

Development of reproductive organs in *Canephora madagascariensis* (Octotropideae - Rubiaceae)

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Background and aims – Ontogenetic studies provide valuable morphological characters to aid interpretation of evolutionary scenarios. But they are rare within the Rubiaceae and the variation in floral ontogenetic patterns in the different Rubiaceae lineages remains underexplored. Here, we provide the first developmental study of the reproductive organs of *Canephora madagascariensis*, a typical representative of the poorly known tribe Octotropideae in most of its characters except for its unique, flattened, assimilating, ‘phylloclade-like’ inflorescences.

Methods – Using SEM and LM we produced a developmental study of inflorescence, flower and fruit in *C. madagascariensis*.

Key results – The inflorescence of *Canephora madagascariensis* is a condensed dichasium. Peduncle, first-order bracts and first-order branches together form a flattened, green, phylloclade-like structure, showing both leaf and stem characters. Initially, the dome-shaped floral apex becomes concave and sepals are formed. Next, from the collar of the concave apex, a tubular corolla with epipetalous stamens grows out. Meanwhile, an inferior bilocular ovary is formed. Per locule, up to seven unitegmic ovules are formed on a U-shaped placenta. Fruits are drupes with one to several fibrous seeds with folded exotesta.

Conclusions – The double-organ identity of the green, flattened part of the inflorescence is due to a modification leading to leaf analogy. The peculiar inflorescence of *C. madagascariensis* constitutes just another state of the character ‘axillary inflorescence’ in Rubiaceae. It results from both reduction and congestion, two trends typical in Rubiaceae inflorescences. Floral cups form the basis of the floral structure in *Canephora*. The inferior ovary is due to the initial formation of a hypanthium, from which calyx lobes, stamen-corolla tube, corolla tube sensu stricto and calyx tube originate through successive outgrowth of underlying annular intercalary meristems.

Key words – *Canephora madagascariensis*, corolla tube, floral development, fruit development, hypanthium, inflorescence, LM, Octotropideae, Rubiaceae, SEM.

INTRODUCTION

Floral developmental data may provide information that can be used to interpret evolutionary hypotheses and relationships among taxa. Ontogenetic characters provide a basis to assign structures to character states or characters and help resolve homoplasy (e.g. Tucker et al. 1993, Ronse Decraene & Smets 2000). However, in the large Rubiaceae family, developmental studies are available for only a few genera, e.g. *Rubia* L. (Payer 1857), *Coffea* L. (Marchand 1864), *Galopina* Thunb. (Ronse Decraene & Smets 2000), *Mitrasacmopsis* Jovet. (Groeninckx et al. 2007) and *Spermacoce* L. (Vaes et al. 2006). As a result, the variation in floral ontogenetic

patterns among Rubiaceae lineages is largely unknown even though it potentially holds important information.

Based on morphological and molecular data, *Canephora* Juss. (De Jussieu 1789) belongs to the poorly known, paleotropical tribe Octotropideae, previously known as Hypobathreae (Robbrecht 1980, Robbrecht & Puff 1986, Robbrecht 1988, Robbrecht et al. 1991, Davis et al. 2007, Tosh et al. 2008) in subfamily Ixoroideae (Bremer & Eriksson 2009) or Cinchonoideae (Robbrecht & Manen 2006) (fig. 1).

The Octotropideae comprise c. 110 described species in 27 genera, most of which only have a single or a few species. Octotropideae have axillary, paired inflorescences, campanulate, hermaphroditic flowers, contorted corolla aestivation,

bilocular ovaries with axile placentation, fleshy fruits with pendulous or horizontally arranged seeds, a wrinkled exotesta with thickened exotesta cells, and, superior or lateral embryo radicles (Robbrecht & Puff 1986, Robbrecht 1988). Between the genera, there is variation in inflorescence structure (sessile, compact, few-flowered versus pedunculate, lax and many-flowered), flower merosity [4–5(–7)-merous], ovule and seed number (1-numerous), and thickenings of the exotesta cells (along radial walls, sometimes also along inner and/or outer tangential walls) (Robbrecht & Puff 1986).

Canephora is endemic to Madagascar. Currently, five species are recognized (Govaerts et al. 2011), but several new species await description (S. Dawson, Royal Botanic Gardens, Kew, UK, pers. com.). *Canephora* is a typical representative of the Octotropideae except for its inflorescence, which is unique within the tribe in that the peduncle “transforms into a flattened, assimilating axis ...” (Robbrecht 1988: 68; fig. 2A–C). The form of the peduncle differs according to the species considered, but in all species, it is flattened and green with the flowers clustered distally (fig. 2A–C).

Here we study *Canephora madagascariensis* as a typical representative of the tribe Octotropideae, but which also has a unique, flattened, assimilating, phylloclade-like inflorescence. By means of a developmental study of inflorescence, flower and fruit, we aim to document in detail the reproductive organs of this species. Questions to be resolved are: what is the morphological origin of the inflorescence, calyx, corolla and gynoecium? How are the fruits and seeds formed? This study is the start of a family-wide floral developmental study, aiming to understand deep morphology and ontogeny and to resolve homology questions in the Rubiaceae.

MATERIALS AND METHODS

Inflorescences, flowers and fruits at different stages of development were freshly collected and fixed in ethanol 70% during fieldwork in Madagascar (De Block et al. 664, BR). Dissection of plant material was done in 70% ethanol under a Leica Wild M3C stereo microscope equipped with a Olympus KL1500LCD cold-light source.

For LM, the material was dehydrated through a graded ethanol series and subsequently embedded in KULZER's Technovit 7100 (based on HEMA, hydroxyethyl-methacrylate). Sections (7 µm thick) were made with a rotation microtome Leica RM2135, using disposable blades (Leica DB80L). They were stained with a 0.1% solution of toluidine blue in aq. dest. Observations were done using an Olympus BX51 microscope equipped with a Color View Soft Imaging System camera.

For SEM, the material was washed in ethanol 70% for 20 minutes and transferred to a 1:1 mixture of DMM/ethanol 70% for 5 minutes and then to 100% DMM (dimethoxymethane) for 20 minutes. Subsequently, the material was critical point dried using liquid CO₂ with a Balzers CPD 030 critical point dryer. The dried samples were mounted on aluminium stubs using carbon adhesive tape and coated with gold with a Balzers SCD 020 sputter coater. SEM images were obtained with a JEOL JSM5800-LV scanning electron microscope. Reference material (SEM) and permanent slides (sections) were deposited at the National Botanic Garden of Belgium.

Illustrations were prepared from the following collections: *Canephora longipedunculata* S.E.Dawson ined.: Madagascar, Tosh et al. 112 (BR) (fig. 2A). – *Canephora madagascariensis* J.F.Gmel.: Madagascar, De Block et al. 664 (BR) (figs 2D & E, 3–14); Razafimandimbison et al. 468

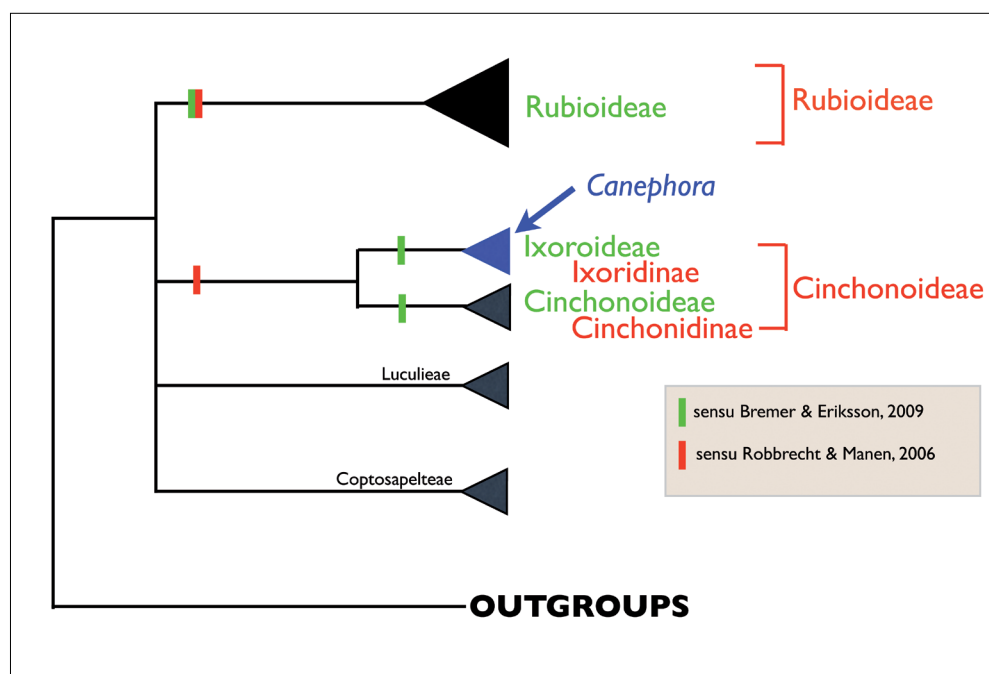


Figure 1 – Simplified cladogram of Rubiaceae after Bremer & Eriksson (2009), who recognised three subfamilies, Rubioideae, Ixoroideae, and Cinchonoideae. According to Robbrecht & Manen (2006), there are only two subfamilies, Rubioideae and Cinchonoideae, the latter comprising supertribes Ixoridinae and Cinchonidinae.

