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Unlocking the secrets of parasitic plants: A comparative study of the development and floral morphoanatomy of *Pholisma* (Lennoaceae)

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ABSTRACT

Pholisma belongs to the family Lennoaceae, classified as obligate root holoparasites. Most existing studies are limited to addressing only *P. arenarium*. In this study, morphoanatomical and developmental aspects of flowers of the three species comprising *Pholisma* are described using histological embedding techniques in Paraplast and LR- White and observations by light microscopy and scanning electron microscopy. Similarities found in the three species include the presence of trichomes on the sepals, each carpel folding independently. Each carpel has transmitting tissue, and an empty cavity is distinguished in the centre of the style. The stigma is crateriform and of the wet type. Sporogenesis, gametogenesis, and floral development follow a similar pattern in the three species. Differences among species include the position of the stamens, pollen morphology, type of inflorescence, and colour of flowers and inflorescences. In *P. arenarium*, the stamens are located below the stigma height, and the pollen is tetracolporate and psilate; in *P. sonorae*, the stamens are at the same height as the stigma, and the pollen is tricolporate and reticulate; and in *P. culiacana*, the stamens are above the stigma height, and the pollen is tricolporate and psilate. The inflorescences in both *P. arenarium* and *P. sonorae* are cymes; the corolla colour is white with purple lines extending from the limb to the corolla tube. In contrast, the inflorescence in *P. culiacana* is a capitulum type, and the corolla colour is white with pink margins. The characters found in the three species of *Pholisma* are compared with some genera comprising the family Ehretiaceae.

1. Introduction

Parasitic plants are organisms that obtain their resources from other plants, called host plants (Musselman and Press, 1995; Heide-Jorgensen, 2008). They acquire these resources through haustorium, structures responsible for the adhesion, invasion, and physiological redirection of resources from the host plant to the parasitic plant (Westwood et al., 2010). One proposed classification of parasitic plants uses certain fundamental stages during their life cycle, recognizing five functional groups: mistletoes, parasitic vines, endoparasites, obligate root parasites, and euphytoid parasites (Teixeira-Costa and Davis, 2021).

The Lennoaceae Solms family belongs to the obligate root holoparasites; in this group, the seeds germinate underground due to chemicals released by the host plant and invade its root system. Furthermore, these holoparasitic plants depend entirely on the host plant from the early stages of their development. They grow underground, anchored to the roots of the host, and only their flowers or inflorescences emerge from the roots of the host and protrude from the external substrate (Teixeira-Costa and Davis, 2021).

Lennoaceae is a small monophyletic holoparasitic family within the order Boraginales Juss. ex Bercht. & J. Presl. Molecular phylogenetic analyses suggested that Lennoaceae is nested within Ehretiaceae (Weigend et al., 2014; Gottschling et al., 2014a). However, branch support was low and relationships of Lennoaceae were not congruent in both analyses. Recently, a genomic phylogenetic analysis recovered to Lennoaceae within Ehretiaceae with high support and as a sister of *Bourreria* P. Browne (Zhang et al., 2020). Despite those results, the Boraginales Working Group holds Lennoaceae at family level because of

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the combination of root parasitic life form, polymerous perianth, and fruit morphology (Luebert et al., 2016; 2024).

In Lennoaceae are included two genera: *Lennoa* Lex. with one species, L. *madreporoides;* and *Pholisma* Nutt. ex Hook. with three species, *P. arenarium, P. culiacana* (Dressler and Kuijt) Yatsk., and *P. sonorae* (Torr. ex A. Gray) Yatsk. (Nickrent, 2020). Lennoaceae is native to the American continent, and its species are distributed in the southwestern United States, northwestern Mexico, and northern South America. They are found in various habitats, ranging from coastal dunes and rocky areas to chaparral scrub and deserts (Yatskievych and Mason, 1986).

Particularly, *P. arenarium* is distributed in Arizona and California in the United States and Baja California, Mexico; *P. sonorae* in Arizona and California in the United States and Sonora, Mexico; and *P. culiacana* in Sinaloa and Sonora, Mexico. The species of *Pholisma* are characterized by being perennial plants having leaves reduced to scales and inflorescences cymosely derived; they are variable; the flowers are 4–10-merous; the calyces are tubular or deeply lobed with glandular trichomes; the corollas are lilac to bluish-purple or pinkish; the stamens are uniseriate; and the ovary has 5–16 carpels. Host plants of *Pholisma* belong to several families, including Euphorbiaceae Juss., Boraginaceae Juss., Asteraceae Bercht. & J. Presl, Polygonaceae Juss., and Hydrophyllaceae R. Br. (Yatskievych and Mason, 1986).

Anatomical and developmental studies on *Pholisma* are scarce. Those that exist are based only on *P. arenarium*, including the work of Copeland (1935), which describes the development, morphology, anatomy, and embryology of the flower, while Drugg (1962) describes the pollen of the Lennoaceae family. Jeiter et al. (2023) describe the development of the flowers and fruits of *P. arenarium*. Embryological studies are fundamental for the reconstruction of the evolutionary history of plants (Endress, 2005). Similarly, floral morphology and development knowledge significantly impact ecology, physiology, and genetics (Kaplan, 2001; Iwamoto and Bull-Hereñu, 2018).

This work describes and compares the morphoanatomy of the flowers and the embryological development of the three species that make up the genus *Pholisma*. Finally, the similarities and differences in morphology, anatomy, and development of the *Pholisma* species are discussed concerning some genera of the Ehretiaceae (Boraginales).

2. Materials and methods

Young inflorescences, bud flowers, and open flowers of each species were collected from three different localities. *Pholisma arenarium* (growing on roots of *Hazardia berberidis* (A. Gray) Greene) was collected in San Quintín, Baja California, Mexico in May 2021; *P. sonorae* (growing on roots *Abronia villosa* S. Watson) in the Gulf of Santa Clara, Sonora, Mexico in May 2021 and *P. culiacana* (on roots of *Jatropha cordata* (Ortega) Müll. Arg., *J. cinerea* (Ortega) Müll. Arg., *Euphorbia gentryi* V.W. Steinm. & T.F. Daniel) in the locality of Nuevo San Miguel, Ahome, Sinaloa, Mexico in September 2020.

Photographs of the three species of *Pholisma* were taken at the collection sites, and inflorescences at different stages of development were collected and fixed in FAA (10:50:5:35, formaldehyde, 95 % ethanol, acetic acid, distilled water, respectively).

After fixation in FAA, the material was dehydrated in a gradual ethanol series in a vacuum chamber and embedded in Paraplast (Sigma-Aldrich) and cut into longitudinal sections 5 μm thick using an American Optical 820 rotary microtome. The sections were stained with safranin-fast green. Additional samples were embedded in medium-grade LR-White (Electron Microscopy Sciences, Fort Washington, PA, USA), cut into longitudinal and transverse Sections 1-1.5 μm thick using a JMT-MT-990 ultramicrotome, and stained with 1 % toluidine blue (Márquez-Guzmán et al., 2016). The histological sections were photographed using an Olympus Provis AX70 optical microscope equipped with an Evolution MP5.1 digital camera.

For scanning electron microscopy (SEM), the samples were dehydrated in increasing ethanol concentrations up to 100 % ethanol, they were brought to a critical point in a dryer (CPD-030 Baltec), mounted onto metallic stubs, and coated with gold in an ionizer (Denton Vaccumm Desk-II). Photomicrographs were obtained using a JEOL JSM-5310LV scanning electron microscope.

To observe the pollen tubes, 1 % decolorised aniline blue was applied to histological sections of the style and observed under an Olympus Provis AX750 fluorescence microscope.

For the analysis of pollen by optical microscopy, acetolysis was performed on the pollen grains of the three *Pholisma* species (Márquez-Guzmán et al., 2016). Finally, the figures were created using Adobe Photoshop 20 (Adobe, Inc).

3. Results

3.1. Morphology of inflorescences and flowers

The plants of *Pholisma* grow clustered along the roots of the hosts they parasitize (Fig. 1A-C). The inflorescences are supported by a long, fleshy inflorescence stem buried in the substrate (sand). The stem of inflorescence has deltoid bracts (Fig. 1D); the stem length varies among species, with *P. arenarium* at 30–40 cm, *P. sonorae* at 60–80 cm, and *P. culiacana* at 10–15 cm. The inflorescence of *P. arenarium* has asynchronous maturation. The different stages of development are scattered along the inflorescence (Fig. 1A), and the flowers develop in irregular double cymes (Fig. 1E). In *P. sonorae*, the inflorescences are flattened (Fig. 1B), and type scorpioid, with centrifugal maturation (Fig. 1F). When the inflorescences are young, their margins are rounded, and the bracts exhibit a compact conformation at the apex; the receptacle where all the flowers are inserted is wide (Fig. 1B). In *P. culiacana,* the inflorescence is a capitulum that becomes mature centrifugally (Fig. 1C, G). In *P. arenarium* and *P. culiacana*, during the early developmental stages, the bracts completely envelop the floral primordia, displaying a compact arrangement among themselves (Fig. 1D).

