Verhuellia is a segregate lineage in Piperaceae: more evidence from flower, fruit and pollen morphology, anatomy and development

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short title: Morphology, anatomy and development in *Verhuellia*

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Abstract

Background and Aims The perianthless Piperales, i.e. Saururaceae and Piperaceae, have simple reduced flowers strikingly different from the other families of the order (e.g. Aristolochiaceae). Recent molecular phylogenies proved *Verhuellia* to be the first branch in Piperaceae, making it a promising object to study the detailed structure and development of the flowers. Based on recently collected material, the first detailed study since 1872 was conducted with respect to morphology, anatomy and development of the inflorescence, pollen ultrastructure and fruit anatomy.

Methods Original Scanning Electron Microscopy (SEM), Transmission Electron Microscopy (TEM) and Light Microscopy (LM) observations on *Verhuellia lunaria* were compared with Piperaceae, Saururaceae and fossils.

Key results The inflorescence is an indeterminate spike with sessile flowers, each in the axil of a bract, developing in acropetal, helical succession. Flowers consist of two (occasionally three) stamens with basifixed tetrasporangiate anthers and latrorse dehiscence by a longitudinal slit. The gynoecium lacks a style but has three to four stigma branches and a single, basal orthotropous, and unitegmic ovule. The fruit is a drupe with large multicellular epidermal protuberances. The pollen is very small, inaperturate, and areolate with hemispherical microechinate exine elements.

Conclusions Despite the superficial similarities with different genera of Piperaceae and Saururaceae, the segregate position of *Verhuellia* revealed by molecular phylogenetics is supported by morphological, developmental and anatomical data presented here. Unitegmic ovules and inaperturate pollen, which are synapomorphies for the genus *Peperomia*, are also present in *Verhuellia*.

Key words: *Verhuellia lunaria*, Piperales, *Peperomia*, *Appomattoxia ancistrophora*, floral development, floral anatomy, fruit morphology, pollen morphology, unitegmic ovule, inaperturate pollen.

INTRODUCTION

Verhuellia is a Piperaceae genus of three species, known from very few collections and localities on Cuba and Hispaniola (Haiti and Dominican Republic). It is a small, herbaceous, saxicolous perennial with monopodial architecture, whorled leaves and perianthless flowers organised in solitary lateral spikes (Fig. 1). These flowers, each subtended by a bract, consist of a gynoecium with three to four stigmas and two tetrasporangiate stamens. With the exception of Schmitz (1872*a*, *b*), who only described some morphological characters based on herbarium specimens, detailed morphological, anatomical or developmental studies have never been conducted. This is probably due to the very limited number of collections and its absence from living collections and also to the presumed close relationship with the genus *Peperomia*.

The absence of some floral organs in the flowers of perianthless Piperales has recently re-enhanced the focus on the representatives of the sister families Saururaceae and Piperaceae, presenting them as an interesting model for studying floral development and evolution (Jaramillo and Kramer, 2007; Arias and Williams, 2008; Madrid and Friedman, 2009). Furthermore, several phylogenetic studies in the order Piperales are now available as a robust framework to interpret character evolution (Wanke et al. 2007*a*, *b*). With the exception of the two Piperaceae genera *Verhuellia* and *Manekia*, floral characters of nearly all genera have been extensively studied in earlier years (Tucker, 1975, 1976, 1979, 1980, 1981, 1982*a*, *b*, 1985; Liang and Tucker, 1989, 1990, 1995; Igersheim and Endress, 1998; Lei and Liang, 1998, 1999; Tucker *et al.*, 1993). In addition, Smith and Stockey (2007) studied the pollen of the four Saururaceae genera. Detailed observations on the fruit morphology in this family are lacking. In Piperaceae, pollen and fruit development have not been studied extensively despite the large number of species.

Verhuellia was generally considered to be part of or at least closely related to *Peperomia* (e.g. Tebbs, 1993), due to its superficial similarities with this genus. However, Wanke *et al.* (2007*b*) recently showed that *Verhuellia* is sister to all other Piperaceae, a position formerly thought to be occupied by *Zippelia* and *Manekia* (Jaramillo *et al.*, 2004; Wanke *et al.*, 2007*a*). As a consequence, the traditional subdivision of the family Piperaceae into Piperoideae and Peperomioideae has been revised, resulting in three subfamilies congruent with the clades recovered by Wanke *et al.* (2007 *b*): Verhuellioideae (with *Verhuellia*), Zippelioideae (with *Zippelia* and *Manekia*) and Piperoideae (with *Piper* and *Peperomia*) (Samain *et al.*, 2008). Figure 2 shows a summary cladogram of the perianthless Piperales.

Given the hypothesis that *Verhuellia* is sister to the four other Piperaceae genera (Wanke *et al.*, 2007*b*), intermediate characters between Piperaceae and Saururaceae could be expected. This is supported by the fact that *Zippelia begoniifolia*, placed in the next branching clade in Piperaceae (Jaramillo *et al.*, 2004; Wanke *et al.*, 2007*a*, *b*), also shows some similarities to Saururaceae (Igersheim & Endress 1998). Tucker *et al.* (1993) and Liang and Tucker (1995) consider these characters as plesiomorphic. *Zippelia* has sometimes been considered a member of Saururaceae (e.g. Blume, 1830; Wu and Wang, 1957; Heywood, 1993) whereas it has never been questioned that *Verhuellia* belongs to Piperaceae.

The aims of this paper are: 1) to present a detailed study on morphology, anatomy and development of the *Verhuellia* inflorescence, flower, pollen and fruit based on fresh material to overcome the lack of knowledge on this genus and 2) to discuss the floral ontogenetic findings in the light of its new position in the family Piperaceae and in relation to available data of other perianthless Piperales, including the presumed fossil relative *Appomattoxia ancistrophora*.

MATERIAL AND METHODS

Plants of *Verhuellia lunaria* (Ham.) C.DC., the type species of the genus, originating from the Dominican Republic were cultivated in the Botanical Gardens of Ghent University and Dresden Technical University. Inflorescences, flowers and fruits of all stages were collected and preserved. Voucher specimens are deposited in the herbaria of Ghent University (GENT) and Dresden Technical University (DR).

Material for Scanning Electron Microscopy (SEM) was prepared by fixation in Kew mix (53% industrial methylated spirit, 37% water, 5% formaldehyde solution and 5% glycerol), followed by dehydration and critical point drying with carbon dioxide in a BAL-TEC CPD 030 critical-point-dryer. Subsequently, samples were fixed to aluminium stubs (Plano GmbH) using a carbon adhesive tape (LEIT-TABS, Plano GmbH), sputter-coated with gold (20 nm) under argon atmosphere using an EMITECH K550 sputter-coater. Images were obtained with a LEO 420 scanning electron microscope at acceleration voltage of 15 kV.

Light microscopy (LM) samples were prepared in 70% ethanol and subsequently gradually transferred to 100% ethanol. The samples were then transferred to LR White Resin, hard grade (London Resin Company Ltd, Reading, England) in a graded LR White Resin/ethanol series using solutions of 25/75, 50/50, 75/25, 100/0 resin/100% ethanol for at least 5 h each. Subsequently, the samples were placed in a closed capsule filled with fresh resin and hardened at 60°C for 48h. Sections of 2 µm were made with a rotation microtome (Microm HM360 Waldorf, Germany) and subsequently stained with 0,1% toluidine blue. The stained sections were fixed on microscopy slides using Eukitt© quick hardening mounting medium (Fluka Chemie GmbH, Switzerland). Observations were done with a light microscope (Leitz Dialux 20, Van Hopplynus, Brussels, Belgium) equipped with a camera (PixeLINK PL-B622CF, Ottawa, Canada) with specially developed software (Microscopica v1.3, Orbicule, Leuven, Belgium).

For TEM pictures pollen of *Verhuellia lunaria* was fixed in 50% alcohol, embedded in Agar Low Viscosity Resin (Agar Scientific Ltd., Stansted, Essex, England); ultrathin sections were poststained using uranyl acetate – lead citrate, $KMnO_4$, and the Thièry Reaction for polysaccharaides. Images were obtained with a Zeiss EM-900 transmission electron microscope. For SEM pictures fixed *Verhuellia lunaria* pollen was rehydrated, critical-point dried, and sputter-coated. Images were obtained with a JEOL JSM 6390 scanning electron microscope.

SEM and LM pictures were also taken from accessions of the genus *Peperomia* for comparison with *Verhuellia* morphology. This material was taken from the living collection of the Botanical Garden of Ghent University.

RESULTS

The inflorescence is initiated in the axil of one of the three to five whorled leaves on the stem (Figs. 3A; 4A, B). Its primordium is surrounded by large, multicellular trichomes (Figs. 4A, B; 5C). The inflorescence is an indeterminate spike with sessile flowers on a filiform axis (Fig. 4C). The inflorescence apex produces bracts in acropetal, helical succession (Figs. 3A; 4A; 5A-C). Each flower primordium appears in the axil of a peltate bract, which originated considerably earlier (Figs. 3A; 4A; 5A-D; 10F). The bract develops large trichomes at its margin, which are especially conspicuous during the young stages of the bract (Figs. 3A; 4A; 5B, C; 6A). Subsequently, two lateral stamen primordia are formed, followed by the gynoecium, which appears as an annular structure (Figs. 5D; 6A, B). Meanwhile, the bract increases in size, overarching the developing flower (Figs. 3A; 4A; 5A-C).

Next, the stigma primordia become apparent on the upgrowing ovary wall (Figs. 6A, B; 8A-C), followed by the differentiation of each stamen into a short filament and a basifixed anther (Fig. 6A). The anthers are tetrasporangiate (Figs. 6A-D; 7A-E). The lateral stamens develop simultaneously. Exceptionally, a third stamen is formed adaxially (Fig. 6B). In immature stamens, the connective ends in a bulge in between the two thecae (Fig. 6A, B). This bulge consists of one large gland (Fig. 7E). At maturity, the stamen is T-shaped as the connective grows out laterally between the pollen sacs in each theca, forming a broad, oblate, saddle-shaped connection between the thecae (Fig. 6C, D; 7A-C). The remnant of the apical bulge, which was prominent in immature stamens, is still visible in mature stamens (Fig. 7B, C). The anthers dehisce latrorsely with a longitudinal slit (Fig. 7B, C). After dehiscence, the anther wall recurves (Fig. 7B, C). Shortly after dehiscence, the stamens are shed.