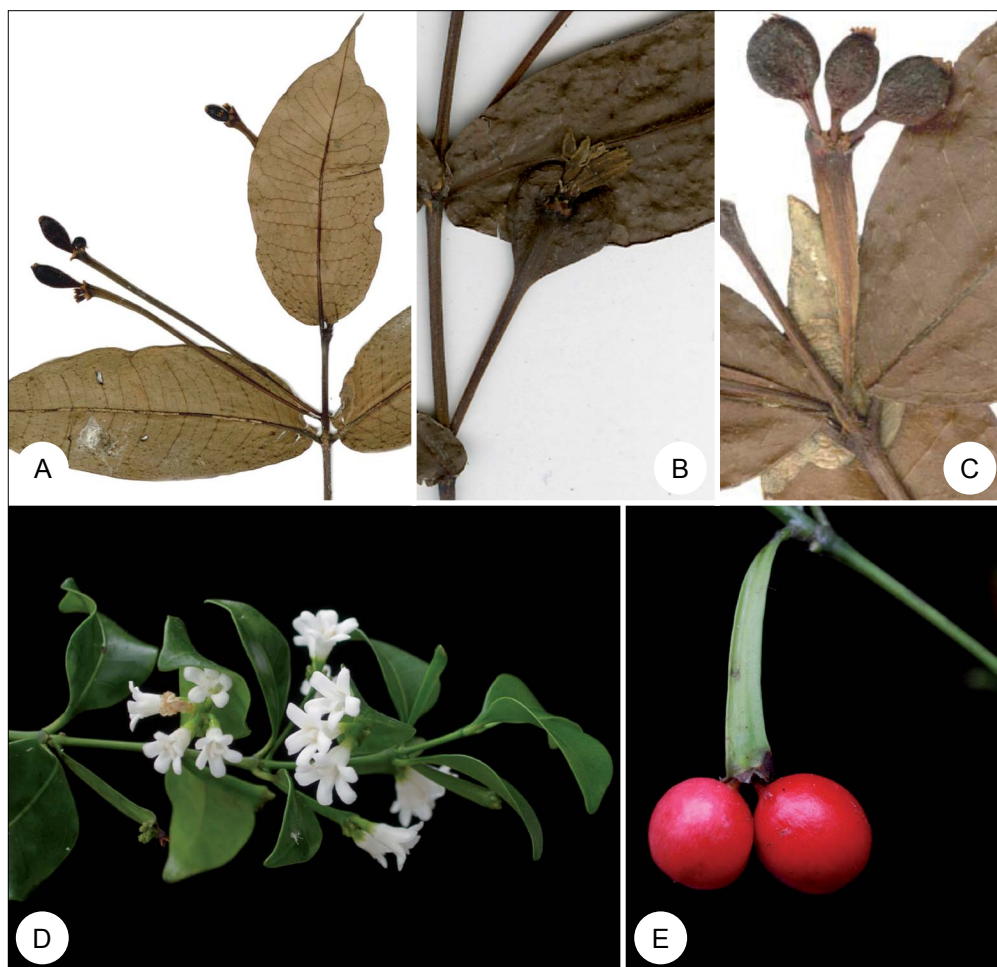


Figure 2 – Photographs of herbarium specimens of species of *Canephora* (A–C) and of plants of *Canephora madagascariensis* (D–E). A, infructescences of *C. longipedunculata*; B, inflorescence of *C. maroana*; C, infructescence of *C. madagascariensis*; D, flowering branch; E, infructescence.

(BR) (fig. 2C). – *Canephora maroana* A.DC.: Madagascar, Capuron 8646-SF (P) (fig. 2B).

RESULTS

Development of the inflorescence in *C. madagascariensis*

In *Canephora madagascariensis* the inflorescences are pedunculate (fig. 2C–E), axillary, and paired at the nodes (figs 3A, B & D, 4A–C). At very young stages, they are completely enclosed by the stipules of the leaves subtending them (figs 3A–C, 4A–C). The adaxial surface of the stipules is densely pubescent and covered with colleters (figs 3A–C, 4A–C). Initially, at a near-primordial stage, the inflorescence buds are undifferentiated (fig. 3E & F). Somewhat later, the basal part of the inflorescence becomes laterally flattened (figs 3C, 4A) and its upper part shows two first-order bracts. These are fused at their bases (figs 3C, 4B) but, more distally, each first-order bract has a free lobe with ciliate margins (figs 3C, 4C). The young inflorescences grow out into flattened, green structures, ranging in length between 1 and 4 cm, with a relatively compact cluster of flowers at the distal end (figs 2C & E, 5C, 11F). While relatively narrow at the

base, the peduncles widen distally. At the height of the first-order bracts they may reach 6 mm in width (figs 2C & E, 5C, 11F, 12E & F).

The number of flowers per inflorescence varies from one to fifteen. A flower cluster essentially consists of a terminal flower, flanked on both sides by a lateral flower (figs 2C, 4E, 5A & C–F). This pattern can be repeated, creating clusters of seven (fig. 6) or fifteen flowers. Usually, the inflorescences are three-flowered, but up to 30% encountered in our study comprised more flowers. Flowering in *C. madagascariensis* is often asynchronous (figs 4E & F, 5A & B).

The first-order bracts enclose the flowers at early developmental stages (figs 3B, 4B & E–H, 5A, 11A–C). Bracts and bracteoles occur in oppositely placed pairs and are basally fused with each other but free in their upper parts. The first-order bracts are laterally flattened and folded, vaulted, green structures (fig. 5B, C & F), which may be torn by the expansion of the growing flowers (fig. 5C). The second-order bracts are positioned adaxially with respect to the flower or flower cluster they subtend (figs 4E & F, 5D–G, 6A & B).

They are well developed centrally in the inflorescence (at the adaxial side of the lateral flower or flower cluster they subtend), but almost absent laterally in the inflorescence (at the abaxial side of the lateral flower or flower cluster they subtend). At early developmental stages, they are fused over most of their height (fig. 5G), but later on, they tear longitudinally (fig. 5F). The third- and fourth-order bracts, in resp.

7- and 15-flowered inflorescences, are more similar in shape to the first-order bracts: they are laterally flattened and folded, triangular structures (see B3, B3' in fig. 6B).