The flowers in the three species of the genus are hermaphroditic and infundibuliform, and pedicels support them. The calyx in *P. arenarium* consists of six flat sepals; the base is white, and the rest is purple covered by white glandular trichomes (Fig. 1H); in *P. sonorae*, the calyx consists of eight cylindrical purple sepals, with abundant elongated, filamentous, and white trichomes that intertwine with each other, forming a dense indumentum (Fig. 1I), giving a woollen appearance to the whole inflorescence (Fig. 1F). The flowers at anthesis protrude above this indumentum; only the base of the sepals in both species is connate respectively, while in *P. culiacana*, the sepals are connate, forming a tube, and only the apices are free and triangular shaped, with pink apex and the rest white. The corolla is sympetalous with free wavy apices in all three species. The petals colour in *P. arenarium* and *P. sonorae* is white, with central purple lines running from the corolla tube to the limbo (Fig. 1J-K), while in *P. culiacana*, it is white with pink apices (Fig. 1L). The androecium in all three species generally consists of six yellow epipetalous stamens; however, their position varies. In *P. arenarium*, anthers are situated below the level of the stigma (Fig. 1J); in *P. sonorae*, they are very close to the level of the stigma (Fig. 1K); and in *P. culiacana*, they are above the level of the stigma (Fig. 1L). The gynoecium is syncarpous; in *P. arenarium* and *P. sonorae,* the ovary is purple, with an elongated style and white stigma (Fig. 1J-K), while in *P. culiacana*, the ovary, style, and stigma are lightly pink (Fig. 1L).

3.2. Flower micromorphology

The micromorphology shows that each epidermis of sepals, petals, and pollen are different. The abaxial and adaxial epidermis of the sepals in *P. arenarium* have stomata and glandular trichomes with a rounded apical cell and a multicellular basal stalk (Fig. 2A-B). In *P. sonorae,* the sepals are cylindrical from the base to the apex and exhibit multicellular and elongated filamentous trichomes, with a rounded apical cell and stomata throughout the surface (Fig. 2C-D). In *P. culiacana*, the sepals

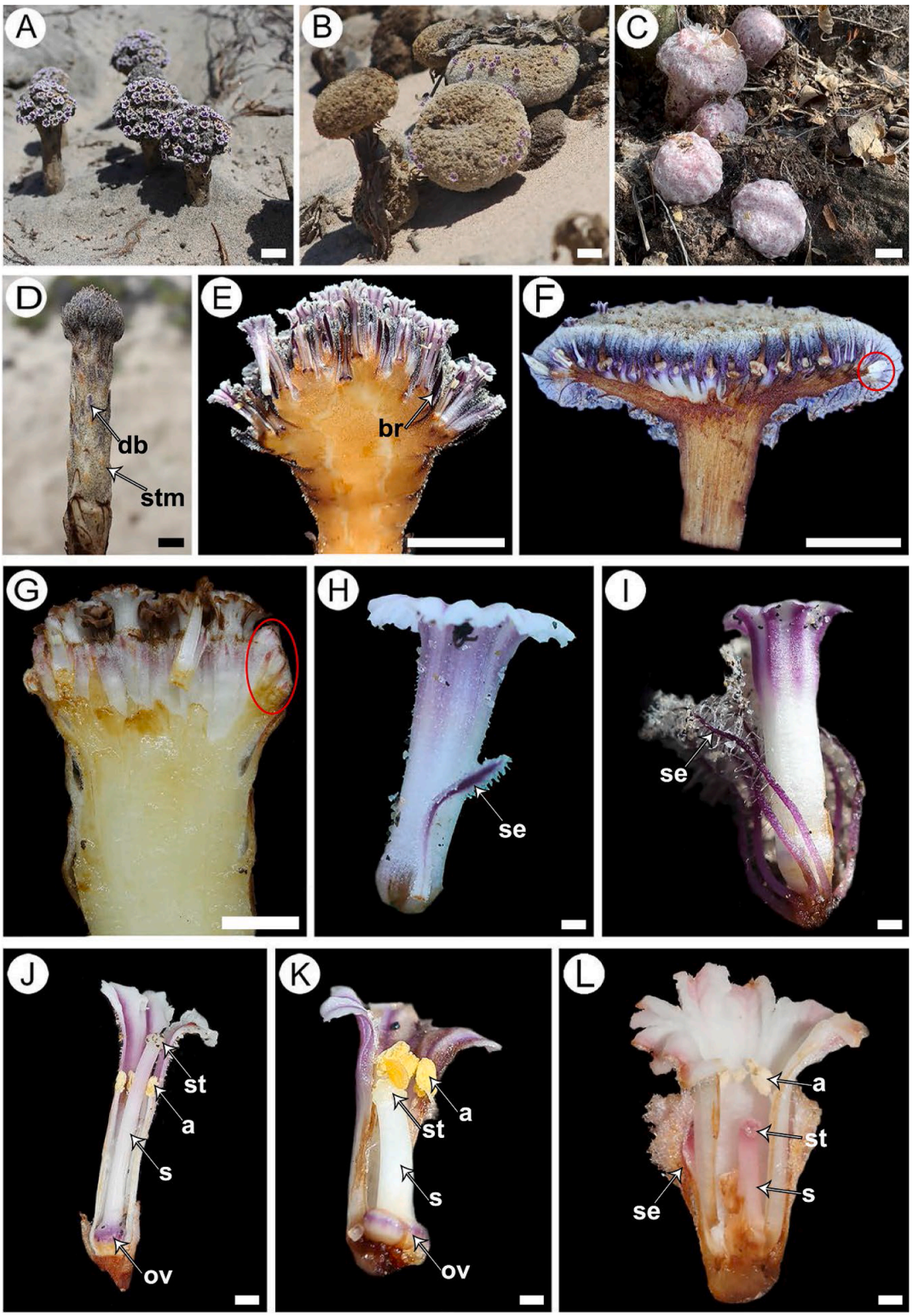

Fig. 1. Morphology of inflorescences and flowers. (A, D, E, H, J) *Pholisma arenarium.* (B, F, I, K) *P. sonorae*. (C, G, L) *P. culiacana.* (A-C) Grouped plants growing on dunes. (D) Stem with deltoid bracts on the surface. (E) Longitudinal section of the inflorescence, bracts can be observed between the flowers. (F) Scorpioid inflorescence, early stages on the periphery (red circle), note the pubescence. (G) Longitudinal section of the inflorescence, early stages on the periphery (red oval). (H-L) Infundibuliform, hermaphroditic flowers, showing the position of the anthers relative to the height of the stigma and sepals with trichomes. Abbreviations: a, anther; br, floral bract; db, deltoid bracts; ov, ovary; s, style; se, sepal; st, stigma; stm, stem. Scales bars = 0.5 cm (A-B), 1 cm (C-G), 1 mm (H-L).