On the annular young gynoecium (Figs. 6B; 8A) generally four stigma primordia develop: one abaxial, one adaxial and two lateral stigmas (Fig. 8B-D). Occasionally, only three stigma branches are present (Figs. 6D; 8E). This variation in stigma number can occur within a single inflorescence. As long as the ovary is open, no ovule development is visible (Fig. 8B, C). The ovule is unitegmic, orthotropous and basal (Fig. 10A-C). The micropyle is situated at the apex of the ovule (Fig. 10C, D). The stigma branches become papillate in later development (Figs. 7A-C; 8F). During maturation of the fruit, the outer surface of the ovary is covered by initially unicellular protuberances that undergo cell divisions (Figs. 6C, D; 7C; 8D-F; 9A, B, E, F; 10A-C, E, F). The fruit develops into a drupe with a relatively thin mesocarp and a stony endocarp and large multicellular protuberances on its wall (Fig. 9C-F; 10E, F). The drupe is situated on a short gynophore, which slightly elongates during maturation of the fruit (Fig. 10A, B, F).

The pollen grains are shed as very small spherical monads only c. 8-10 µm in diameter (Fig. 11A, B). Pollen is inaperturate and areolate with evenly distributed hemispherical microechinate exine elements (Fig. 11A-D). These exine elements are separated by narrow channels, forming a negative reticulum (Fig.11C, D). Sometimes, as an artificial result of pollen expansion, these channels become conspicuously broader, and in such cases one or more apertural regions are mimicked (Fig. 11B, C). However, the absence of any intine thickening in ultrathin sections demonstrates the absence of apertures (Fig. 12A, B). The pollen wall consists of an ektexine (a massive and prominent tectum with very low columellae and a thin foot layer), an evenly thin endexine and an evenly thin intine (Fig. 12A, B). Extremely small very inconspicuous orbicules have been observed (not shown).

DISCUSSION

Wanke *et al.* (2007*b*), based on molecular data, showed that *Verhuellia* and *Peperomia* are not closely related and this is supported by this study. Our morphological, anatomical and ontogenetic observations of flower, pollen and fruit in *Verhuellia lunaria* show that the genus is a clearly distinct lineage within Piperaceae and perianthless Piperales. An overview of important characters for Piperaceae and Saururaceae is given in Table 1.

The acropetal, helical succession of the initiation of bracts subtending the flowers in *Verhuellia* is comparable to the inflorescence development of all other Piperaceae studied. As in all other Piperaceae, there is a shift in position of the floral primordia with respect to their subtending bract as well as a considerable plastochron between the initiation of the bract and the floral primordium (Tucker, 1980; 1982*a*, *b*; Liang and Tucker, 1995). In contrast, Saururaceae have a so-called common floral and bract primordium, which is a synapomorphy for the family (Tucker, 1975, 1981, 1985; Liang and Tucker, 1989). In many Piperaceae and Saururaceae investigated, trichomes around or on the developing and mature spikes as well as on the bracts and gynoecia occur in a wide range of shapes and sizes (unicellular, multicellular, pearl glands, etc.) (e.g. Tucker, 1975, 1976, 1982*a*, *b*; Fig. 11A, B). The function of these trichomes remains unknown. However, in his study of *Peperomia magnoliifolia*, Vogel (1998) suggested that the bract trichomes attract pollinators through secretion of small quantities of sugars.

All *Verhuellia* flowers observed are bisexual. However, the stamens are shed relatively soon after anthesis, which could explain why de Candolle (1866) reported "female" flowers at the base of the inflorescence (see also Schmitz, 1872*a*). In addition, de Candolle (1866) described hermaphrodite flowers in the middle of the inflorescence and male flowers at the apex. However, this may be based on a misinterpretation as the flowers develop in acropetal succession: the stamens are already shed in the basal "female flowers", while they still cover the small, developing gynoecia in the distal "male flowers". The arrangement of floral organs, number and order of initiation are highly variable among the perianthless Piperales investigated (see all abovementioned references, especially the floral diagrams in Tucker *et al.*, 1993; Liang and Tucker, 1995 and the ancestral state reconstruction in Wanke *et al.*, 2007*b*). Nevertheless, these characters are usually constant within Piperaceae and more or less variable in Saururaceae. However, being a member of Piperaceae, *Verhuellia* proves to be an exception to this rule as I) the number of stamens and stigma branches can vary between 2-3 and 3-4 respectively (Fig. 6A-D), II) the initiation sequence of the stigma branches is variable (Fig. 8B vs. C) and III) in the case of three stigma branches, their position is not fixed (Figs. 6D vs. 8E). In contrast, in *Gymnotheca* (Saururaceae), the number of stigma branches and stamens also can vary but it usually is the abaxial stigmatic branch that is missing. In addition, the abaxial median stamen also is usually absent in these flowers (Liang and Tucker, 1989).

Despite this aberrant floral development in *Verhuellia*, the zygomorphic symmetry of its flowers is similar to all other perianthless Piperales (Figs.7B, C; 11A, B). The genus also shares the pairwise initiation of stamens and stigma branches and the simultaneous development of each pair of organs with most other Piperaceae and Saururaceae (Fig. 5D, although the two stamens in *Peperomia* as well as the second stamen pair in some *Piper* sometimes show disparity in size throughout development, Fig. 13B; Tucker 1980, 1982). The two stamens arise at the same position as the first two stamens in *Piper* and the only two stamens in *Peperomia* and a few other *Piper* species (Figs. 7A-C; 13A, B; Tucker, 1980, 1982; Lei and Liang, 1998). The development of a flower with three stamens has not been observed, as only one mature flower with three stamens was captured (Fig. 6B). However, it can be assumed that this third adaxial stamen in the median sagittal plane does not develop exactly at the same time as the two lateral stamens, but slightly later, similar to the third stamen in *Houttuynia* (Saururaceae; Tucker, 1981) and in the dioecious species of *Piper* (Lei and Liang, 1998), which occupies the same position in the flower. Similar to most *Piper* species, the anthers of *Verhuellia* are tetrasporangiate and dehisce latrorsely with longitudinal slits whereas *Peperomia* anthers are bisporangiate and open extrorsely with longitudinal slits (Fig. 7B, C vs. Fig. 13A-D). The laterally extended connective is not present in *Peperomia* (Fig. 11C, D)

In *Verhuellia*, as well as in *Zippelia*, *Piper* and Saururaceae, the gynoecium begins development as an annular structure that could be the result of a congenital fusion of several carpels. Another possible explanation could be a complete ontogenetic reorganisation resulting in an ontogenetically acarpellate gynoecium primordium. In both interpretations, the meristems from which the stigma branches develop can be considered to be carpel tips. In the second interpretation, the stigma primordia then can be seen as remnants of congenitally fused carpels. The upper part of the carpels can still be seen in the three or four rims on the fruit (Fig. 9A-D). In *Peperomia*, the ovary also originates from an annular primordium, but this can be understood as the ascidiate base of a single carpel (Fig.13B; Tucker, 1980).

Similar to all other Piperaceae, the gynoecium is superior and unilocular with a single basal ovule (Igersheim and Endress, 1998). As in all other perianthless Piperales studied, the ovule is orthotropous. The ovules of *Verhuellia* and *Peperomia* are characterized by a single integument in contrast with the other representatives of the family, which are bitegmic (Igersheim and Endress, 1998). This can result in the formulation of two hypotheses about the origin and evolution of unitegmy and bitegmy in Piperaceae: I) the ovule of ancestral Piperaceae was unitegmic and bitegmy evolved in the common ancestor of Zippelioideae and in *Piper* and underwent a reversal to unitegmy in *Peperomia* or II) ancestral Piperaceae were characterized by bitegmic ovules and unitegmy evolved independently in *Verhuellia* and *Peperomia*. Given that the outgroups to Piperaceae are bitegmic, hypothesis II, which assumes two origins of unitegmic ovules (two steps), is clearly more parsimonious than hypothesis I, which requires one origin of unitegmic ovules at the base of the perianthless Piperales, followed by a reversal to bitegmy in the common ancestor of Zippelioideae and Piperoideae, and another reversal to unitegmy in Peperomia (three steps).

In *Verhuellia*, a young gynoecium shows some superficial similarities to that of *Zippelia* and *Saururus*, including the presence of four stigma branches and the verrucose surface of the outer wall (Fig. 9A-D). However, the protuberances on the ovary of *Zippelia* at anthesis develop into glochidiate hairs (Liang and Tucker, 1995), whereas the ovary of *Saururus* is characterized by warty ridges (Tucker, 1976), showing no similarity to the multicellular protuberances on the ovary wall of *Verhuellia*. Another character shared by *Verhuellia*, *Zippelia* and *Saururus* is the four recurved free papillate stigmas.

Large multicellular protuberances on the fruit wall of *Verhuellia* were also observed by Schmitz (1872*b*) and are unique for the genus. The fruit wall of many *Peperomia* species in contrast also is provided with protuberances but these are sticky and unicellular papillae. It can be supposed that the protuberances have a function in epizoochory, but histochemical studies and detailed field observations are required to give conclusive statements about this.

Pollen of *Verhuellia* resembles *Peperomia* pollen in being inaperturate and very small. However, *Verhuellia* pollen is distinctively characterized by its ornamentation: evenly distributed, uniquely formed exine elements consisting of ± hemispherical microechinate aggregates (Fig. 11A-D, resembling mace-heads, the medieval cutting weapons). Pollen of

Peperomia is characterized by a less conspicuous ornamentation with unevenly shaped verrucae with few, tiny microechini (Fig. 14A, B). The inaperturate pollen of *Verhuellia* and *Peperomia* contrasts with the sulcate pollen in other perianthless Piperales (see Table 1). Remarkably, pollen of the genus *Aristolochia*, another member of Piperales, is also inaperturate, whereas the pollen of its closest relatives are (mono)sulcate (Asaroideae, Hydnoraceae and *Lactoris*). Hence, parallel to the origin and evolution of unitegmy in Piperaceae, two hypotheses about the occurrence of inaperturate pollen in Piperales can be made: I) ancestral Piperales pollen was inaperturate and the various aperturate morphologies have evolved in the different groups, II) ancestral Piperales had a particular type of aperturate pollen and inaperturate pollen and the various aperturate pollen types developed in the different Piperales lineages. The interpretation of their evolution is somewhat uncertain because of uncertainty on the positions of *Lactoris* and Hydnoraceae, but given the sulcate condition in Canellales, Saururaceae, and most Piperaceae, hypothesis II is probably much more parsimonious than loss of the sulcus at the base of Piperales followed by several reappearances within the order. In basal magnoliids inaperturate pollen is infrequent, however not rare; beside the mentioned examples it is found also in, e.g., Laurales (Furness *et al.*, 2002). Apart from the difference in pollen ornamentation, *Verhuellia* also contrasts with *Peperomia* in the presence of orbicules. Very inconspicuous orbicules have been observed on the tapetum of *Verhuellia* whereas orbicules are not known from *Peperomia*.