Anatomy of the peduncle in *C. madagascariensis*

In a transverse section of a mature peduncle, a thicker central part in between two lateral 'wings' can be distinguished

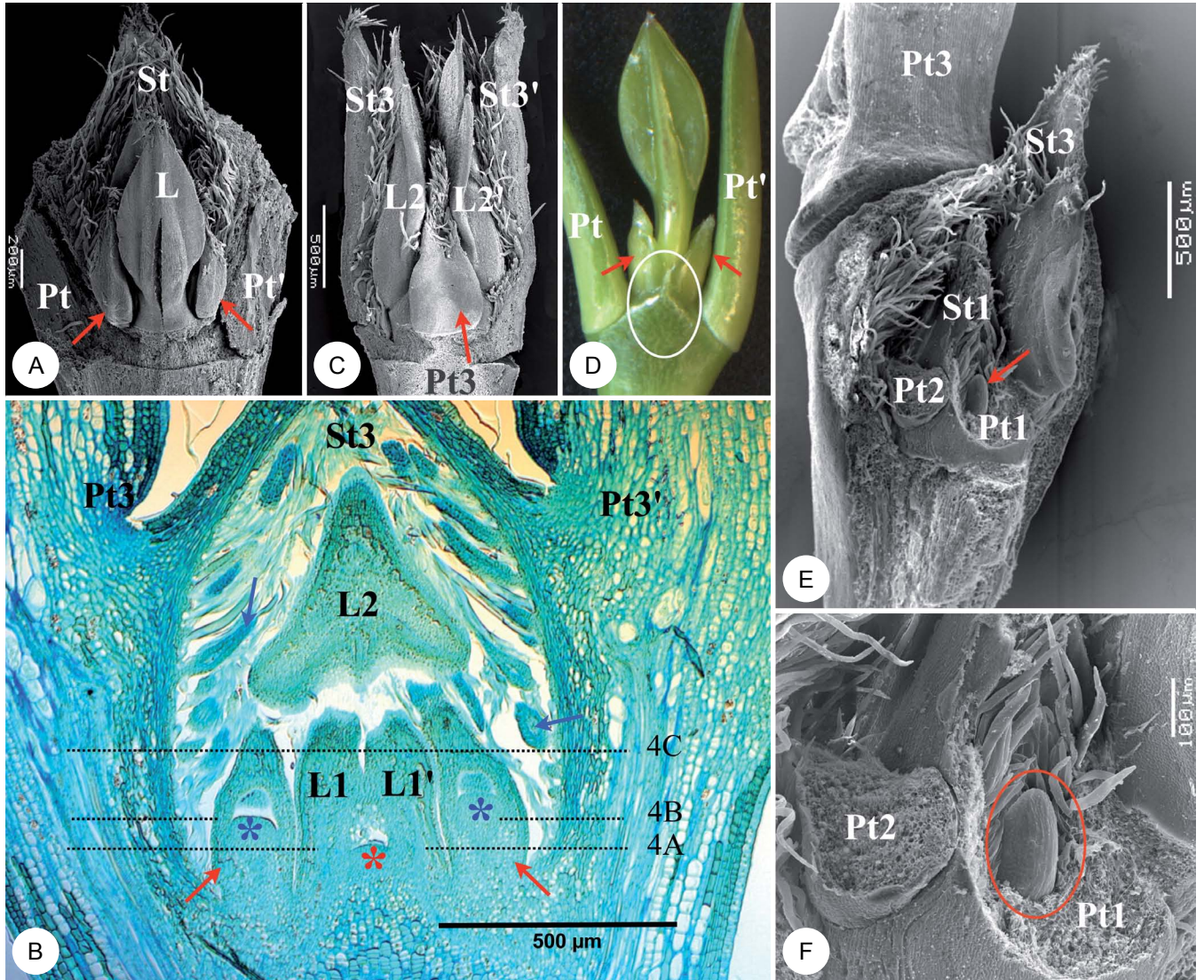


Figure 3 – SEM (A, C, E, F), LM (B) and macrophotographic (D) images of the apical part of an axis in *C. madagascariensis*. A, distal part of vegetative axis, with one interpetiolar stipular sheath removed. In the background, the adaxial, densely pubescent surface of the alternate stipular sheath can be seen. Two young inflorescences (arrowed) develop, each in the axil of a leaf of which only the petiole is visible (Pt, Pt'). Central in the image, a younger leaf pair (higher node) can be seen; B, longitudinal median section through the apical part of a vegetative axis. The apex of the axis (red *) is visible in between a pair of distal leaves (L1, L1'). Central in the image, a part of a leaf (L2) of the leaf pair of the second distalmost node, alternating 90° with the distal leaf pair, can be seen. In the axils of the petioles (Pt3, Pt3') at the third distalmost node, two young inflorescences (arrowed in red) occur (see also fig. 4A–C). The adaxial surfaces of the stipular sheaths (St3) bear trichomes and mucus-exuding colleters (blue arrows). The indications '4A', '4B', and '4C' refer to corresponding transverse LM sections in figure 4; C, Same as in 'A', with a shift of 90° (stipular sheaths partially removed). One young inflorescence is visible (arrowed) in the axil of a removed petiole (Pt3); D, successive developmental stage: two young inflorescences (arrowed), each in the axil of a leaf. Encircled is one interpetiolar stipule of the pair of stipules corresponding to 'St' in 'A'; E, opened-up apical part of an axis, showing a very young, still undifferentiated inflorescence (arrowed); F, detail of E.

Abbreviations: 1, distalmost node; 2, second distalmost node; 3, third distalmost node. L, leaf; Pt, petiole; St, interpetiolar stipular sheath; red * (asterisk), vegetative apex; blue *, generative apex; ' indicates an opposite same structure.

(fig. 7A). While the peduncle is clearly flattened, there is no conspicuous dorsal/ventral side (fig. 7A). In the central part, from the centre to the periphery, the following tissues can be distinguished: a central parenchyma surrounded by a massive, almost completely closed, band of xylem (2–7 radially arranged cell-layers), which in turn is surrounded by a band of phloem of only a few cell-layers thick (fig. 7B). The vascular bundle as a whole is flattened, and surrounded by an

almost continuous fibrous sheath of one or two cell-layers and by a dense parenchyma (fig. 7B). At the outer edge of this parenchymatous tissue, 8–12 fibrous strands are present (fig. 7A & B). Immediately below the epidermis, there is a band of tannin-filled cells, consisting of three to four cell-layers, regularly interrupted by air lacunas linked with stomata. The epidermis consists of a single cell-layer with a thick cuticula and stomata (fig. 7A–C). The wings of the