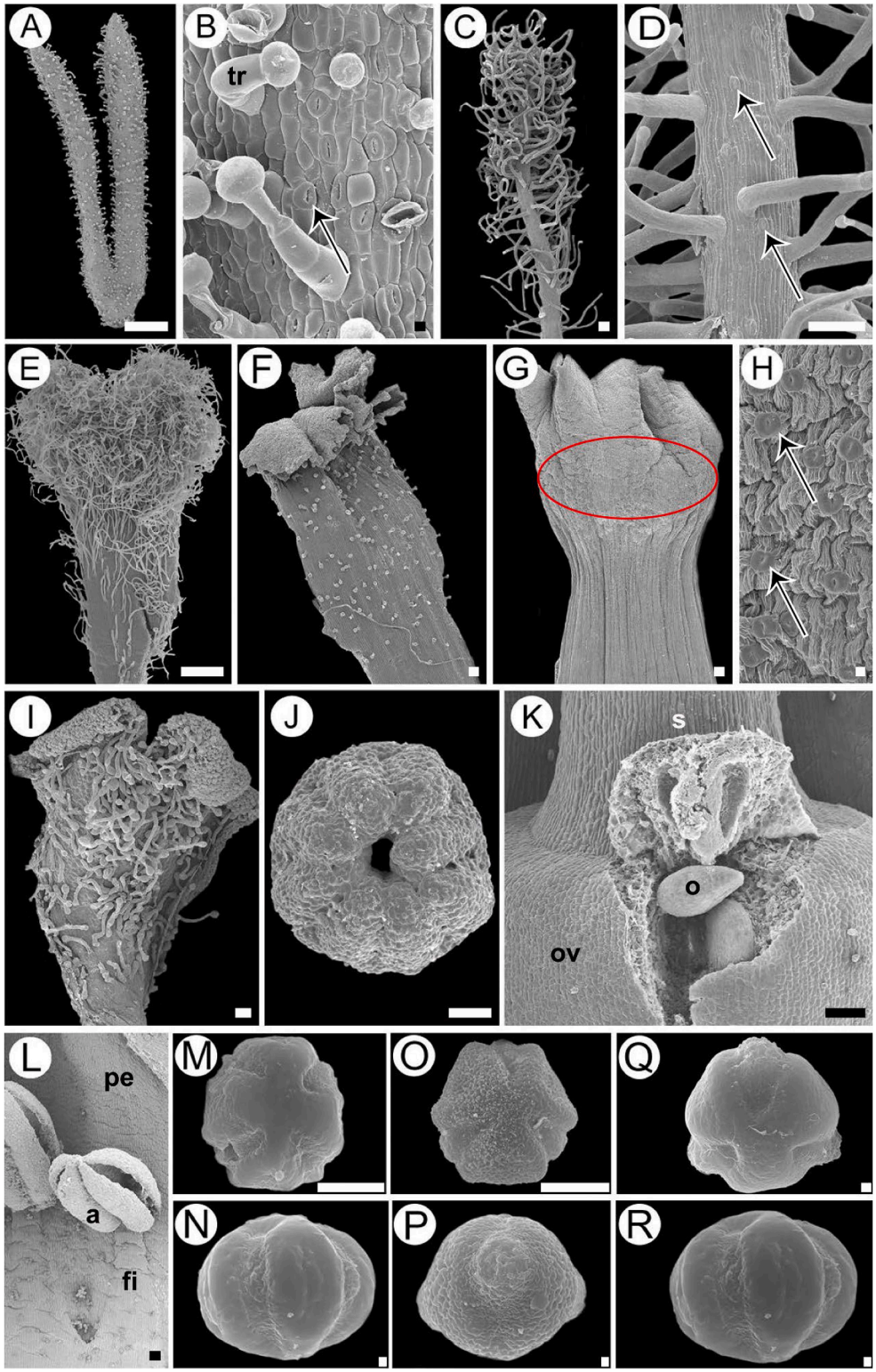

Fig. 2. Flower micromorphology. (A-B, F, K-N) *P. arenarium*. (C-D, G-H, J, O-P) *P. sonorae*. (E, I, Q-R) *P. culiacana*. (A) Sepals connate at the base. (B) Close-up of the abaxial face of the sepal, the epidermis with cells of straight, smooth, and slightly swollen cell walls; glandular trichomes and stomata (arrow) can be observed on its entire surface. (C) Apex of the cylindrical sepal with elongated glandular trichomes. (D) Close-up of the sepal, stomata are observed (arrows). (E) Flattened sepal covered by filamentous trichomes on the abaxial zone. (F) Segments of the sympetalous corolla, with short glandular trichomes on the abaxial epidermis. (G) Middle and apical part of the sympetalous corolla in abaxial view, only stomata are found (red oval). (H) Close-up of the epidermis with slightly sinuous and striated cell walls with stomata (arrows). (I) Petal, with filamentous trichomes with glandular apex on the abaxial epidermis. (J) Crateriform stigma in frontal view. (K) Close-up of the ovary showing ovules and the base of the merged styles. (L) Anthers with longitudinal dehiscence, filaments adnate to the corolla. (M-N) Tetracolporate pollen grain with psilate exine. (O-P) Tricolporate pollen grain with reticulate exine. (Q-R) Tricolporate pollen grain with psilate exine. Abbreviations: a, anther; fi, filament; o, ovule; ov, ovary; pe, petal; s, style; tr, trichomes. Scales bars = 1 mm (A, E), 10 μm (B, H, M, O), 100 μm (C-D, F-G, I, J-L), 1 μm (N, P-R).

are flatted, having an apex with heart form; the abaxial epidermis has filamentous trichomes, being more abundant at the apex (Fig. 2E). Regarding the corolla, the petals of *P. arenarium* have irregularly distributed short glandular trichomes on the abaxial epidermis (Fig. 2F), while *P. sonorae* has only stomata (Fig. 2G-H), and *P. culiacana* has elongated glandular trichomes with a rounded apical cell; trichomes are distributed from the apex up to three-quarters of the base of the petals (Fig. 2I).

The three species of the genus have syncarpous gynoecium consisting of a crateriform stigma (Fig. 2J), a style formed by independently fused carpels but with a single-style appearance and an ovary containing flattened and ovoid ovules (Fig. 2K). The stamens have the filament adnate to petals, and only the top of the filament is free; the anthers are bilobed, basifixed, oblong, and exhibit longitudinal dehiscence (Fig. 2L). While the pollen micromorphology differs among species, in *P. arenarium*, the pollen grains are tetracolporate, with psilate exine (Fig. 2M, N, S1A, D); in *P. sonorae*, pollen grains are tricolporate, with reticulate exine (Fig. 2O, P, S1B, E); and in *P. culiacana*, pollen grains are tricolporate, with psilate exine (Fig. 2Q, R, S1C, F); however, the shape of the pollen in the three species of the genus is subspherical (Fig. 2N, P, R).

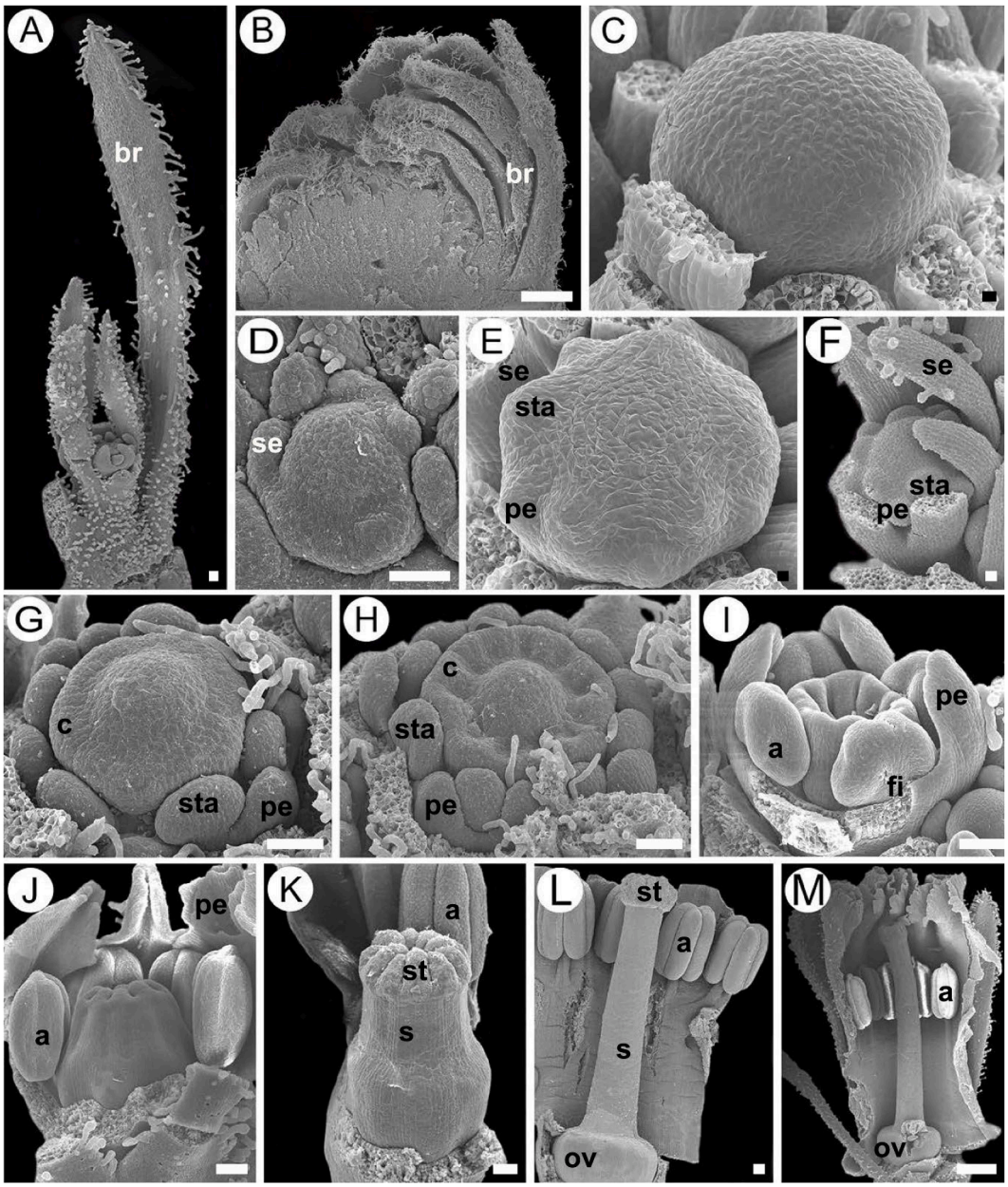

Fig. 3. Flower development through SEM. (A, F, I, J, M) *P. arenarium.* (D, G, H) *P. sonorae* (B, C, E, K, L) *P. culiacana.* (A) Floral bud protected by a bract. (B) Floral primordia protected by bracts forming an involucre on the margin of the inflorescence. (C) Floral meristem in dome shape. (D) Close-up of a floral bud showing sepal primordia. (E) Emergence of petal primordia, stamen primordia arise alternately with petal primordia. (F) Sepals begin trichome formation. (G) Carpel primordia begin to differentiate. (H) Elongation of all floral whorls. (I) Growth of carpel margins inward forming the ovary without septa formation; anthers become bilobed. (J) Styles formation; petal apices become lobed and trichomes appear on the abaxial surface. (K) Styles elongation and stigma formation. (L) Elongation of floral whorls. (M) Flower in anthesis. Abbreviations: a, anther; br, floral bract; c, carpels; fi, filament; ov, ovary; pe, petal; s, style; se, sepal; st, stigma; sta, stamens. Scales bars = 100 μm (A, D, F-L), 1 mm (B, M), 10 μm (C, E).