Finally, the very distinct ornamentation of *Verhuellia* pollen allows comparison with presumed fossil members of Piperales. According to Friis *et al.* (2006), *Appomattoxia ancistrophora* is most closely related to members of Piperales. However, Doyle and Endress (2010) state in a phylogenetic study of a morphological data set for extant basal angiosperms to assess the relative parsimony of placements of early angiosperm fossils that *Appomattoxia* is more parsimoniously placed near Chloranthaceae or *Amborella* than in Piperales. Smith and

Stockey (2007) already showed that *Appomattoxia* pollen differs from Saururaceae pollen. Pollen of *Appomattoxia ancistrophora* also differs from *Verhuellia* pollen in dimension, aperture condition, pollen wall stratification and pollen ornamentation (Friis *et al.*, 1995). It is much larger (16-19 µm), it is sulcate (*Verhuellia* pollen is inaperturate), it has a thick foot layer and a continuous tectum (*Verhuellia* shows a thin foot layer and a strictly discontinuous tectum), and its ornamentation is verrucate-rugulate and microechinate.

To conclude, observations on the morphology, anatomy and development of inflorescence, flower, pollen and fruit of *Verhuellia lunaria* confirm that the genus is a clearly distinct lineage within Piperaceae. It does not show any intermediate characters between Saururaceae and Piperaceae and there are only superficial similarities with any other specific group within perianthless Piperales. The unitegmic ovule and the inaperturate pollen of *Verhuellia* and *Peperomia* appear to be either an independent parallel evolution or a reversal to the ancestral state. *Appomattoxia ancistrophora* pollen is different from the pollen of *Verhuellia*.

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FIG. 1. General habit of *Verhuellia lunaria*, grown in greenhouse conditions.

FIG. 2. Simplified cladogram of perianthless Piperales (after Wanke et al., 2007*b*).

FIG. 3. SEM images of the most important floral structures in *Verhuellia lunaria* discussed in the text. (A) Young spike developing in leaf axil. The axis is coloured blue, bracts and bract primordia are green and the young flower is yellow. The arrow points to a trichome on the adaxial side of the bract. (B) Detail of the spike with developing flowers. One flower, with its two developing stamens and its gynoecium in primordial stage, is coloured pink. The floral bract has been removed (arrowed). (C) Mature flower with green coloured bract, yellow stamens, with opened thecae in blue, and purple gynoecium. The arrow points to the tapetum that is visible because the anther wall has recurved. Abbreviations: a, anther; B, bract; co, connective; F, flower primordium; f, filament; gy, gynoecium; ps, pollen sac; *, apex of spike.

FIG. 4. SEM images of inflorescence development in *Verhuellia lunaria*. (A) Young spike with developing floral bracts with young flowers in their axils (one young flower with its subtending bract is encircled). A young leaf develops next to the spike. Multicellular trichomes (arrowed) surround the spike. The older leaves on the main stem are removed. (B) View of a stem node with two older leaves still present and the immature spike (encircled) in the axil of the removed leaf. Multicellular trichomes (arrowed) surround the spike. (C) Lateral view of the maturing spike shortly before anther dehiscence. The peltate bracts partially cover the nearly mature anthers which, in turn, cover the young gynoecia. Abbreviations: FL, foliage leaf; *, apex of spike.

FIG. 5. SEM images of inflorescence and floral development in *Verhuellia lunaria*. (A) Apical view of the spike apex and bract and young flowers in successive stages of development. (B) Lateral view of the spike apex with developing bracts partially covering flowers at successive stages of development. In the most developed floral primordia visible, two stamen primordia and the gynoecium primordium are present. On the margins of the bracts, trichomes are present. (C) Lateral view of a spike at the same developmental stage as in (B). The youngest flowers and bracts are encircled. Multicellular trichomes are present at the base of the spike. (D) Apical view of a developing flower with two lateral stamens and the gynoecium primordium (floral bract removed). Abbreviations: a, anther; B, bract; F; flower; f, filament; fa, floral apex; s, stamen; *, apex of spike.

FIG. 6. SEM images of floral development in *Verhuellia lunaria*. (A) Flower with bract removed and developing tetrasporangiate anthers. The two thecae of each anther are visible and the connective is beginning to develop as a small bulge. On top of the developing ovary, adaxial and abaxial stigma primordia are apparent (encircled). Trichomes are visible on the

remaining base of the bract. (B) Close-up of developing flower before closure of the ovary, with the subtending bract removed, three stamens and possibly three stigma primordia. The third stamen is positioned opposite the bract, which has been removed. (C) Almost mature flower with four stigmas (encircled) and its subtending bract. In the stamens, the connective has broadened and its distal part is still recognizable as a small bulge in between the two thecae. The arrow points to a developing protuberance on the ovary wall. (D) Flower with three stigmas (encircled) at the same developmental stage as in (C). Abbreviations: B, bract; co, connective; f, filament; gy, gynoecium; ov, ovary; ps, pollen sac; s, stamen; th, theca.

FIG. 7. SEM and LM images of floral development in *Verhuellia lunaria*. A-C: SEM images of stamens at anthesis from early (A) to late (C). (A) Flower with its subtending bract shortly before anther dehiscence. The gynoecium has four receptive stigmas. (B) Flower with its subtending bract after anther dehiscence. The anther wall has recurved, exposing the tapetum. The connective is broadening and flattening. (C) Flower with bract after anther dehiscence and with four withering stigmas. The connective forms a broad, oblate, saddle-shaped connection between the thecae. (D) Transverse section through an anther showing the four pollen sacs. (E) Longitudinal section through a flower and inflorescence axis; in each stamen, one of the pollen sacs is visible. The connective ends in a bulge in between the two thecae and this bulge contains a single large oil cell. Abbreviations: co, connective tissue; f, filament; ps, pollen sac.

FIG. 8. SEM images of gynoecium development in *Verhuellia lunaria*. (A) The adaxial and abaxial stigma branches start to develop from the annular gynoecium base (encircled). (B) Beginning closure of the ovary (encircled). The adaxial stigma branch develops first, followed by the two lateral ones. The abaxial stigma branch is the last to develop. The four stigma

branches are arrowed. (C) Ovary closed. The three most developed stigma branches are arrowed. (D) All four stigma branches distinct. (E) Gynoecium with three stigmas at the same stage as in (D). (F) Mature gynoecium with four recurved receptive stigmas with papillose surface. One multicellular protuberance is arrowed. Abbreviations: sg, stigma branch.

FIG. 9. SEM images of fruit development in *Verhuellia lunaria*. (A) Lateral view of immature fruit with bract; the four papillate stigmas are still visible (encircled) and each stigma branch is decurrent on the fruit wall as a longitudinal rim. The initially single-celled wart-like protuberances on the fruit wall have started to divide (arrowed). (B) Lateral view of immature fruit with four papillate stigmas (encircled) and bract, seen from the adaxial side. One protuberance is arrowed. (C) Mature fruit with three stigmas and three rims. The fruit is still attached to the inflorescence axis and the multicellular protuberances on the fruit wall are fully developed. (D) Mature fruit, fallen from the inflorescence axis. (E) Detail of a single-celled fruit wall protuberance. (F) Detail of a mature protuberance after cell division.

FIG. 10. LM images of sections through developing gynoecium and fruit of *Verhuellia lunaria*. The stamens are shed. (A) Longitudinal section through the mature gynoecium, two stigmatic branches are visible. The micropylar area is arrowed. The gynoecium stands on a short gynophore on the axis of the inflorescence (in transverse section). (B) Longitudinal section through the ripening fruit with the single ovule. The gynophore has elongated and the fruit wall protuberances are fully developed (arrowed). (C) Detail of a ripening fruit showing the single central ovule with micropyle formed by the single integument. (D) Detail of the micropylar area (micropyle arrowed), showing the single integument and the apical part of the nucellus. (E) Transversal section through mature fruit, with the single ovule. One of the multicellular protuberances on the fruit wall is arrowed. (F) Maturing fruit in the axil of the

bract. Large glandular cells are present in the bract and the stigmatic area. The arrow points to the micropyle. Abbreviations: B, bract; gp, gynophore; mp, micropyle; o, ovule; ov, ovary; nc, nucellus; sg, stigma; st, stigma branch; tg, integument.