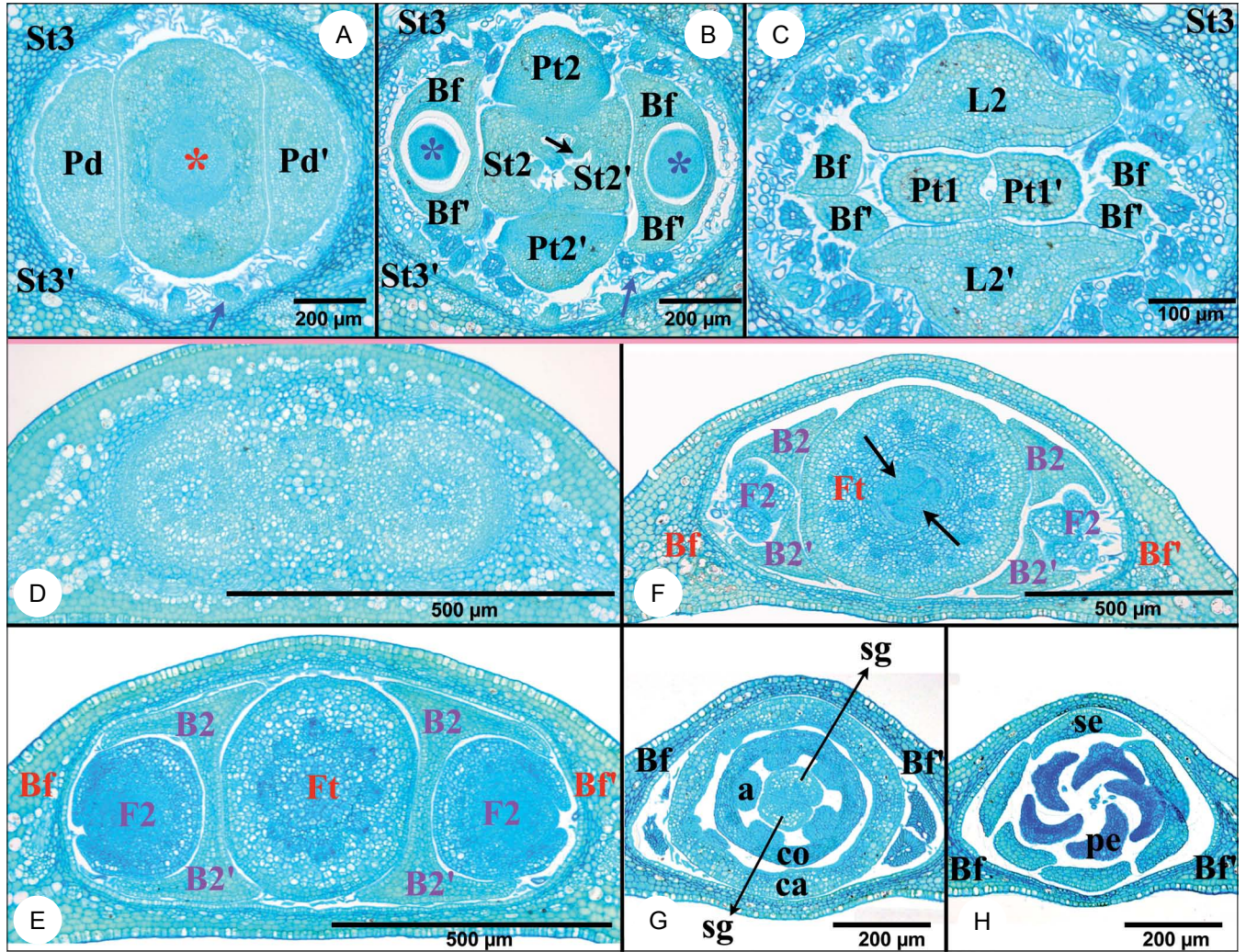


Figure 4 – LM images of successive transverse sections through the distal part of a vegetative axis, as indicated in fig. 3B (A–C), and of successive transverse sections through a developing inflorescence comprising three flowers (D–H). A, section at the base of the stipules at the third distalmost node. Colleters are arrowed in blue. The stipule pair (St3, St3') encloses the tip of the vegetative axis (red *), which is flanked by two inflorescences (at the level of the peduncle), each in the axil of a leaf (out of the image); B, section just above the vegetative apex. Centrally, the pair of stipules at the second distalmost node (St2, St2'), with trichomes and colleters (arrowed in black) on the adaxial surface, is visible, flanked by the corresponding petioles (Pt2, Pt2'). Left and right, there are sections through the tips of the inflorescences (* in blue), each enclosed in a pair of first-order bracts (Bf, Bf'); C, section through the blades of the leaf pair at node 2. The petioles of the leaves at node 1 are visible centrally. Of the inflorescence, only the free upper parts of the first-order bracts can be seen; D, section through a peduncle just below the first-order bracts; E, section through the base of the terminal flower (Ft). At the adaxial side of each lateral flower (F2), a section through the basally fused second-order bracts is visible. The lateral flowers are sectioned obliquely; F, section at the level of the placentas (arrowed) of the developing terminal flower and the free upper parts of the second-order bracts; G, section at the level of the corolla tube of the terminal flower; H, section above the stigmas, showing the sepals and petals of the terminal flower. The petal lobes are contorted clockwise.

Abbreviations: 1, distalmost node; 2, second distalmost node; 3, third distalmost node. a, anther; Bf, first-order bract; B2, second-order bract/bracteole; ca, calyx; co, corolla tube; F2, lateral flower; Ft, terminal flower; L, leaf; Pd, peduncle; pe, petal; Pt, petiole; se, sepal; sg, stigma; St, interpetiolar stipular sheath; ' indicates an opposite same structure.

peduncle are filled with irregularly shaped parenchyma cells, interspaced with many intercellular air spaces. A few vascular bundles and fibrous strands occur in this parenchyma tissue (fig. 7C).

Development of the flower in *C. madagascariensis*

The differentiation of the floral primordium starts with the unequal formation of sepal lobes. Two sepal lobes at opposite sides of the floral primordium develop first, the remaining sepals appear successively (figs 8A & B, 11A). The number of sepal lobes is typically five (figs 5C, 8D), but flowers with four and six (figs 4H, 8C) sepal lobes also occur. At

later stages, the calyx consists of a short calyx tube and more or less equally developed sepal lobes with ciliate margins (fig. 8D). Meanwhile, the floral apex has become concave. On its annular margin, five (or very rarely four or six) petal primordia originate simultaneously, alternating with the sepals (figs 8B, 9A, 11B). Alternating with the petals, five (or very rarely four or six) stamens develop adaxially on the collar of the concave floral apex (fig. 9B & C). At this stage, the central (concave) part of the floral apex is undifferentiated (fig. 9B & C) and the petals acquire a contorted aestivation (figs 4H, 9B & E). Basally on the adaxial surface of the calyx tube, one or a few colleters are visible opposite the petals (figs 9E, 11D). Next, two stigmas appear in the

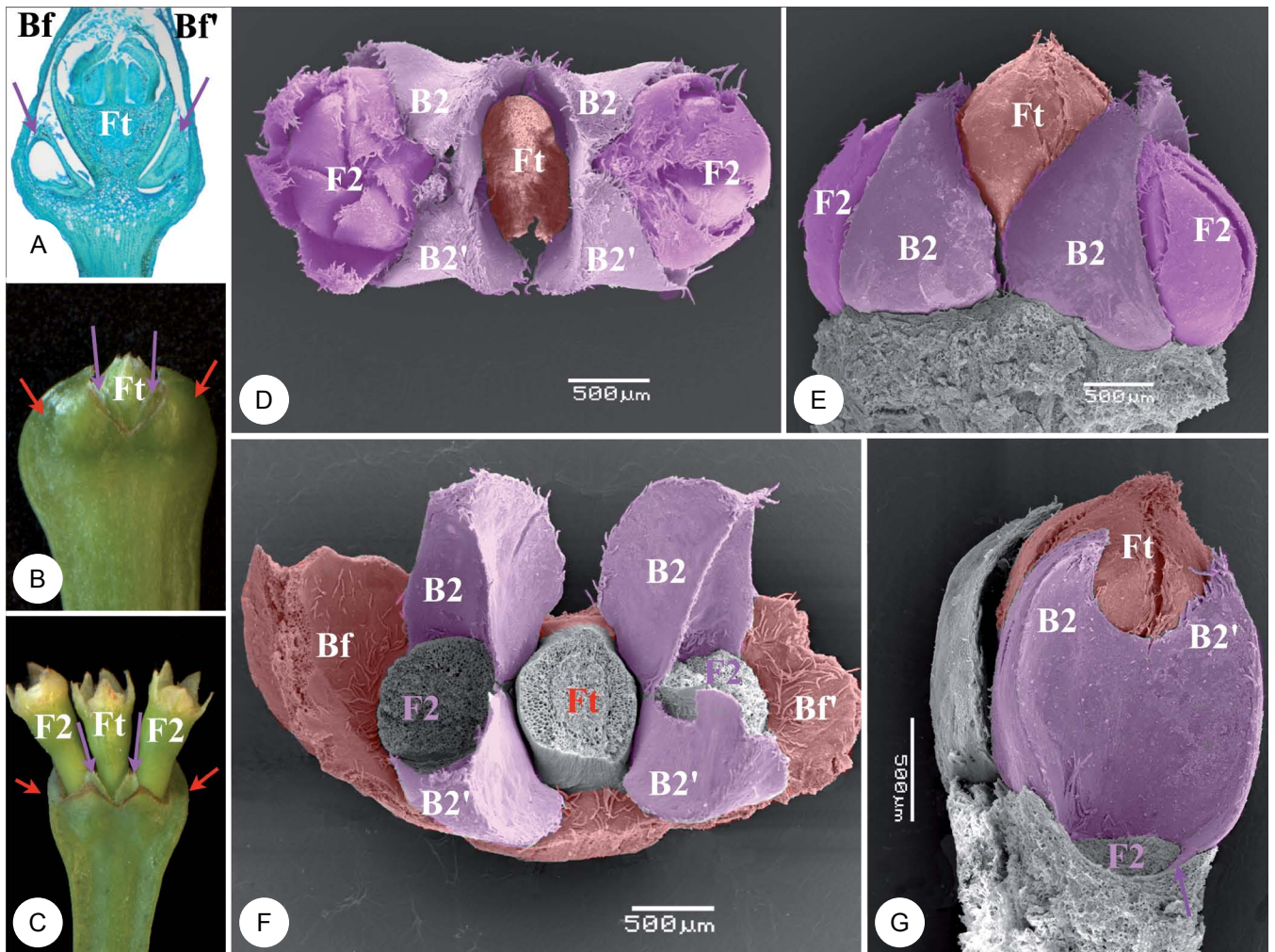


Figure 5 – LM (A), macrophotographic (B–C) and SEM (D–G) images of the distal part of an inflorescence with three flowers in *C. madagascariensis*. A, longitudinal section through a young inflorescence (flower cluster still enclosed by the first-order bracts), showing the terminal flower and two less-developed lateral flowers (arrowed); B, developing inflorescence, with first-order bracts arrowed in red, second-order bracts in purple; C, inflorescence after flowering, with first-order bracts arrowed in red, second-order bracts in purple; D, apical view of a young inflorescence, with a flower cluster consisting of a removed terminal flower (Ft) and two lateral flowers (F2) in the axils of the first-order bracts (not visible). Each lateral flower has two bracteoles (B2, B2'); E, idem as in 'D', lateral view. The terminal flower is visible here; F, apical view of semi-mature inflorescence, all flowers removed but first- and second-order bracts visible; G, side view of young inflorescence with lateral flowers removed, showing fused second-order bracts (bracteoles of the lateral flower F2). These are well developed on the adaxial side of the flower they subtend, but reduced on the abaxial side (arrowed).