3.3. Comparative flower development

Bracts cover the flower primordia in *P. arenarium* and *P. culiacana*, but their position varies. In *P. arenarium*, the bracts develop between the flowers (Fig. 3A), while in *P. culiacana*, additional bracts are positioned at the periphery of the inflorescence as an involucre (Fig. 3B). Each flower primordium in the three species of the genus is dome shaped (Fig. 3C), with the first flower whorl to differentiate being the sepals (Fig. 3D). The floral apex develops into a pentagonal or hexagonal shape. Subsequently, the petals begin to differentiate, alternately concerning the sepal and stamen primordia (Fig. 3E). The flower apex begins to flatten after the emergence of the petal primordia; the young sepals enclose it; simultaneously, on the surface of the sepals, glandular trichomes begin to develop, and the stamen primordia arise alternately, internally, and adnate to the petals (Fig. 3F). During the formation of the anthers, the petal apices bend inward, and the margins of the floral apex

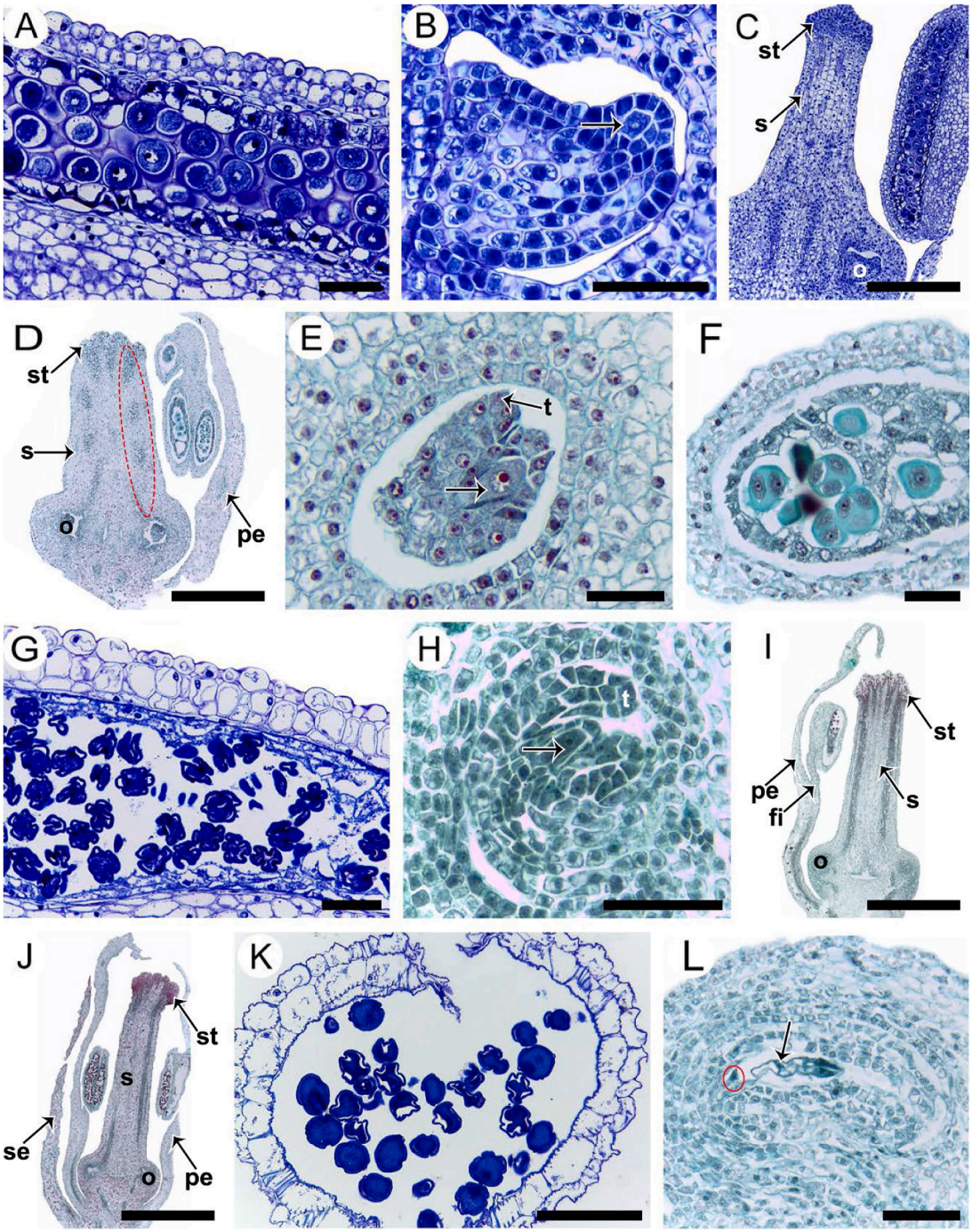

Fig. 4. Flower anatomy during the development of *Pholisma*. (A-E, G-H, J) *P. arenarium*. (I) *P. sonorae*. (F, K-L) *P. culiacana*. (A) Anthers containing microspore mother cells. (B) Ovule primordium at archesporial cell stage. (C) The stigma and style begin to differentiate, and ovule primordia emerge. (D) Elongating styles and stigma, the series of transmitting tissue (red oval). (E) Ovule at the megaspore mother cell stage. (F) Anthers at the stage of tetrad of microspores surrounded by callose. (G) Anther with young pollen grains. (H) Ovule at functional megaspore stage. (I) Initiation of stigmatic papillae development. (J) Flowers in anthesis with mature pollen grains and ovules. (K) Mature bicellular pollen grains. (L) Ovule with mature embryo sac. Abbreviations: fi, filament; o, ovule; pe, petal; s, style; se, sepal; st, stigma; t, integument. Scales bars = 320 μm (C-D, I-J), 80 μm (A-B, E-H, K-L).

start to elevate, forming a shallow depression, which will give rise to the carpel primordia; however, a dome shaped structure remains in the centre (Fig. 3G). The carpels continue elongating apically and are distinguished as fused laterally; their margins elongate towards the centre of what will be the ovary without forming septa (Fig. 3H). This base of the gynoecium widens, while in the androecium, the filaments begin to differentiate, and the anthers become bilobed (Fig. 3I). The carpel apices grow in length, and their margins close independently, forming a stylar structure through the independent fusion of the carpels, leaving an opening in the centre representing a false stylar canal. The upper zone of the corolla tube and the petal lobes begin to grow over the developing anthers, while the petal apices become lobulated and wavy (Fig. 3J). Subsequently, the ovary widens; the merged styles elongate; and the carpel apices form the crateriform stigma (Fig. 3K). The fused styles continue elongating, reaching a greater height than the ovary, and the stigma widens (Fig. 3L). Finally, in the mature flower, the corolla completely covers the sexual whorls, consisting of connate petals with highly lobulated apices and imbricate aestivation. The androecium with stamens adnate to the petals; the anthers supported by filaments that remain free only close to the base of the anther; the rest of the filament remains adnate to the corolla; the syncarpous gynoecium is formed by an ovary with a long compound style and crateriform stigma (Fig. 3M).

3.4. Flower anatomy during development

There is asynchrony between the development stages of the male and female gametophytes among the three species of *Pholisma*. When the anthers contain microspore mother cells (Fig. 4A), the ovule is in the stage of the archesporial cell (Fig. 4B). At this stage, both the stigma and style begin to differentiate (Fig. 4C). Subsequently, the style begins to elongate, and the series of transmitting tissue along each carpel from the ovary to the stigma begins to be distinguished (Fig. 4D); the megaspore mother cell arises from the ovule primordium (Fig. 4E) at the tetrad stage within the anthers (Fig. 4F). During the early pollen grains stage (Fig. 4G), the ovule is in the functional megaspore stage (Fig. 4H). The stigmatic papillae develop on the flowers' stigma, and the style continues elongating (Fig. 4I). Finally, in the last stage of flower development (Fig. 4J), the anthers contain mature pollen grains (Fig. 4K), and the ovule contains the Polygonum-type embryo sac (Fig. 4L).

3.5. Flowers in anthesis

3.5.1. Gynoecium

The three species of *Pholisma* exhibit a syncarpous and multicarpellary gynoecium. The stigmas are of the wet type, with elongated stigmatic papillae containing abundant cytoplasm connected to the individual tract of transmitting tissue of each carpel (Fig. 5A), which close independently, forming a ring of fused carpels and giving the impression of a single style with a central canal. In the centre of the stigma, a hollow is distinguished, varying in diameter among the three species of *Pholisma*, with the widest diameter found in *P. culiacana*, followed by *P. arenarium*, and *P. sonorae* has the most minor diameter (Fig. 5B-D). This canal extends throughout the styles until the ovary). All three species of the genus have merged style (since each carpel is independent. Each style is elongated and has an inner and an outer epidermis of the originating carpel. The inner epidermis forms the internal canal. Between the two epidermises are isodiametric parenchymal cells without intercellular spaces between them, one vascular bundle per carpel, and a tract of transmitting tissue. Along the merged style, the transmitting tissue forms a ring of independent tracts with equidistant distribution between them, consisting of isodiametric cells of smaller size compared to the parenchymal cells comprising the style and dense cytoplasm characteristic of secretory cells (Fig. 5E). Pollen tubes grow through each of the transmitting tissue tracts (Fig. 5F).