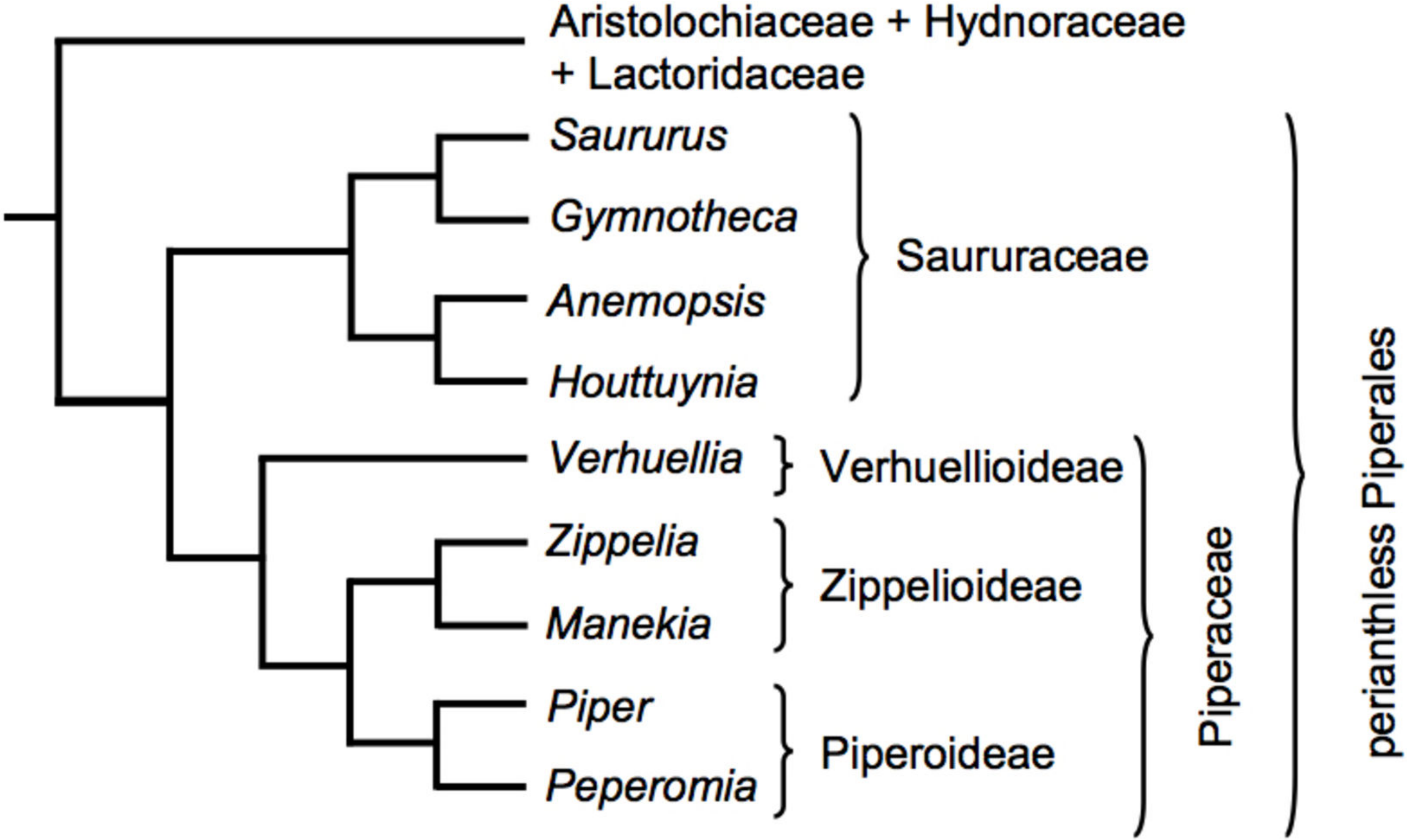
FIG. 11. SEM images of pollen morphology of *Verhuellia lunaria*. (A) Pollen grain with evenly distributed isolated microechinate exine elements. Note the channels are uniformly very narrow. (B) Pollen grain with artificially broad channels between the microechinate elements, mimicking a furrow-like aperture. (C) Close-up of pollen surface, with microechinate exine elements and a channel mimicking an aperture. (D) Close-up of pollen surface with a very narrow channel.

FIG. 12. TEM images of pollen ultrastructure of *Verhuellia lunaria*. (A) Pollen wall stratification: with ektexine (prominent tectum, short columellae, thin foot layer), meandering highly electron-dense endexine and medium-dense, evenly thin intine. (B) Pollen in cross section. The intine (this is the layer below the highly electron-dense endexine) is meandering, however never distinctively thickened, thus indicating an inaperturate condition. Abbreviations: cm, columella; en, endexine; fl, foot layer; in, intine; T, tectum.

FIG. 13. SEM and LM images of inflorescence and flowers of *Peperomia*. (A) Lateral view of a part of the inflorescence of *P. trifolia* with flowers partially sunken in pits in the spadix rachis. One flower and its subtending bract are coloured pink. The anthers are bisporangiate and the gynoecium has one apical penicillate stigma. (B) Close-up of a flower of *P. wolfgang-krahnii* (pink). The annular ovary wall is still rising. The floral bract has been removed (arrowed). (C) Longitudinal section of a flower of *P. macrostachya*, with two lateral bisporangiate stamens and a cylindric ovary. A conspicuous gland (arrowed) is present near

the stigmatic area. (D) Longitudinal section through a stamen of *P. prostrata* showing the two pollen sacs with the pollen grains. The surface of the anther wall is characterized by cuticular ridges (arrow). Abbreviations: B, bract; f, filament; g, gynoecium; ov, ovary; ps, pollen sac; s, stamen; sg, stigma.