Abbreviations: B2, second order-bract/bracteole; Bf, first-order bract; F2, (scar of removed) lateral flower; Ft, (scar of removed) terminal flower; ' indicates an opposite same structure. Colour code: red, terminal flower and its two bracteoles, which act as first-order bracts subtending the second-order branches; purple, second-order branches (lateral flowers and their bracteoles).

centre of the floral apex (figs 9D & F, 11C). They are fused basally (figs 9F, 10B, 11D), forming a style that eventually becomes as long as the corolla tube (fig. 11F). On top of the style two stigmatic lobes develop, their abaxial surfaces pubescent (fig. 10B). Simultaneously, each stamen differentiates into an anther and a very short filament, inserted upon the corolla below the corolla lobes (figs 10A & B, 11D). A ring-shaped nectary develops around the base of the style (fig. 9G). At this stage, an inferior ovary has been formed (figs 9G, 10A & B, 11E). Meanwhile, the introrse anthers, each consisting of two bilocular thecae, elongate and their bases become sagittate in shape (fig. 9G). Simultaneously, the stamens are raised by the growing stamen-corolla tube (figs 9C & H, 11D). At this stage, trichomes develop along the petal margins (figs 9F & G, 10B). At maturity, the dorsimedifixed, subsessile anthers are inserted at the throat of the corolla tube in the sinuses between the corolla lobes (figs 9H, 11F). The stigma consists of two stigmatic lobes, which

spread at anthesis (fig. 11F). Their adaxial surfaces are papillose, their abaxial surfaces moderately pubescent (fig. 11F).

The inferior ovary is bilocular (figs 9G, 10B, 11 E & G), the locules are separated by a septum in the same plane as the section plane in between the two stigma lobes (fig. 10B). Even at very early developmental stages, the ovary wall is distinct from the surrounding tissue (figs 9G, 10B, 11E). Its cells are smaller and more densely stained and tannin-filled cells are completely absent whereas they occur sporadically in the surrounding tissue (as well as in a continuous layer just below the epidermis). In each locule, a U-shaped placenta is formed (fig. 10), upon which, successively, up to seven unitegmic ovules develop (fig. 10, 11G). In a mature ovary, the attachment of the placenta is to the upper half of the septum (apical placentation, not shown here). The ovules are partially embedded in the placental tissue (fig. 11G).

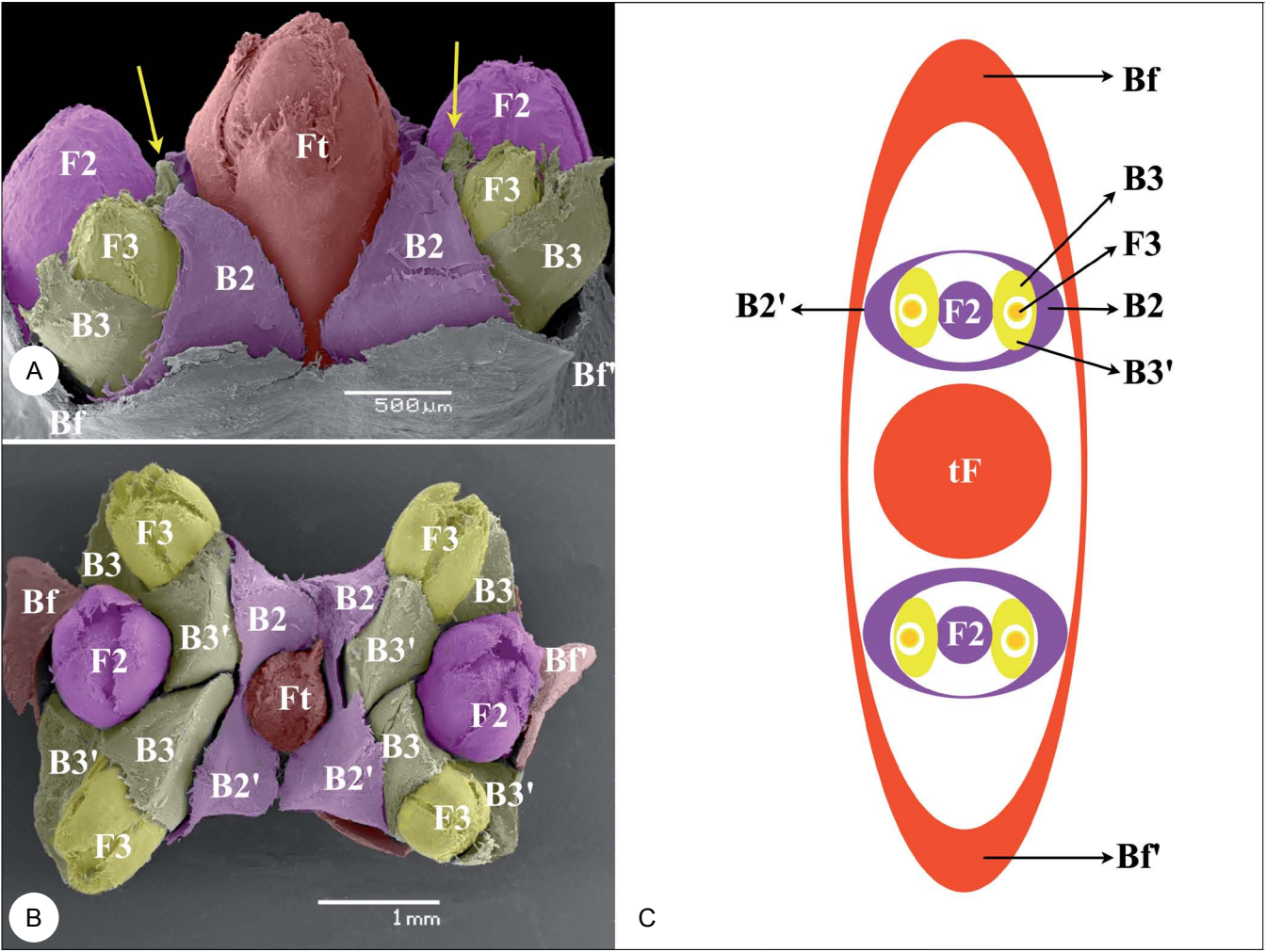


Figure 6 – SEM micrographs (A–B) of the distal part of an inflorescence with third-order branching (seven flowers) in *C. madagascariensis*, and a theoretical outline of such an inflorescence (C). A, lateral view, with arrows indicating bracteoles of third-order flowers and first-order bracts not coloured; B, idem as in ‘A’, apical view; note the absence of the abaxial part of the second-order bracts (bracteoles of F2); C, inflorescence diagram. Abbreviations: B, bract/bracteole; Bf, first-order bract; F, flower; Ft, (scar of removed) terminal flower; ' indicates an opposite same structure. Numbering according to the order of branching. Colour code: red, terminal flower and its two bracteoles; purple, second-order branches; yellow, third-order branches.

Mature flowers and fruits are shortly pedicellate (figs 2C & E, 5C, 11F, 12E & F). The green calyx is inconspicuous compared to the white campanulate corolla (< 1 cm long), and the calyx tube inconspicuous compared to the calyx lobes (figs 5C, 11F). Stamens are epipetalous (figs 9H, 11F). Surrounded by a nectary, a thin, long style reaches the base of the anthers (fig. 11F). The fused bases of the stigma lobes are surrounded by the anthers, the spreading upper parts of the stigma lobes are situated just above the throat of the flower (fig. 11F).