The ring, formed by each tract of the transmitting tissue, extends to the carpels at the ovary level, where two campylotropous ovules develop per carpel. The ovules exhibit parietal placentation, as even though the margins of each carpel extend towards the locule of the ovary, they never reach the centre, thus not forming complete septa but rather false septa in some areas (Fig. 5G). Each ovule arises laterally in the carpel tissue forming the ovary wall. The number of carpels, and therefore of ovules, varies among the three species. In *P. arenarium*, 18–22 ovules were recorded, while in *P. sonorae* 20–24 and *P. culiacana* 18–24 ovules. The ovules are in the upper third of the ovary. The ovary wall consists of the inner and outer epidermis and approximately 4–5 layers of irregularly shaped and sized parenchymal cells between them, without intracellular spaces and with large vacuoles, small nuclei, and vascular bundles (Fig. 5H).

3.5.2. Androecium

The stamens are adnate to the petals, and a vascular bundle innervates each one. The apex of the filament is short, composed of parenchymal cells with central nuclei, and the anther consists of two parallel lobes (thecae) connected by a broad parenchymatous connective tissue. Each anther lobe has two microsporangia separated by a septum of connective tissue consisting of around five layers of parenchyma (Fig. 5I).

3.5.3. Perianth

The petals of the three species are connate; the cells between the abaxial and adaxial epidermis are parenchymatous, with variable shapes and sizes and some intercellular spaces between them, and a vascular bundle runs along the entire length of each petal. The adaxial epidermis forming the limb and corolla tube presents rounded, vacuolated cells of smaller size compared to the rest of the cells that make up the petal. The papillose adaxial epidermis looks like osmophores. At the same time, glandular trichomes develop on the abaxial epidermis, varying in shape, size, and distribution according to the species of *Pholisma* (Fig. 5J-K). The sepals are formed by adaxial and abaxial epidermis formed by small cells. Between the two epidermises, there is vacuolate parenchymatous tissue innervated by a vascular bundle. Both epidermises have glandular trichomes, with variations in distribution, shape, and size among the three species (Fig. 5L).

3.6. Megasporogenesis and megagametogenesis

Megasporogenesis and megagametogenesis are identical for the three species of *Pholisma*. Megasporogenesis begins with the ovule primordium (Fig. 6A), where an archesporial cell surrounded by a nucellar protodermis initiates within the nucellus, along with the commencement of an incipient integument (Fig. 6B). Subsequently, the ovule primordium curves, revealing the integument covering the monolayered nucellus, and the archesporial cell expands and functions directly as a megaspore mother cell (Fig. 6C). The megaspore mother cell undergoes meiosis I to produce a dyad of megaspores. After meiosis II, a linear tetrad of megaspores results (Fig. 6D). The chalazal megaspore becomes the functional megaspore, and the integument becomes multilayered (Fig. 6E). It surrounds the nucellus to form the micropyle; at this stage, a small funiculus is distinguished. Fully developed ovule is almost sessile because the funicle is short, campylotropous, unitegmic, and tenuinucellate, and they harbour a Polygonum-type embryo sac comprising three antipodal cells, a central cell with two polar nuclei, and an ovocellular apparatus formed by the egg cell and two synergids (Fig. 6F).

3.7. Microsporogenesis and microgametogenesis

Microsporogenesis and microgametogenesis follow a similar pattern in the three species of *Pholisma*. Initially, the anther has only parenchymatous tissue, which is a fundamental tissue delimited by a protodermis and an incipient vascular bundle in the centre of the parenchyma. Subsequently, the anther comprises two parallel lobes (thecae) united by a broad parenchymatous connective tissue and a

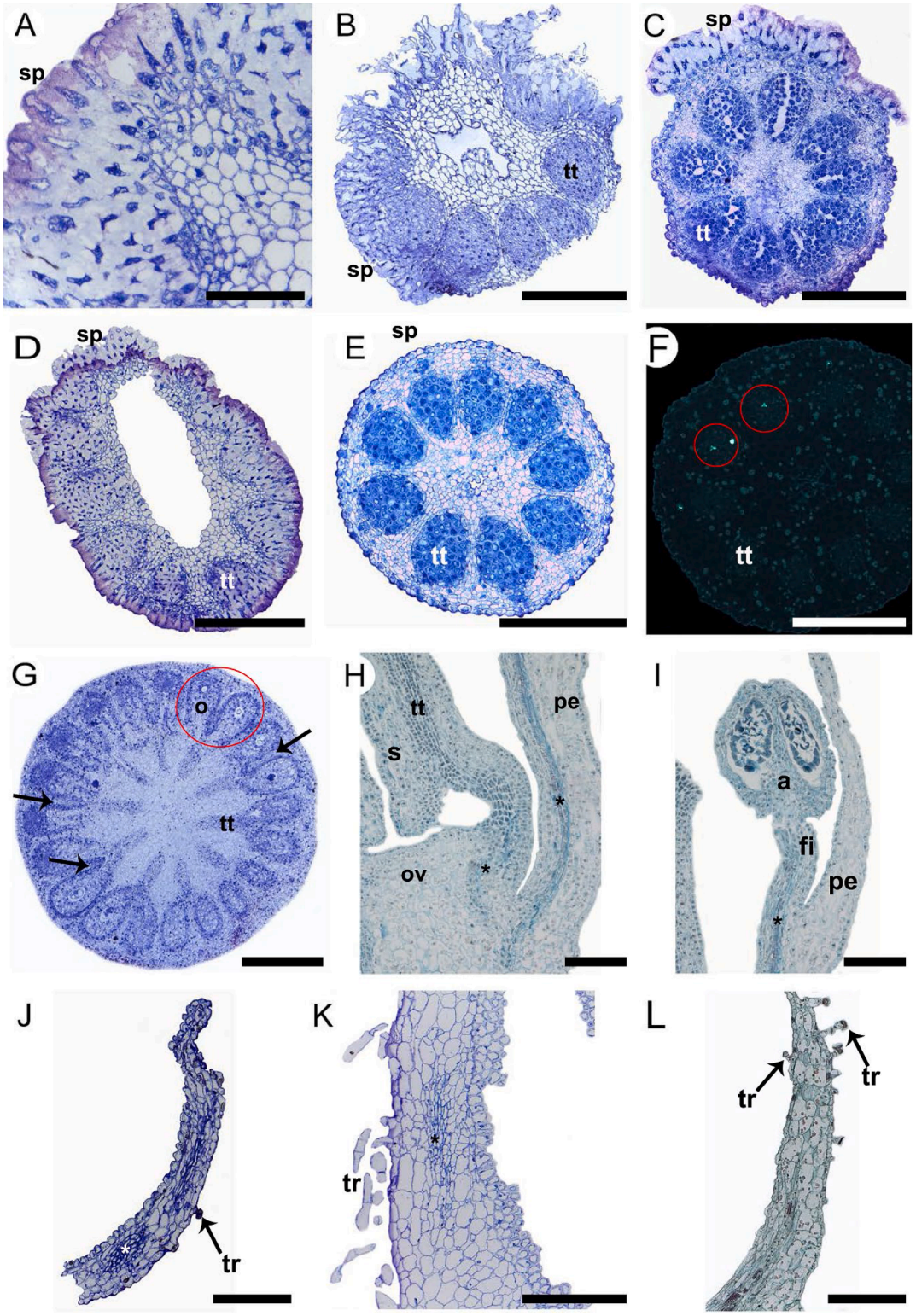

Fig. 5. Anatomy of the floral whorls of *Pholisma* species. (B, F, L) *P. arenarium*. (C, E, G-J) *P. sonorae.* (A, D, K) *P. culiacana.* (A) Close-up of the stigma, showing stigmatic papillae with exudate and transmitting tissue. (B-D) Wet stigmas with secretory papillae and dense cytoplasmic transmitting tissue. (E) Mid-level section of the style, showing the ring of fused styles and the transmitting tissue of each. (F) Pollen tubes (red circles) growing through the series of transmitting tissue. (G) Cross-section of the ovary, showing two ovules per carpel (red circle), the tracts of transmitting tissue, and the false septa of the unilocular ovary (arrows). (H) Long section of ovary and corolla. The ovary shows the transmitting tissue tract. (I) Epipetalous stamen, supplied by a vascular bundle (asterisk) adnate to the petal. (J-K) Petals, with abaxial epidermis bearing trichomes, and papillose adaxial epidermis like osmophores. (L) Sepal with trichomes. Abbreviations: a, anther; fi, filament; o, ovule; ov, ovary; pe, petal; s, style, sp, stigmatic papillae; tr, trichomes; tt, transmitting tissue; Scales bars = 320 μm (B-L), 80 μm (A).