FIG. 14. SEM images of pollen morphology of *Peperomia rubella*. (A) Pollen grain with verrucate ornamentation; the verrucae are covered with few, tiny microechini. (B) Close-up of pollen surface. Note the different size of verrucae.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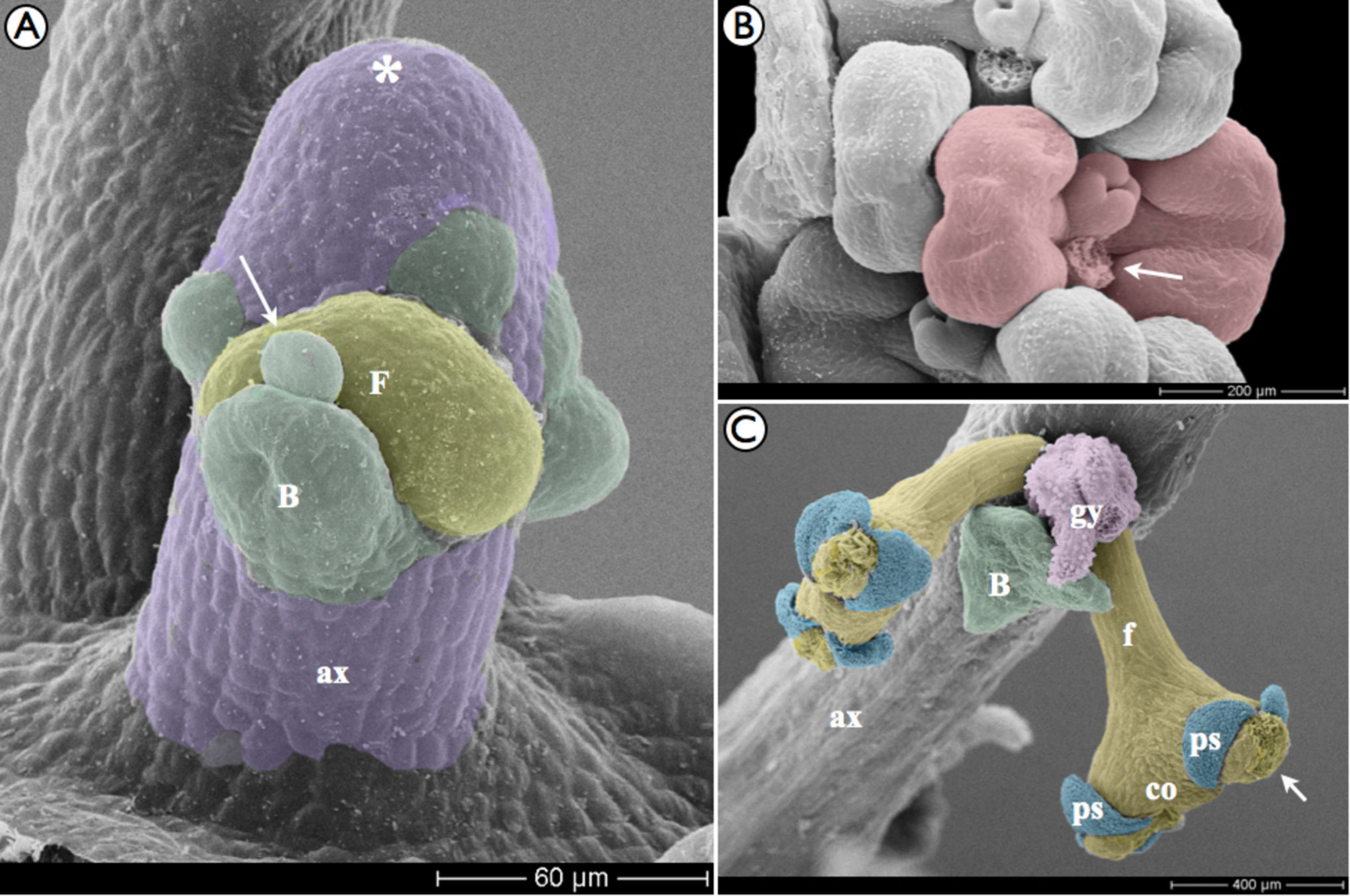
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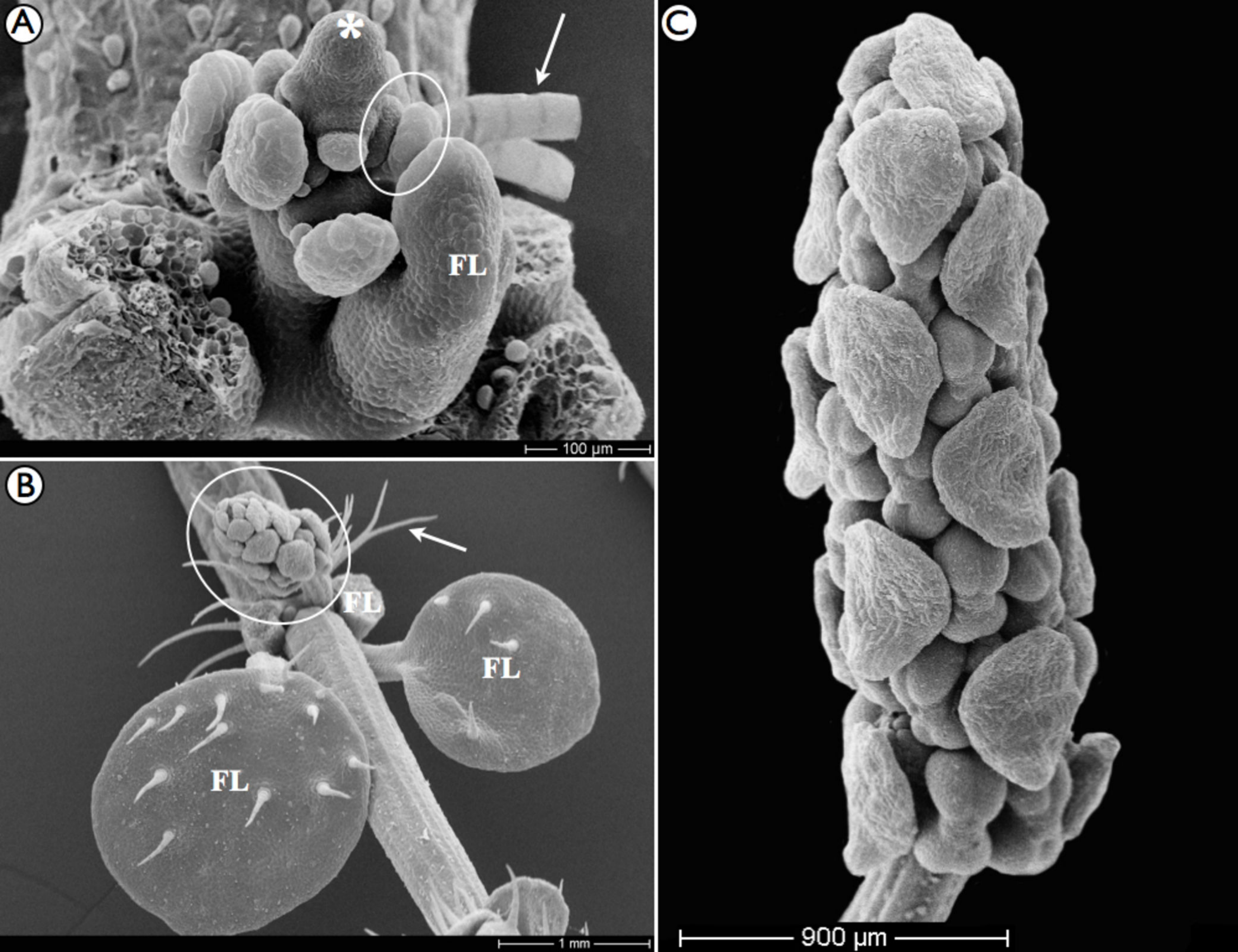
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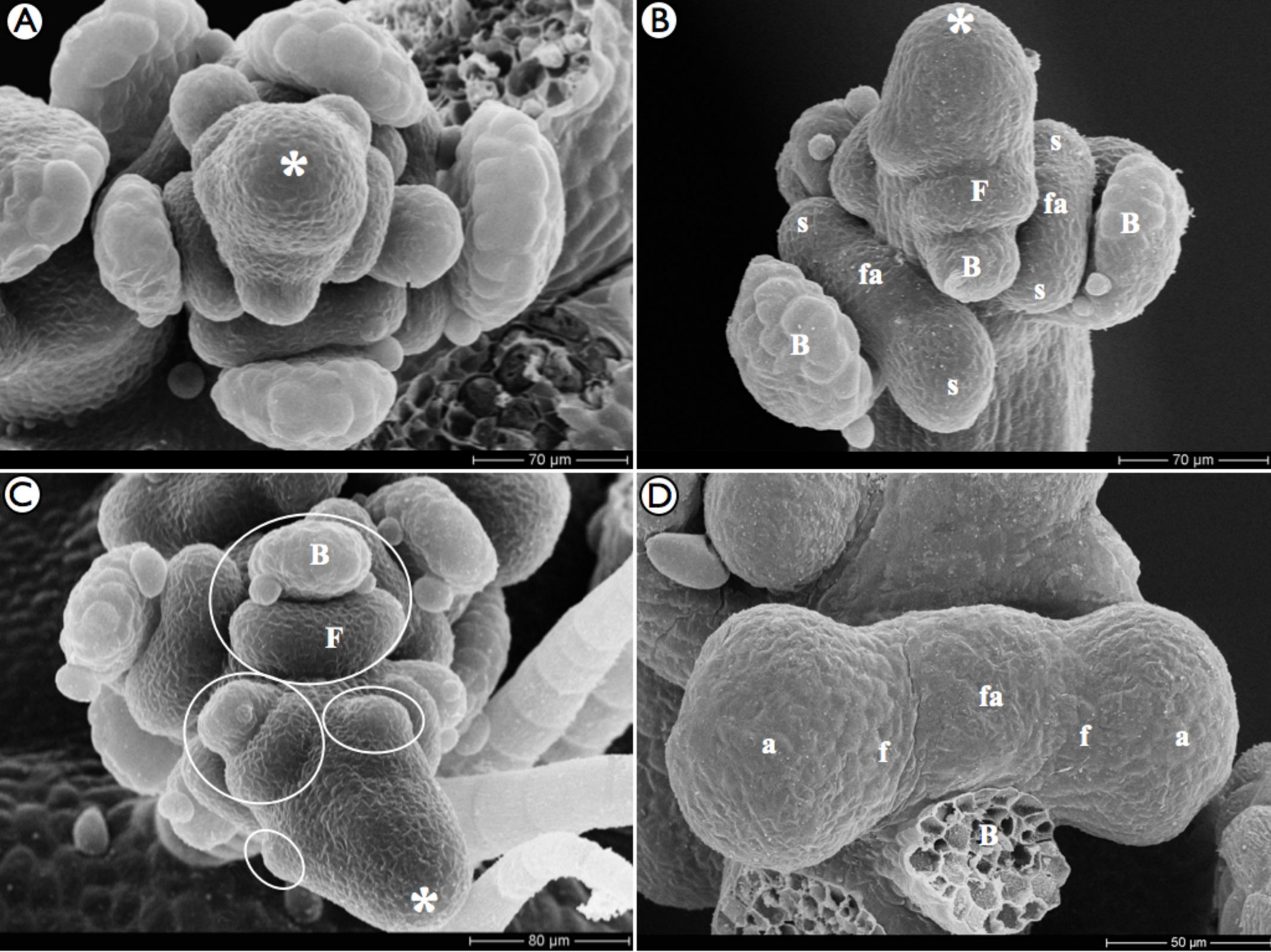