Development of fruit and seed in *C. madagascariensis*

After anthesis, corolla and style are shed and only calyx and ovary persist. At this stage, the inferior part of the flower develops into a red berry, crowned by the remains of the sepal lobes (fig. 12 E & F). Usually, a single fruit develops per inflorescence; more rarely up to three flowers per inflorescence

reach the fruiting stage (fig. 2E). During fruit formation, only some of the ovules develop into seeds (one to three per placenta), while the other ovules eventually abort (figs 12 B–D, 13B). This may result in an unequal development of the two locules in the fruit (fig. 13B). Fruits usually contain one to six seeds. With the seeds increasing in size, the placenta and developing seeds fill up the entire locule (figs 12G, 13B). In ripe fruits (spherical or ellipsoid in shape, ≤ 1 cm in diameter), the seeds are pendulous from a massive apical placenta (fig. 12C & D).

The seeds of *C. madagascariensis* are c. 3×4 mm in size, laterally flattened (fig. 13C & D) but their shape depends greatly on the available space in the locule, and hence, on the number of seeds per fruit. The hilum is linear and superficial (fig. 13D). The embryo is small (c. 1 mm long in mature seeds) and the radicle is superior or lateral towards the septum (not shown). The seeds are fibrous in appearance

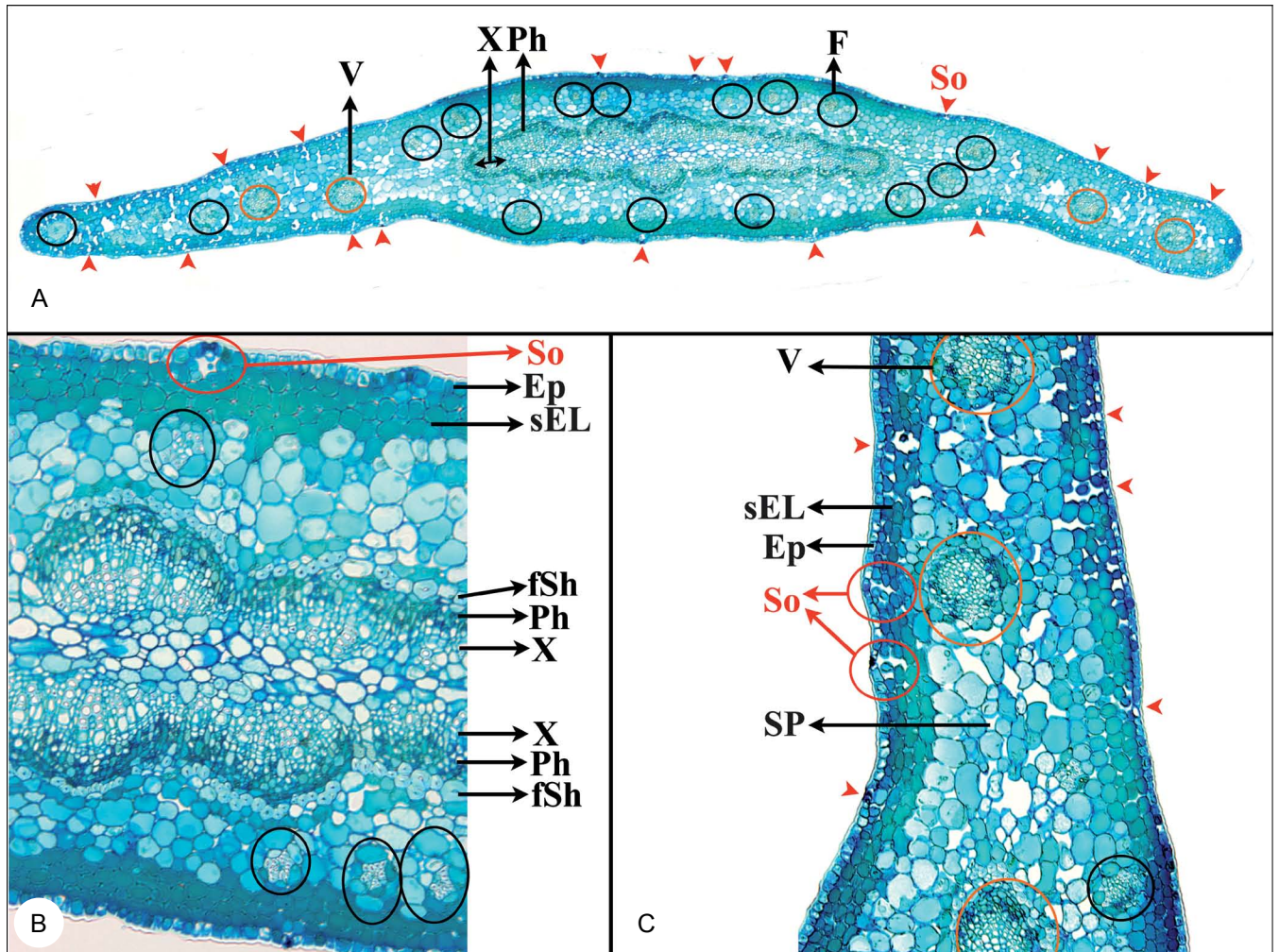


Figure 7 – LM image of transverse sections through the peduncle in *C. madagascariensis*. A, transverse section of distal part of the peduncle. The wider central part consists of a central parenchyma and a massive, flattened vascular bundle surrounded by parenchyma containing fibrous strands. The slender wings are filled with ‘spongy’ parenchyma throughout which fibrous strands (encircled in black) and a few vascular bundles (encircled in orange) are present. Stomata are present on both surfaces (encircled in red or indicated by red arrowheads); B, detail of the central part with vascular bundle; C, detail of a wing.

Abbreviations: F, fibrous strand; fSh, fibrous sheath; Ep, epidermis; Ph, phloem; sEL, sub-epidermal layer; So (and red arrowhead), stoma; SP, spongy parenchyma; V, vascular bundle; X, xylem.

(fig. 13C & D) and the seed coat is folded (fig. 13). The seed coat is exotestal and consists of one cell layer. The thickenings of the exotesta cells are plate-like and occur along the entire height of the anticlinal (radial) walls (fig. 13E–G). The endotesta consists of several layers of parenchyma-like cells. Some of these cell-layers remain intact in the folds of the exotesta (figs 13F & G) but the other endotestal cell-layers are crushed by the development of the endosperm. This is visible in a cross-section as a thin amorphous, heavily stained

layer (fig. 13F & G). The folding of the exotesta starts at a very young developmental stage (fig. 13A, B & E).

DISCUSSION

Inflorescence and peduncle

In the past, the flattened, green, assimilating peduncle and the compact inflorescence in *Canephora* were not always clearly understood. In his protologue, De Jussieu (1789)