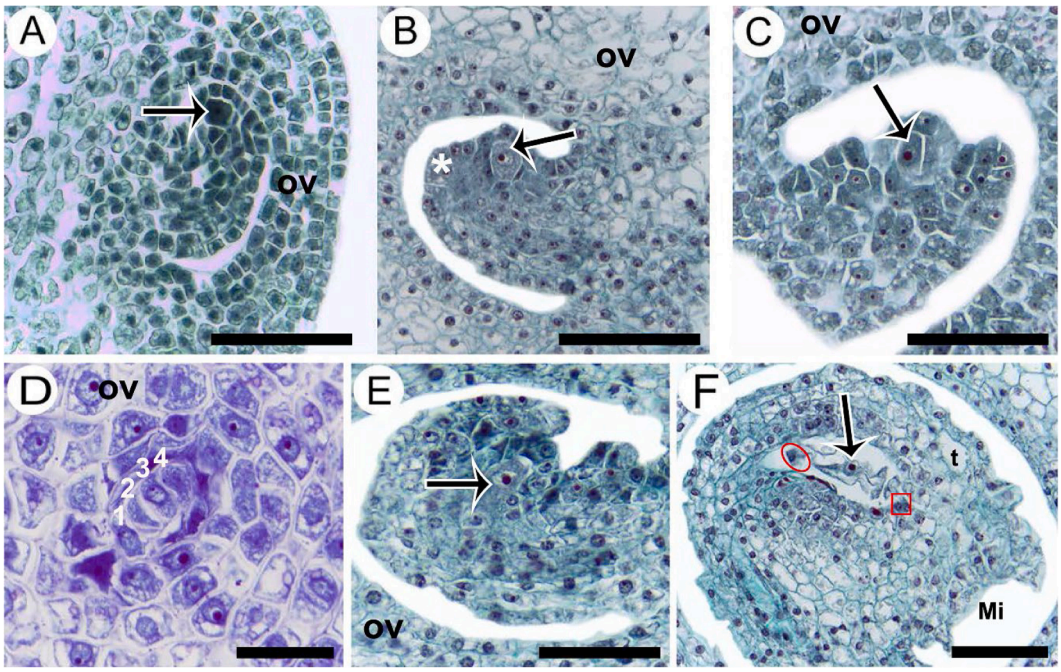

Fig. 6. Megasporogenesis and megagametogenesis. (C-D) *P. arenarium.* (A) *P. sonorae.* (B, E-F) *P. culiacana.* (A-B) Ovule primordium with archesporial cell (arrow) and integument primordium (asterisk). (C) Ovule at megaspore mother cell stage (arrow) and elongation of the integument. (D) Linear tetrad of megaspores. Each megaspore is numbered (1, 2, 3, 4). (E) Functional chalazal megaspore (arrow), the integument grows to form the micropyle and ovule curvature, the funicle is short. (F) Mature campylotropous ovules with very short funicle and Polygonum-type embryo sac, antipodes cells (red circle), central cell (arrow), ovocellular apparatus (red square). Abbreviations: Mi, micropyle; ov, ovary; t, integument. Scales bars = 80 μm (A-C, E-F), 30 μm (D).

vascular bundle. Each anther lobe has two microsporangia, each with a monolayered protodermis and archesporial cells (Fig. 7A). Below it, periclinal divisions occur to form a primary parietal layer in a subepidermal position and archesporial tissue. The cells of the parietal layer divide periclinal, forming two secondary parietal layers, the outer and inner, which surround the sporogenous cells (Fig. 7B). The cells of the two parietal layers divide periclinal and form the endothecium and a stratum of middle layer, and the other parietal layer form a stratum of the middle layer and the tapetum; therefore, the anther wall is of the basic type. Sporogenous cells proliferate (Fig. 7C). The layers of the anther wall increase in size, the innermost having dense cytoplasm. Five layers form the anther wall: the epidermis, a subepidermal endothecium, a two-layered middle layer, and the tapetum. The microspore mother cells increase in size, their cytoplasm is dense, and nuclei become evident preparing for meiosis and are surrounded by callose, and the tapetal cells become vacuolated with dense cytoplasm (Fig. 7D). At this time, the microsporangia may be found in different developmental stages, indicating asynchronous development. While in one of the microsporangia, the microspore mother cells entered meiosis, forming four nuclei that share the same cytoplasm (early tetrads of microspores), in others microsporangia, tetrahedral microspores surrounded by callose had already formed (Fig. 7E). At this stage, the binucleated secretory tapetum is evident. Subsequently, some microsporangia with free microspores (or young and unicellular pollen grains) can be observed with thin cell walls because they have been released from callose and have irregular shapes. In others, meiosis is concluding, and there are tetrads of microspores. The anther wall at this stage consists of an epidermis, cubical endothecium cells, middle layers, and active secretory tapetum (Fig. 7F). As callose degrades, the microspores or young pollen grains become free in the pollen sacs. The middle layers begin to collapse; the tapetal cells are rectangular and have dense cytoplasm, and their walls break to incorporate their content into the pollen sacs. The microsporangia development is synchronous at this time (Fig. 7G). The unicellular pollen grains, which begin to increase in size; their cytoplasm becomes dense, and colpi become evident. The endothecium cells are cubical, in some of them, thickenings begin forming in their walls, the tapetum cells are collapsed (Fig. 7H). The septa between each pair of microsporangia will begin to degrade, as well as the tapetum and middle layer, so that each pair of pollen sacs unite and force the dehiscence line to break, as both the epidermis are very thin. The epidermis is persistent, and all the endothecium cells develop thickenings in their walls; the pollen grains are bicellular. Finally, in the last stage of development, the septum between each pair of microsporangia degrades, and the stomium breaks (Fig. 7I).

4. Discussion

The Lennoaceae parasitic plants lack a visible vegetative body above the substrate when they emerge from the root of the host and the sand, and only flowers or inflorescences can be detected. In this sense, the morphology of inflorescences and flowers of the studied species facilitate the recognition of each species of *Pholisma*, and it will be important in attracting pollinators and their sexual reproduction. However, the embryology characteristics and development patterns appear to be homogeneous.

Recently, molecular analysis has demonstrated that the Lennoaceae family is related to the Ehretiaceae family of the Boraginales order (Zang et al., 2020). Therefore, the characteristics described in this study of *Pholisma* are compared with some genera that comprise the Ehretiaceae family (*Bourreria, Ehretia* P. Browne, and *Tiquilia* Pers.) to identify similarities among them and thus support the recently proposed phylogenetic relationship with morphological, embryological, and anatomical data.

4.1. Inflorescences and flower morphology

Within the Ehretiaceae family, the only flower trait shared among its species is the more-or-less bifid style (Gottschling et al., 2014a). However, this is one of the differences between Ehretiaceae and *Pholisma* (Lennoaceae) because in all three species, the carpels are fused from the