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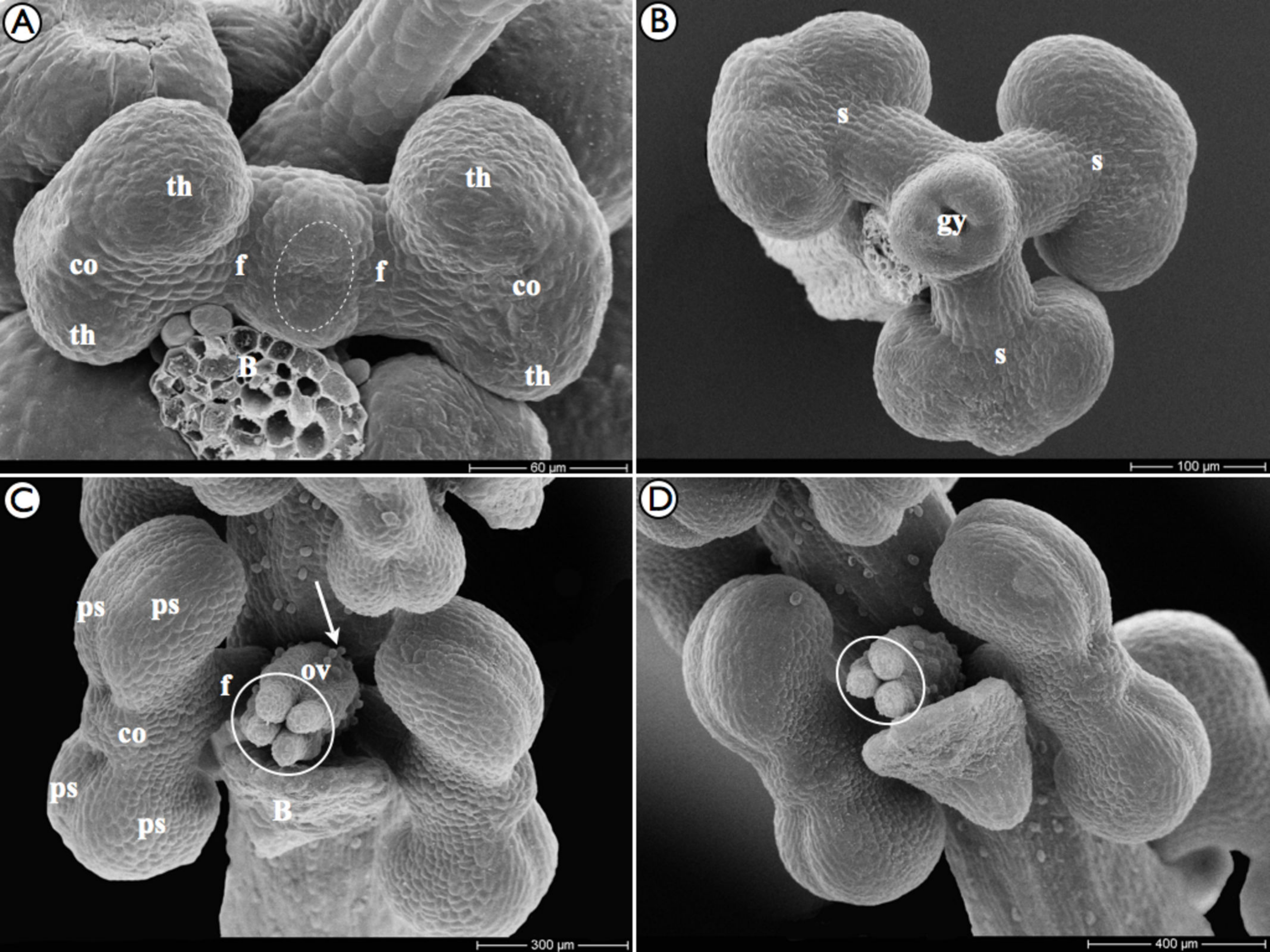
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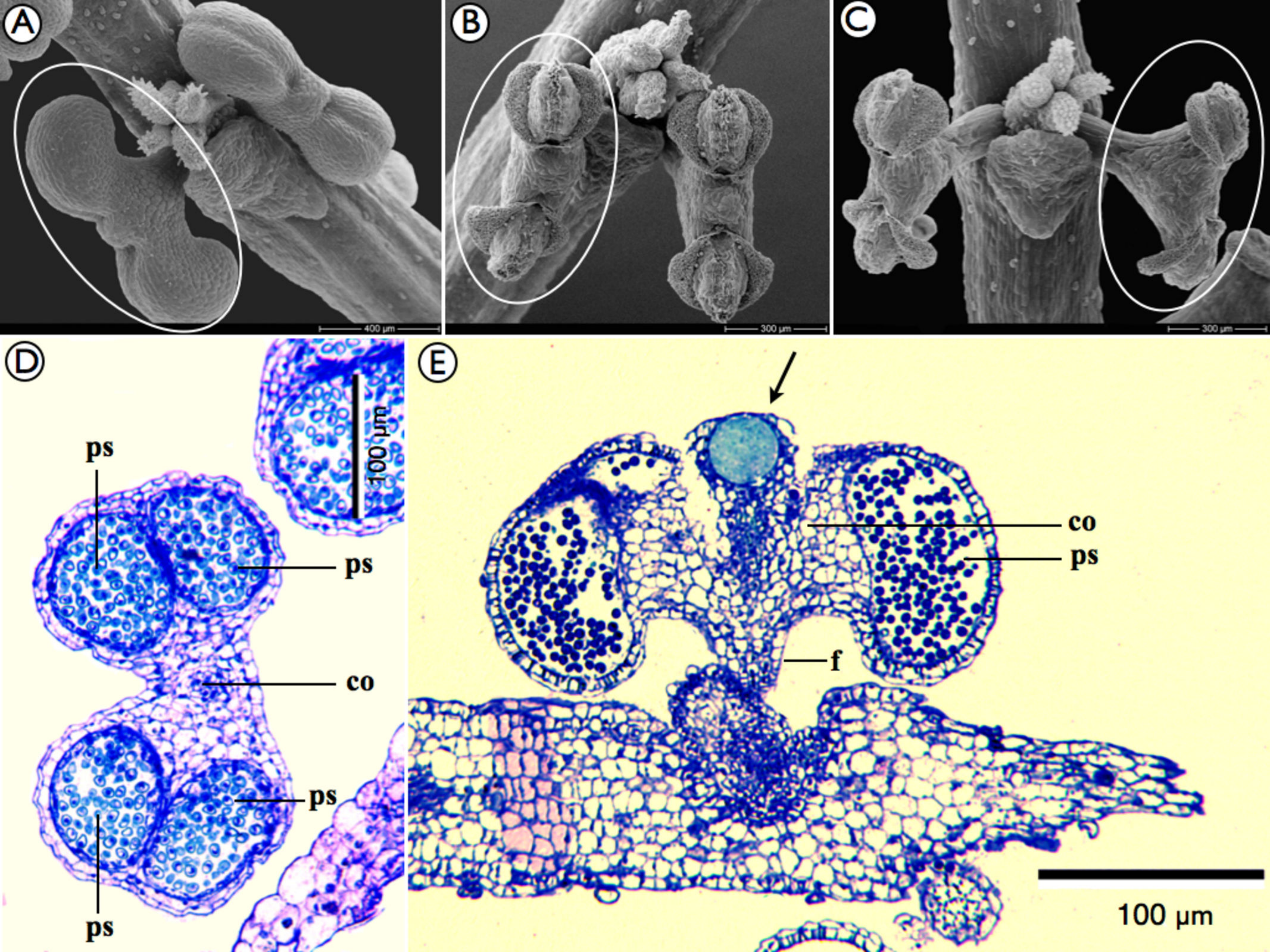
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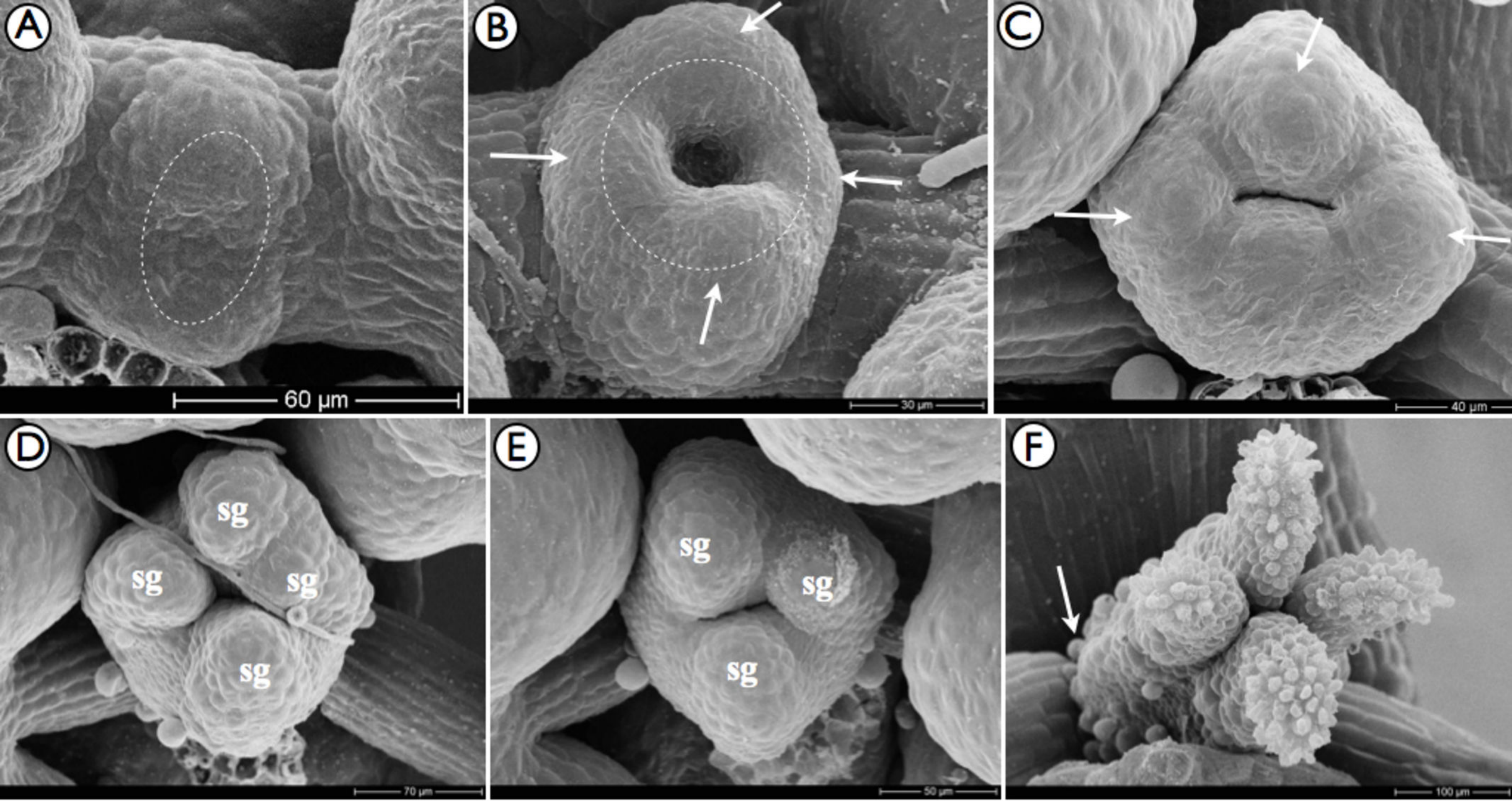
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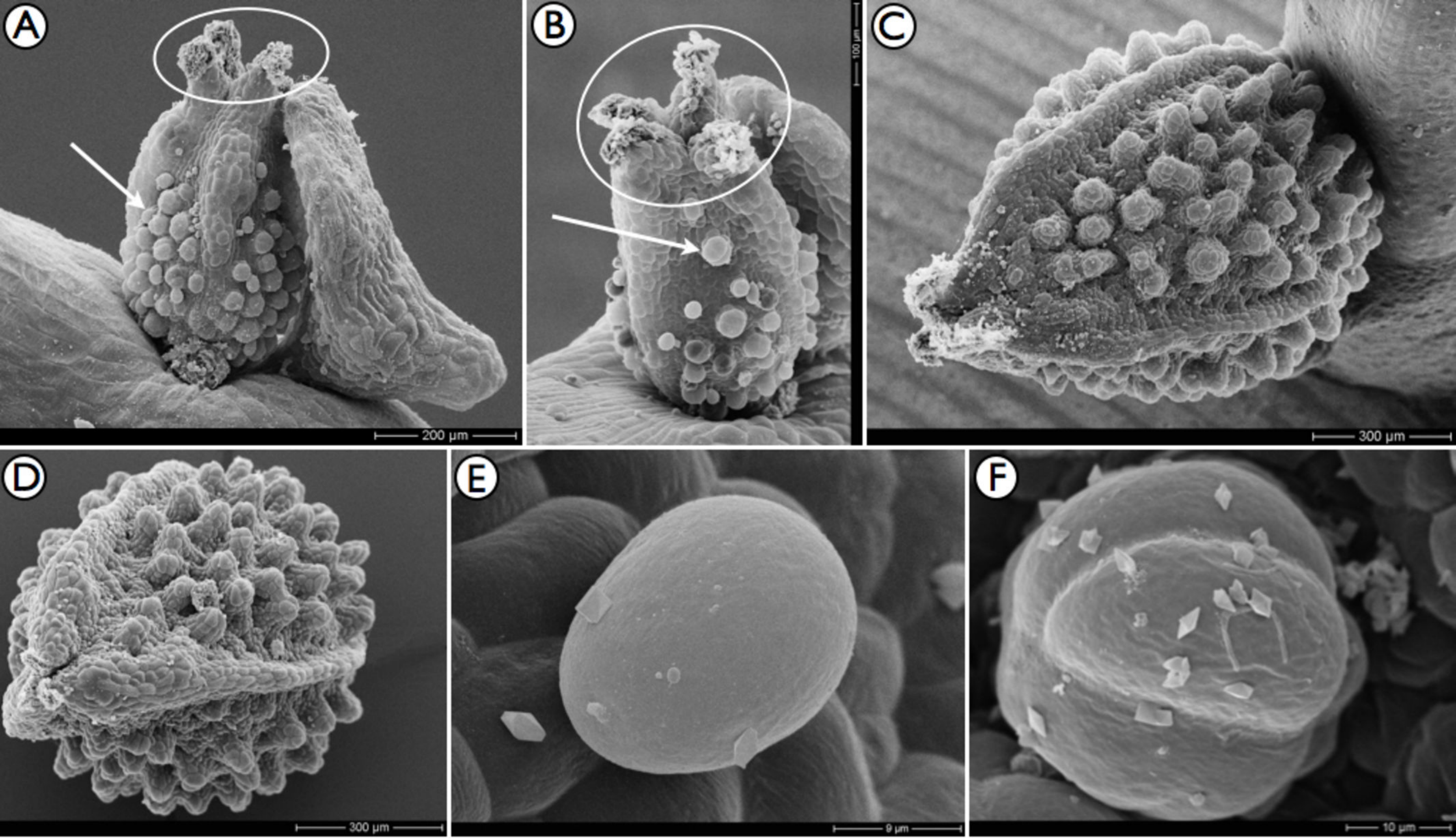
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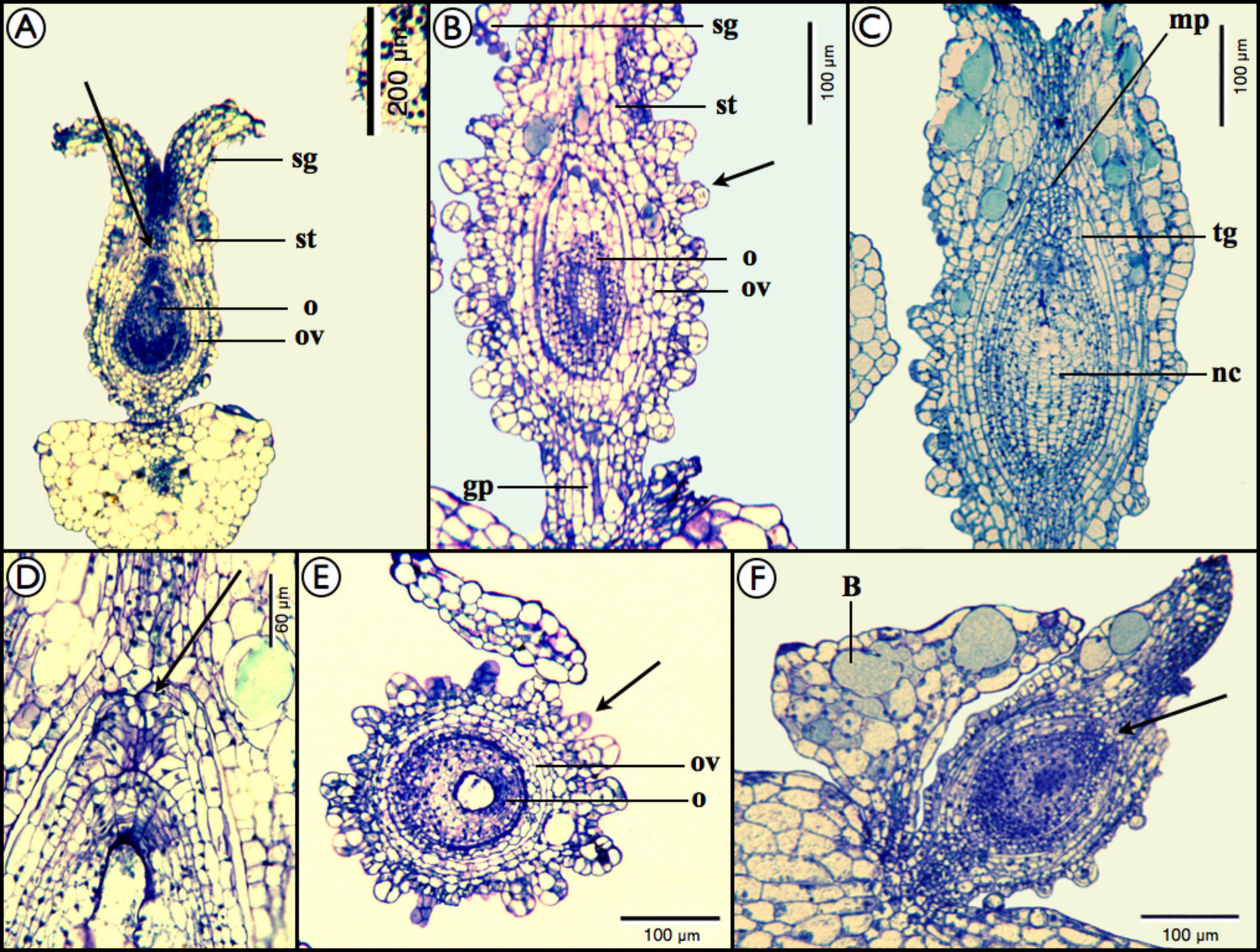
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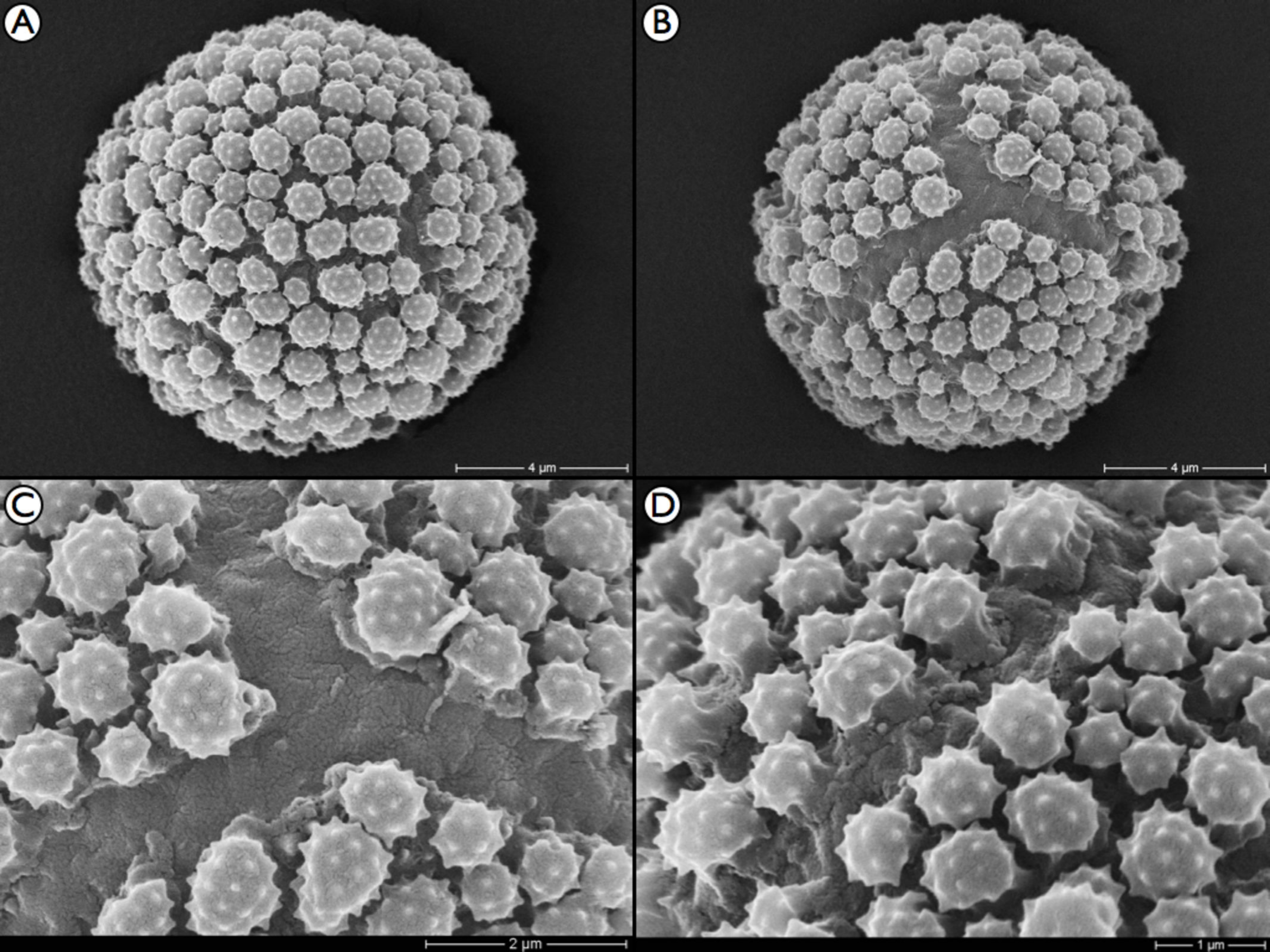