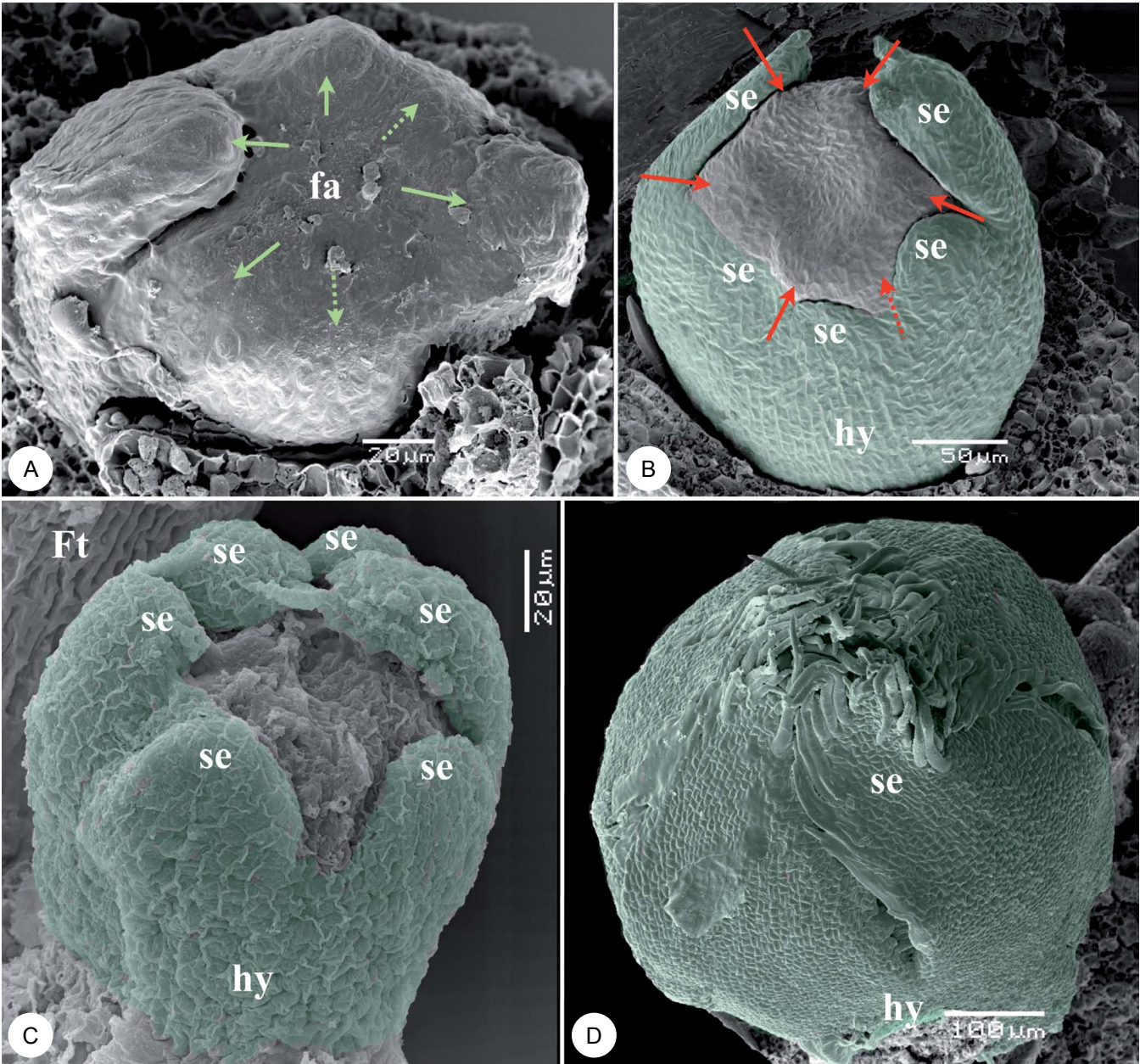


Figure 8 – SEM micrographs of early developmental stages of flowers in *C. madagascariensis*. A, early, mostly undifferentiated flower with disciform apex. Unequally developing sepal lobes are visible (arrowed, dotted arrows indicate putative sepal primordia); B, successive developmental stage with concave floral apex, on which petal primordia originate (arrowed in red), surrounded by the developing calyx (atypical flower with six petal primordia); C, successive developmental stage in atypical flower with six sepals; D, successive developmental stage, with ciliate sepals enveloping the inner parts of the flower. Abbreviations: fa, floral apex; Ft, terminal flower; hy, hypanthium; se, sepal. Colour code: green, hypanthium/calyx.

wrote that the peduncles in *Canephora* are thickened at base and apex and end in a common cup-shaped calyx enclosing 3–6 campanulate flowers. The name he proposed for this genus, *Canephora*, means ‘basket bearer’, and refers to this inflorescence structure (Roccas 1995). Other authors described the inflorescences as positioned: “... sur le sommet d’un

axe commun cladodiforme” (Baillon 1879: 200), or, “borne in the hollowed apex of a phylloclade, and surrounded by a common involucre” (Wernham 1911: 79) and the flowers as “kopfig zu 4–7 zusammengedrängt, auf der Spitze eines phyllodinen Blütenstieles” (Schumann 1891: 81, figure 29D & E). Also, the peduncle was sometimes interpreted as a

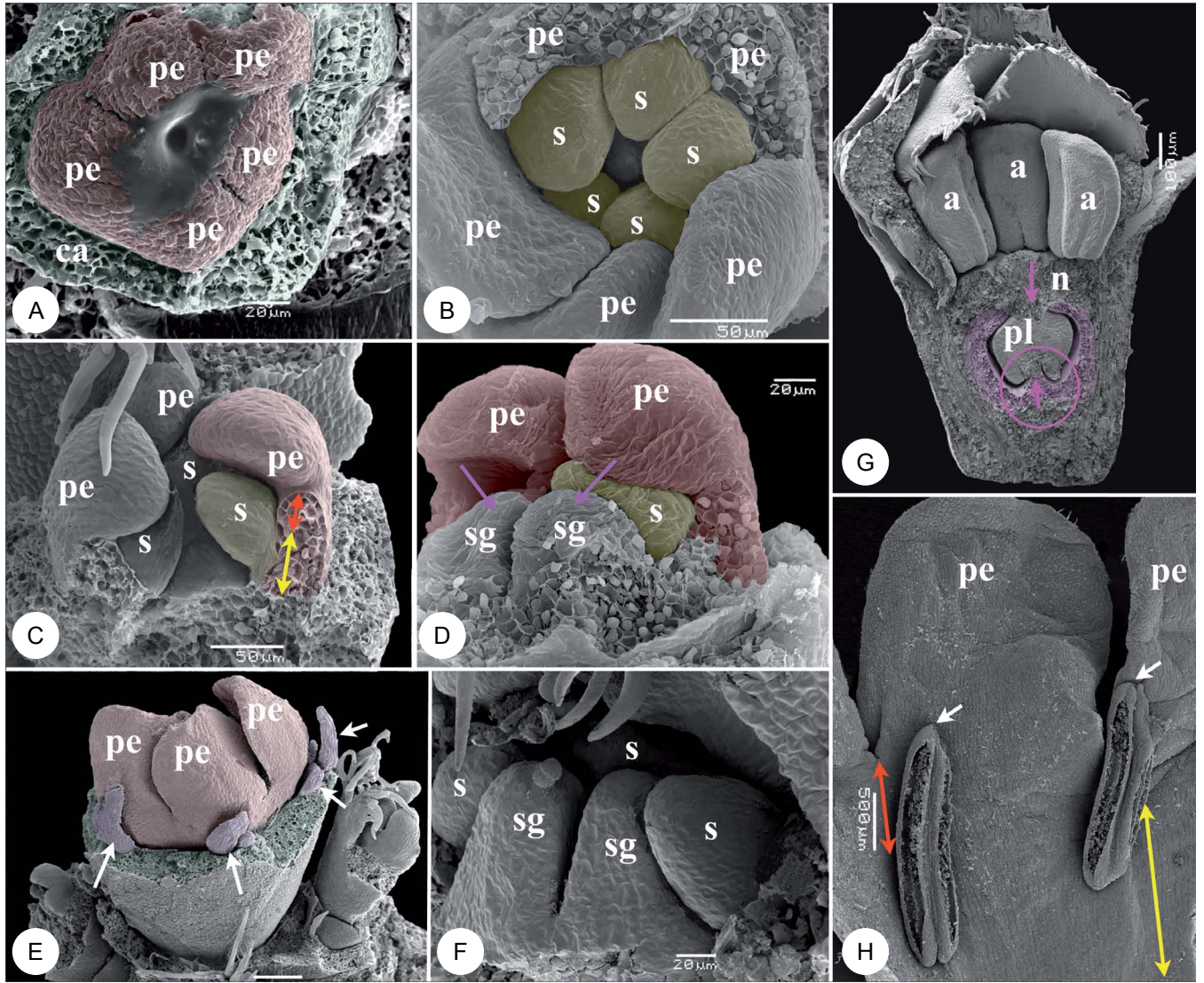


Figure 9 – SEM micrographs of the development of the inner floral whorls in *C. madagascariensis*. A, five petal primordia (red) on the collar of the concave floral apex, surrounded by the calyx (green, removed); B, apical view on developing corolla and androecium, young stamens alternating with clock-wise contorted petals; C, same as in ‘B’, lateral view of longitudinal section. A stamen-corolla tube is formed by upward growth (yellow double arrow) from the base, raising petals and stamens. Above the insertion of the stamens, a corolla tube sensu stricto is formed (red double arrow); D, centrally, two stigmas (arrowed) develop; E, external view of a developing corolla (red), calyx removed (scar coloured green). Colleters (purple, arrowed) are present opposite the petal lobes; F, same stage as in ‘E’, inner view. A short style with two developing stigma lobes is present in the centre of the flower, surrounded by stamens; G, longitudinal section through a developing flower (perpendicular to the septum, compare with fig. 10A). The style/stigmas are removed, only a developing nectary remains visible. Anthers are being formed. In the lower part of the flower, an inferior gynoecium is visible with a distinctive gynoecium wall (purple), and two locules with developing placentas. Only one leg of each U-shaped placenta is visible. The arrows (purple) indicate portions of the septum. Encircled are the basal portion of the septum and the scars resulting from the removal of the second leg of each U-shaped placenta; H, mature stamens (raised by the developing stamen-corolla tube, yellow double arrow), inserted at the base of the corolla tube sensu stricto (red double arrow) in the sinuses of the corolla lobes. The stamens have longitudinally opening pollen sacs, a sagittate base, and, on top of each anther, a small apiculus (arrowed).

