercellular spaces are generated, indicating a degeneration process after pollination. Further ultrastructural analyses are needed to confirm these interpretations.

CONCLUSIONS

The species of the traditionally recognized Trichocereeae, Browningieae and Notocacteae, the former two currently placed in Cereeae on the basis of molecular data, share a general pattern of gynoecium syncarpy and structure. Furthermore, ovule characteristics described in this study, such as integuments, micropyle, funiculus and placentation, are widespread and, therefore, characterize Cactaceae. In contrast, ovule curvature is variable, being anatropous, amphitropous or campylotropous. Here, we report the exceptional development of a nucellar beak in two *Echinopsis* spp. for the first time in the family.

Taking into account all the evidence available to date, the semi-closed style with a multilayered PTTT forming a compitum can be recognized as a conserved trait for the traditional tribes Trichocereeae and Browningieae and a trend in the family in general. The closed style condition may be relatively rare, since so far it has only been reported for Notocacteae and Phyllocacteae. The stigmatic surface is variable at the family level, but rather uniform among the studied genera of Trichocereeae. The stigma may be covered with uniseriate or multiseriate stigmatic papillae or a combination of both types; in some genera, the papillae accumulate tannins. The path of the pollen tubes is constant in the species studied with the same spatial distribution of PTTT. Style and stigma structure and histology, including epidermal morphology, should be explored in more detail in other species, since they appear to be relevant to the morphological characterization of clades of genera and to play a role in setting trends in the current phylogenetic classification of Cactoideae.

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DATA AVAILABILITY

The data underlying this study are contained within the article.

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