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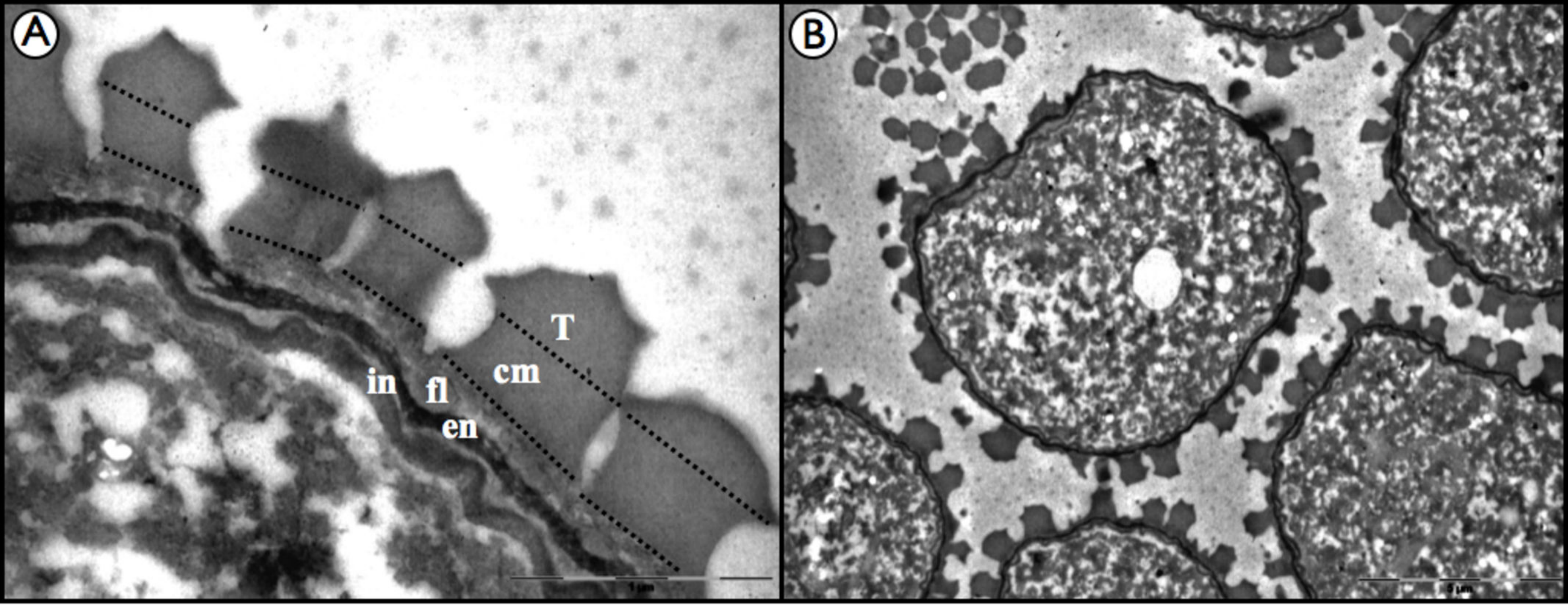
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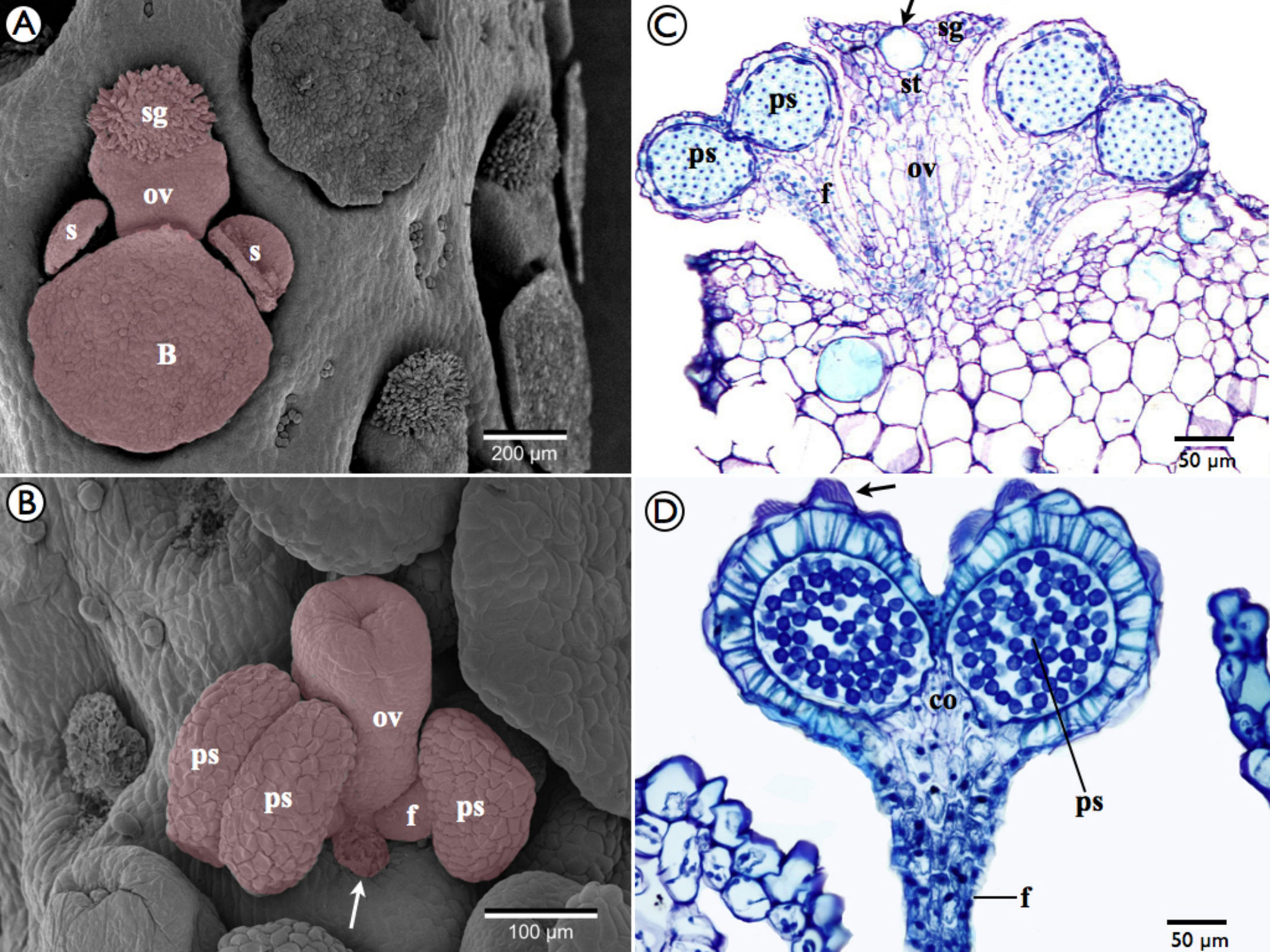
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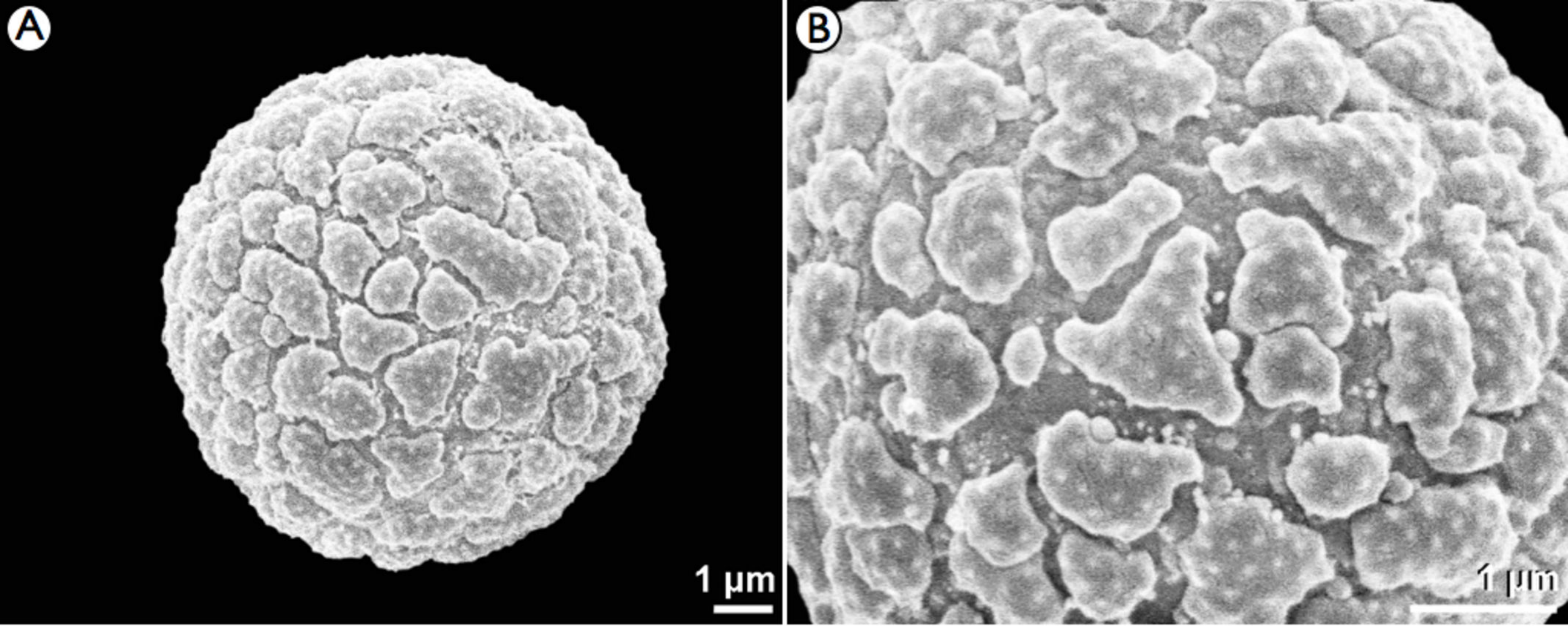
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TABLE 1. Overview of the most important characters of the five genera of Piperaceae and its sister family Saururaceae (from Samain, 2008).