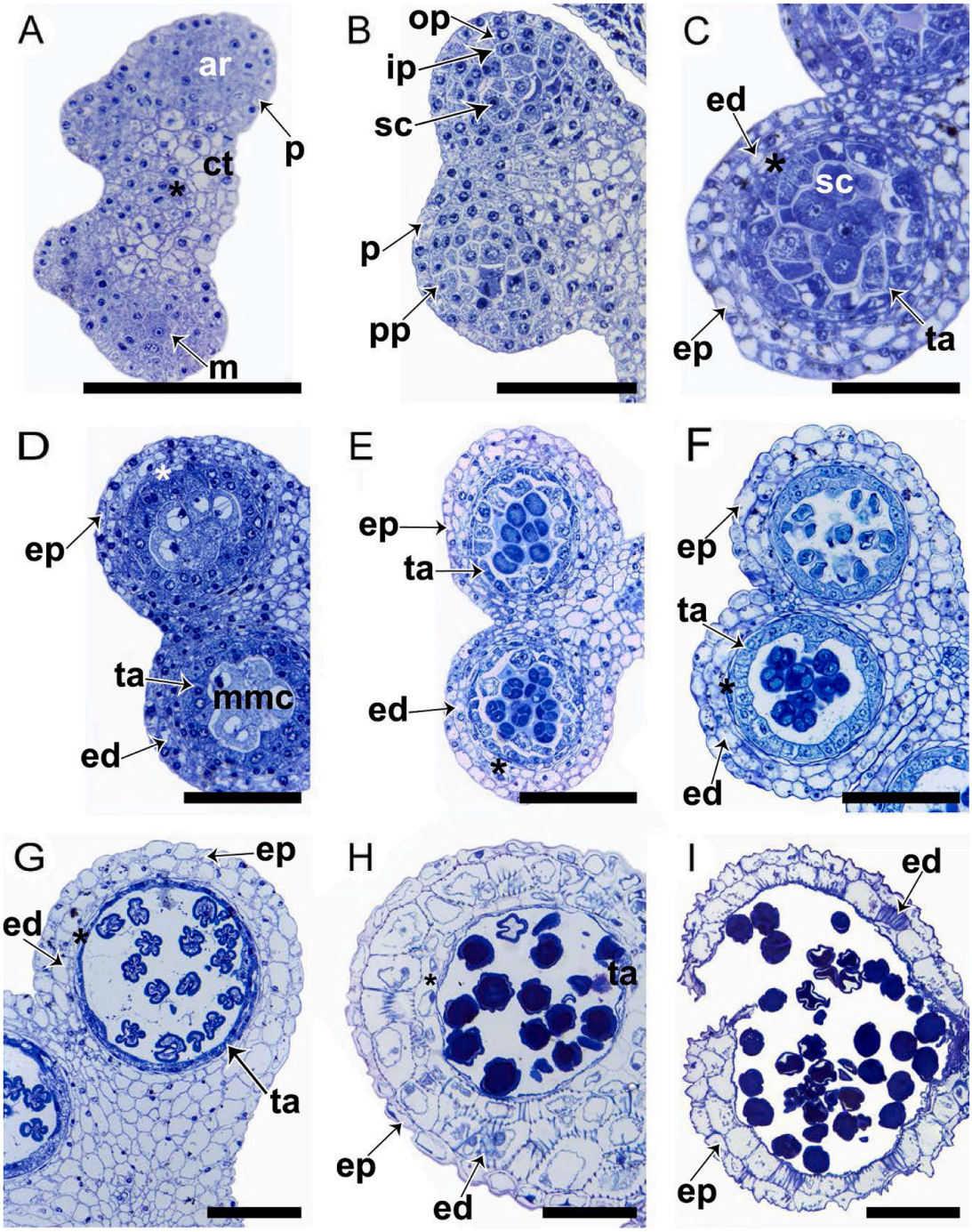

Fig. 7. Microsporogenesis and microgametogenesis. (A, C, E) *P. culiacana.* (B, D, F, G, I) *P. arenarium.* (H) *P. sonorae.* (A) Tetrasporangiate anther with archesporial cells. (B) Close-up of a theca, showing outer and inner parietal layers. In the centre of each microsporangium the sporogenous cells. (C) Anther wall formed of five layers. (D) Detail of the anther wall at the microspore mother cell stage. (E) Anther wall at tetrad stage, showing developmental asynchrony between microsporangia. (F) One microsporangium at tetrad stage, surrounded by callose, and the other with free microspores; highly active tapetum. (G) Close-up of the anther wall at young pollen grain stage, at this stage microsporangia are synchronous. (H) Endothecium begins to form lignified thickenings, tapetum collapsed, and pollen grains with reserves. (I) Dehiscent anther, the septum between each pair of microsporangia degrades and the anther wall formed only by epidermis and endothecium. Abbreviations: ar, archesporial cells; ct, connective tissue; ed, endothecium; ep, epidermis; ip, inner secondary parietal layer; m, microsporangium; mmc, microspore mother cells; op, outer secondary parietal layer; p, protodermis; pp, parietal layer; sc, sporogenous cells; ta, tapetum; *, middle layers. Scales bars = 320 μm (A), 80 μm (B-I).

base of the ovary, styles until the apex of the stigma. The inflorescences of *Bourreria* are terminal or subterminal, and the flowers are arranged in corymbiform cymes. In contrast, in *Ehretia*, they are terminal from cymose to paniculate, and in *Tiquilia*, the flowers are highly condensed, forming scorpioid inflorescences. Particularly in *Pholisma* can be observed both determinate (in *P. arenarium* and P. *sonorae*) and indeterminate (in *P. culiacana*) inflorescences, with those of the *Tiquilia* being more similar to *P. sonorae.*

Some characteristics of the flowers of *Pholisma* species are shared with the compared genera of the Ehretiaceae family. They have perfect flowers with actinomorphic symmetry, with petals congenitally fused into a tube, and the lobes (apical part of the petals) are free only at the

distal part of the limb. The stamens adnate to the corolla tube; the anthers are dorsifixed, with longitudinal dehiscence; and the free portions of the filaments are generally short. However, particularly in *Ehretia*, the anthers are oblong to ellipsoid (Miller, 1989; Gottschling, 2004; Gottschling and Miller, 2007; Gottschling et al., 2014a).

The sepals of *Bourreria* flowers are congenitally fused into a tube at the base, while the lobes are free at the apex and have a triangular shape (Gottschling, 2004). In *Ehretia*, the calyx consists of five imbricated or open sepals with triangular, ovate, or deltoid lobes and may be pubescent or glabrous (Miller, 1989). In all three species of *Pholisma*, the calyx is pubescent; however, the distribution and shape of the trichomes vary. Similarly, the shape of the sepals is different for each *Pholisma* species. In *P. arenarium*, the sepals are connate only at the base, linear in shape, and both epidermises develop glandular trichomes. Similarly, in *P. sonorae*, the trichomes are present on both epidermises but have an elongated and filamentous shape; the sepals are cylindrical and connate at the base, while in *P. culiacana*, the calyx is fused into a tube; the lobes are triangular and free at the apex, and the abaxial epidermis of the calyx has filamentous trichomes, being more abundant at the apex.

However, some differences found are the position of the anthers, which are exerted in most species of *Bourreria* and *Ehretia* (Miller, 1989; Gottschling, 2004; Gottschling and Miller, 2007), while in *Tiquilia*, generally, the anthers do not protrude from the corolla (Gottschling et al., 2014b), as is the case with all *Pholisma* species. Regarding the colour of the petals, many species of Ehretiaceae have white corollas (Gottschling et al., 2014a); however, in *Tiquilia*, some species have pink (*T. greggii* (Torr. & A. Gray) A.T. Richardson) and blue (*T. dichotoma* (Ruiz & Pav.) Pers.) corollas (Gottschling et al., 2014b). Both *P. sonorae* and *P. arenarium* have white corollas with purple lines running from the tube to the apex of the limb, while in *P. culiacana*, the corolla colour is white with pink margins. The shape of the corolla is the same in all three *Pholisma* species; they have infundibuliform corollas, while in *Bourreria*, the corolla can be infundibuliform, hypocrateriform, or tubular (Campos-Ríos, 2005); in *Ehretia*, it is tubular or campanulate; and in *Tiquilia*, it is infundibuliform or campanulate (Gottschling et al., 2014a). Similarly, the stigma in all three *Pholisma* species is wet type and crateriform, while in *Bourreria, Tiquilia*, and *Ehretia*, the stigma is bifid and papillose (Miller, 1989; Gottschling, 2004; Gottschling et al., 2014a). Within the genus *Pholisma*, the pollen morphology varies. In *P. arenarium*, it is tetracolporate with psilate exine; in *P. sonorae*, it is tricolporate with reticulate exine; and in *P. culiacana*, it is tricolporate with psilate exine. Previously, pollen in *P. arenarium* has been described as tricolporate, tetracolporate, or pentacolporate, with tetracolporate being the most common form, with a sculpturing forming a latimurate reticulum (Drugg, 1962), while in the genus *Bourreria*, it is predominantly tricolporate. However, there are also species with tetracolporate pollen, with ornamentation ranging from warty to rugulate, punctate, scabrous, or psilate (Campos-Ríos and Alfaro, 2003); in *Ehretia*, the pollen is prolate and tricolporate (Miller, 1989; Gottschling, 2004).

4.2. Trichomes

All three species of *Pholisma* present trichomes on the surface of the sepals and petals of their flowers. Particularly in species inhabiting coastal dunes, these trichomes could play a crucial role as a protective layer. Their functions include reducing wind speed near stomata, thereby decreasing water loss through transpiration and preventing wind-driven sea spray from reaching plant tissues and causing damage (Ariza and Belmonte, 1985). Additionally, the presence of trichomes acts as a filter by reflecting and scattering light, protecting the plant surface from direct sunlight, which in turn reduces light stress on the plant (Hesp, 1991; Liakopoulos et al., 2006; Ciccarelli et al., 2009; Skelton et al., 2012; Ciccarelli and Bona, 2022). All studied species have glandular trichomes. It has been reported that this type of trichome releases its contents in response to mechanical damage or after stimulation by light radiation. Some of the reported components in *Otanthus maritimus* Hoffmanns. & Link (Asteraceae), a species found in coastal dunes, include santolina triene, camphor, and artemisyl acetate, which have demonstrated antimicrobial properties and repellent/lethal effects on insects (Romeo et al., 2007; Ciccarelli et al., 2009). Similarly, glandular trichomes in halophyte plants have been observed to play a crucial role in removing excess salts from the plant (Voronkova et al., 2008). Although the proper function of the trichomes present in the studied species remains to be demonstrated, it should not be ruled out that some of the mentioned functions may occur in Lennoaceae.