	Verhuellia	***Manekia***	***Zippelia***	***Piper***	***Peperomia***	**Saururaceae**
Number of species	3	4-5	1	± 2000	± 1600	4 genera/6 species
Distribution	Cuba and Hispaniola	Central and South America	Southeast Asia	pantropical with highest diversity in America and Asia	pantropical with highest diversity in America	East Asia and North America
Life form	perennial	perennial	perennial	perennial	perennial, rarely annual	perennial
Growth form	terrestrial; herbaceous	terrestrial; woody root climber	terrestrial; small shrub	terrestrial; woody (small trees, shrubs, lianas), rarely herbaceous	epiphytic or terrestrial; herbaceous	terrestrial (somethimes aquatic); herbaceous
Androecium	stamens 2, tetrasporangiate	stamens 4, tetrasporangiate	stamens 6, tetrasporangiate	stamens (1-) 2-6(-10), tetrasporangiate	stamens 2, bisporangiate	stamens 3-8, adnate to gynoecium, tetrasporangiate
Gynoecium	carpels 3-4, ovary unilocular	carpels 4-5, ovary unilocular	carpels 3-4, ovary unilocular	carpels 2-4, ovary unilocular	carpels 1(-2), ovary unilocular	carpels 3-4, ovary unilocular
Number of ovules	1	1	1	1	1	(1)2-many
Number of integuments	1	2	2	2	1	2
Pollen	inaperturate	monosulcate	monosulcate	monosulcate	inaperturate	monosulcate, trichotomo-sulcate (*Houttuynia*)
Fruit	drupe	drupe	drupe	drupe	drupe	capsule