4.3. Flower development

Flower development is very similar among the three species of *Pholisma*. Some differences are observed compared to *Bourreria succulenta* Jacq., the flower apex is flat at the onset of development, and the sepal primordia protrude on edge around the apex, with petals developing simultaneously with the sepals. In contrast, in species of the genus *Tiquilia* (*T. conspicua* (I.M. Johnst.) A.T. Richardson, *T. dichotoma, T. elongata* (Rusby) A.T. Richardson, *T. greggii, T. latior* (I.M. Johnst.) A.T. Richardson, *T. nuttallii* (Hook.) A.T. Richardson, *T. paronychioides* (Phil.) A.T. Richardson and *T. plicata* (Torr.) A.T. Richardon); the flower apex has the shape of a pentagonal receptacular plate, with sepals developing around the pentagonal plate, followed by petals and stamens (Gottschling et al., 2014b; Jeiter et al., 2023). In *Pholisma* species, the flower apex has a dome shape, and sepals develop in a proximal position concerning the bract, followed by the subsequent development of petals.

Both in *B. succulenta* and *Ehretia dicksonii* Hance, the primordia of stamens and petals cover the flower apex before the initiation of carpel primordia development, contrary to what happens with *Pholisma* species, where carpel primordia are distinguished, as stamen and petal primordia never cover the flower apex. Meanwhile, in the genus *Tiquilia*, the formation of carpel primordia starts later, developing when stamen and petal differentiation has occurred (Gottschling et al., 2014b; Jeiter et al., 2023). Similarly, the number of carpel primordia varies, with two in *B. succulenta* and *Ehretia dicksonii*; in *Pholisma*, this number exceeds 10 (approximately). Among the similarities found in flower development between *Pholisma* species and the genera *Bourreria* and *Tiquilia* is that the sepals exhibit valvate aestivation and complete their development before anthesis, while the corolla shows imbricate aestivation (Gottschling, 2004; Gottschling et al., 2014b; Jeiter et al., 2023).

4.4. Flower anatomy

In all three species of *Pholisma*, the ovules are in the upper third of the ovary, similar to *B. succulenta* (Jeiter et al., 2023). Similarly, *Pholisma* flowers have approximately 10–12 carpels, with two ovules developing per carpel, resulting in 20–24 ovules in *P. sonorae* and *P. culiacana,* and 24 in *P. arenarium*. The ovules exhibit parietal placentation, with the two ovules of each carpel separated by false septa due to the intrusion of ovary wall tissues. In contrast, the flowers of the genera *Bourreria, Tiquilia,* and *Ehretia* have two carpels, each with two ovules and axile placentation. In these genera, the two ovules of each carpel are also separated by false septa and basal septa, meaning that two ovules from different carpels are closer to each other than those within an individual carpel (Gottschling, 2004; Gottschling et al., 2014a; Jeiter et al., 2023). This latter condition does not occur in any *Pholisma* species, which is supported by seed development, where each seed is surrounded by a lignified endocarp, as reported by Campos-González (2024) for all Lennoaceae species. Another shared characteristic in all three *Pholisma* species is the presence of transmitting tissue tracts, extending from the stigma to the level of the funicles. This characteristic has also been reported in *Bourreria* (Gottschling, 2004), *Ehretia dicksonii* (Jeiter et al., 2023), and species within the genus *Tiquilia* (Gottschling et al., 2014b).

4.5. Megasporogenesis and megagametogenesis

All three species of *Pholisma* exhibit campylotropous, unitegmic, and tenuinucellate ovules. Previous studies on *P. arenarium* have reported an anatropous and epitropous ovule (Copeland, 1935). Whereas in species of the genera *Bourreria, Ehretia,* and *Tiquilia*, the ovules are anatropous but share the characteristic with *Pholisma* of being unitegmic and tenuinucellate (Khaleel, 1977; Rao and Rao, 1984; Gottschling, 2004; Gottschling et al., 2014a, b). The embryo sac is of the Polygonum type in all species of *Pholisma* as well as in the genera *Bourreria* and *Tiquilia* (Gottschling, 2004; Gottschling et al., 2014b). In *Ehretia* species, the embryo sac is generally Polygonum type, although the Allium type is occasionally also present (Khaleel, 1977; Rao and Rao, 1984). The ovule primordium in all species of *Pholisma* is composed of the archesporial cell surrounded by a nucellar protodermis. This configuration differs from what has been reported for *Bourreria*, where it has been described that the archesporial cell is surrounded by parietal cells (thus being an ovule of the crassinucellate type) and the nucellar epidermis (Gottschling, 2004). Similarly, in *Ehretia* species, it has been reported that the archesporial cell is surrounded by a parietal cell (Khaleel, 1977; Rao and Rao, 1984). In *Tiquilia*, the nucellar epidermis and parietal cells surround the archesporial cell (Gottschling et al., 2014b). Another shared characteristic among *Pholisma* species is the linear arrangement of tetrads after meiosis. For this characteristic, it has been reported that in *P. arenarium*, the megaspore tetrad has a T shape (Copeland, 1935), while in the genus *Ehretia*, tetrads are linear or T-shaped (Khaleel, 1977; Rao and Rao, 1984).

4.6. Microsporogenesis and microgametogenesis

The anthers of the genus *Pholisma* are tetrasporangiate, and the development of the anther conforms to the basic type, according to Davis' classification (1966). The tetrads are tetrahedral, and mature pollen grains are bicellular. Before anthers become dehiscent, the anther wall consists of four layers: epidermis, endothecium, middle layers, and secretory tapetum. During the dehiscence stage, the epidermis persists; the endothecium develops thickened walls, and the middle layers and tapetum collapse. This unique development pattern sets *Pholisma* apart from other genera, such as *Bourreria, Ehretia,* and *Tiquilia*, which exhibit different number of anther wall layers at maturity (Khaleel, 1977; Rao and Rao, 1984; Gottschling, 2004; Gottschling et al., 2014a, b). Particularly in *Ehretia*, the development of the anther wall conforms to the dicotyledonous type; the tetrads are tetrahedral, decussate, and isobilateral; and at maturity, the anther wall consists of a fibrous endothecium, a layer of middle layers, and glandular tapetum. The pollen grains are bicellular at the dehiscence stage, with a thick, smooth, and grooved exine (Khaleel, 1977; Rao and Rao, 1984).

5. Conclusions

This work significantly contributes to the expansion of our knowledge of the entire genus *Pholisma* of the holoparasitic Lennoaceae. By proving detailed descriptions of the morphology of inflorescences and flowers, flower development, and embryology, this study fills a crucial gap in our understanding, particularly since most previous research has focused solely on *P. arenarium*.

Our research has identified several similarities among the three species of *Pholisma* in terms of inflorescence and flower morphology. The stems exhibit deltoid bracts, crateriform stigmas, basifixed, adnate, and oblong bilobed anthers. Floral development reveals floral meristems in a dome shape, with a pentagonal or hexagonal floral apex, and valvate and imbricate sepal and petal aestivation, respectively. Floral anatomy shows syncarpous and multicarpellary gynoecium, wet stigmas, parietal ovule placentation, and false septa formation. Embryological characteristics include campylotropous, unitegmic, and tenuinucellate ovules, basic type anther wall development, tetrahedral microspore tetrad, and secretory tapetum. These thorough observations provide a strong foundation for our conclusions.

In contrast, some differences were observed among the three species in sepals, petals, corolla colour, inflorescence type and maturation, stamen position relative to stigma height, and pollen morphology. Sepals in *P. arenarium* are linear and connate only at the base, with glandular trichomes and stomata; in *P. sonorae*, they are cylindrical, connate only at the base with elongated, filamentous trichomes and stomata; and in *P. culiacana*, they are connate forming a tube with filamentous trichomes. The abaxial epidermis of petals in *P. arenarium* has glandular trichomes, in *P. sonorae* it has stomata, and in *P. culiacana* it has glandular trichomes. The corolla color in *P. arenarium* and *P. sonorae* is white with purple central lines, whereas in *P. culiacana* it is white with pink apices. Inflorescence in *P. arenarium* is an irregular double cyme with asynchronous maturation; in *P. sonorae* it is a scorpioid cyme with centrifugal maturation; in *P. culiacana* it is a capitulum with centrifugal maturation. Stamens in *P. arenarium* are positioned below the stigma height; in *P. sonorae,* they are very close to the stigma level; in *P. culiacana,* they are positioned above the stigma. Also, pollen is tetracolporate with psilate exine in *P. arenarium*, tricolporate with reticulate exine in *P. sonorae*, and tricolporate with psilate exine in *P. culiacana*.

Pholisma shares characteristics with Ehretiaceae, including cymose inflorescences, perfect flowers, infundibuliform flowers, epipetalous stamens, dorsifixed anthers with longitudinal dehiscence, transmitting tissues in the form of channels and tetrasporangiate anthers.

Comprehensive morphoanatomical and developmental studies of the *Lennoa* species are essential for a thorough characterization of the entire Lennoaceae. This study not only addresses important aspects of the reproductive biology of these parasitic plants but also underscores the need for further research on pollination, fruit and seed development, and dispersal in these species.

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CRediT authorship contribution statement

Gabriela Delgado-Pérez: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Daniel Sánchez:** Writing – review & editing, Supervision, Methodology, Formal analysis, Conceptualization. **Pactli F. Ortega-González:** Writing – review & editing, Methodology, Investigation, Formal analysis, Conceptualization. **Sonia Vázquez-Santana:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare no conflicts of interest.

Data availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.flora.2024.152567.

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