Abbreviations: a, anther; ca, calyx; n, nectary; pe, petal lobe; pl, placenta; s, stamen; sg, stigma.

branch, e.g. by Richard (1829) and Wernham (1912: 453): “borne in the hollowed apex of an axillary flattened branch”.

The inflorescence in *C. madagascariensis* is essentially dichasial (figs 5 & 6). Flowers are shortly pedicellate and have two bracteoles. These bracteoles may act as bracts, each subtending a higher-order flower or partial inflorescence (figs 4–6). Depending on growth conditions, higher-order clusters may blossom, which explains the varying number of flowers in inflorescences. The few-flowered and compact inflorescences of *Canephora* are an example of both reduction and congestion, two trends typical in Rubiaceae inflorescences (Weberling 1977, Robbrecht 1988).

The bracts/bracteoles are oppositely positioned pairs and are fused to each other at their bases. At early developmental stages the first-order bracts completely enclose the flower clusters (figs 3B–D, 4B & E–H, 5A, 11A–C). As a result of

the growth of the flowers, they are damaged but remain persistent. The “common cup-shaped calyx” (De Jussieu 1789), the “common involucre” (Wernham 1911) and the “hollowed apex” (Wernham 1912) all refer to this pair of first-order bracts. The second-order bracts are somewhat aberrant. Because of the lack of space in the narrow confines of the first-order bracts, the abaxial parts of the second-order bracts are reduced, giving the impression of adaxially positioned bracts. Possibly, however, a remnant of the abaxial part may be present (fig. 5G). A similar ontogenetic pattern was observed in the distantly related Loranthaceae (Wanntorp & Ronse Decraene 2009).

The use of terms such as “phylloclade” (Wernham 1911), “cladode” (Baillon 1879, Robbrecht 1988), and “phyllode” (Schumann 1891) in the inflorescence descriptions for *Canephora* refer to the green, flattened, assimilating part

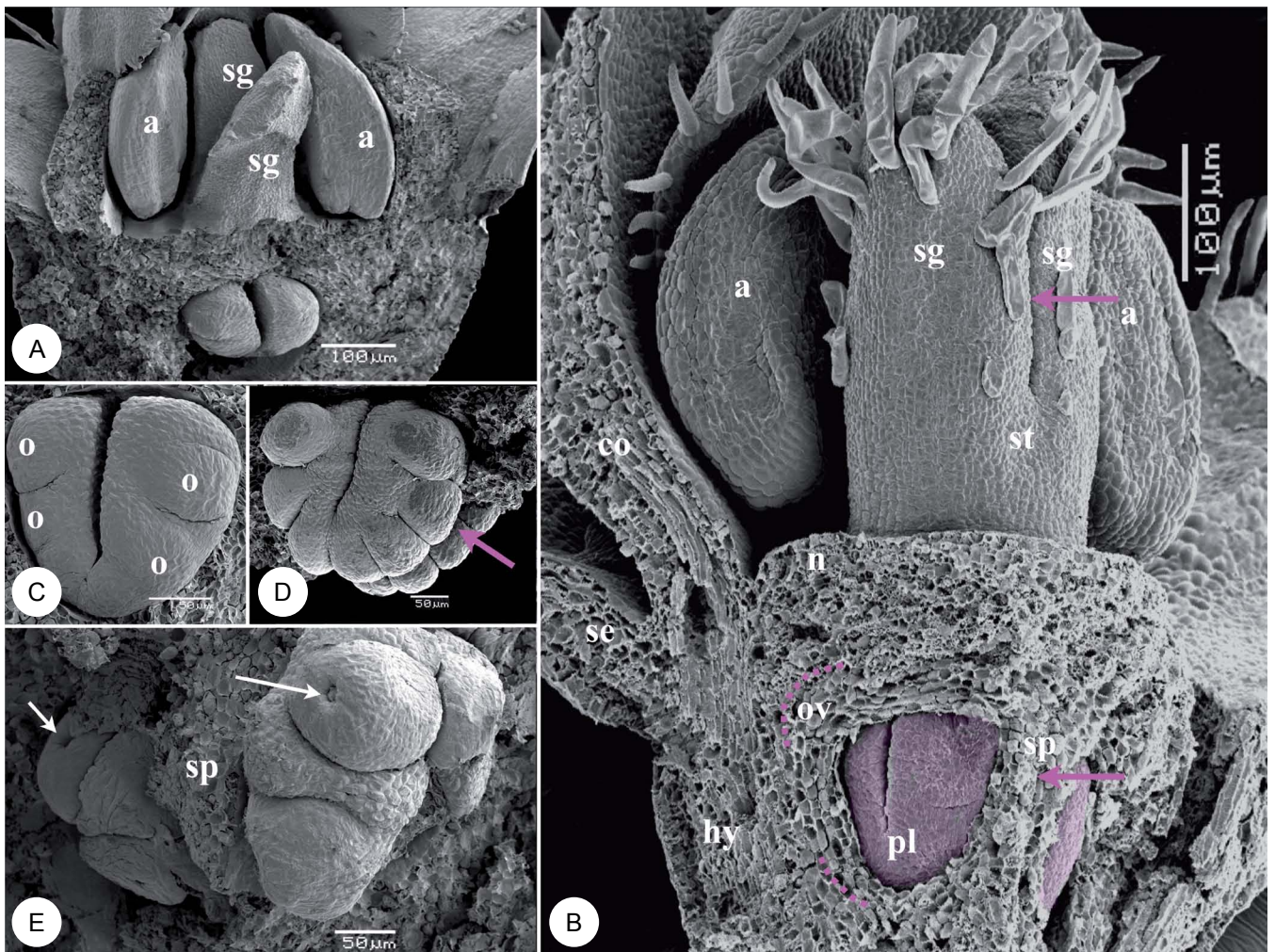


Figure 10 – SEM micrographs of the development of the gynoecium in *C. madagascariensis*. A–B, lateral view of successive developmental stages of a longitudinally opened developing flower (parallel to the septum, compare with fig. 9G). ‘B’ shows that the septum is in the same plane as the section plane in between the two stigma lobes (purple arrows). At each side of the septum, there is a U-shaped placenta (purple) on which ovule primordia are initiated. The ovary wall is distinct from the outer tissue (border between both tissues indicated by dotted lines); C–D, successive developmental stages of a U-shaped placenta (view parallel to the septum, indicated by a purple arrow in ‘D’); E, developing ovules with micropyle becoming visible (arrowed).

Abbreviations: a, anther; co, corolla; hy, hypanthium; n, nectary; o, ovule; ov, ovary wall; pl, placenta; se, sepal; sg, stigma; sp, septum; st, style.

of the inflorescence. In literature, the terms ‘phylloclade’ or ‘cladode’ seem to be defined in many different ways, depending on the author (such as Cooney-Sovetts & Sattler 1986, Hickey & King 2000, Simpson 2010), but they always refer to structures which possess both leaf and stem characters. Some of the confusion may be due to the fact that it is often not clear whether the term is used functionally or purely morphologically descriptive.

In the past, the double-organ identity of ‘phylloclade’-like structures lead to the question whether these structures

are leaf- or stem-derived, causing controversy about their morphological homology assessment (e.g. Sattler 1992, Hirayama et al. 2007, Nakayama et al. 2012). The peduncle in *Canephora* shows both leaf and stem characters. Anatomically, the central part of the peduncle is stem-like. Young stems in *C. madagascariensis* have a single, massive, flattened vascular bundle (Wernham 1912: 455, figure 2), which is similar in structure to the one in the central part of the peduncle (fig. 7A). The wings flanking the central part are filled with irregularly shaped parenchyma cells, interspaced with

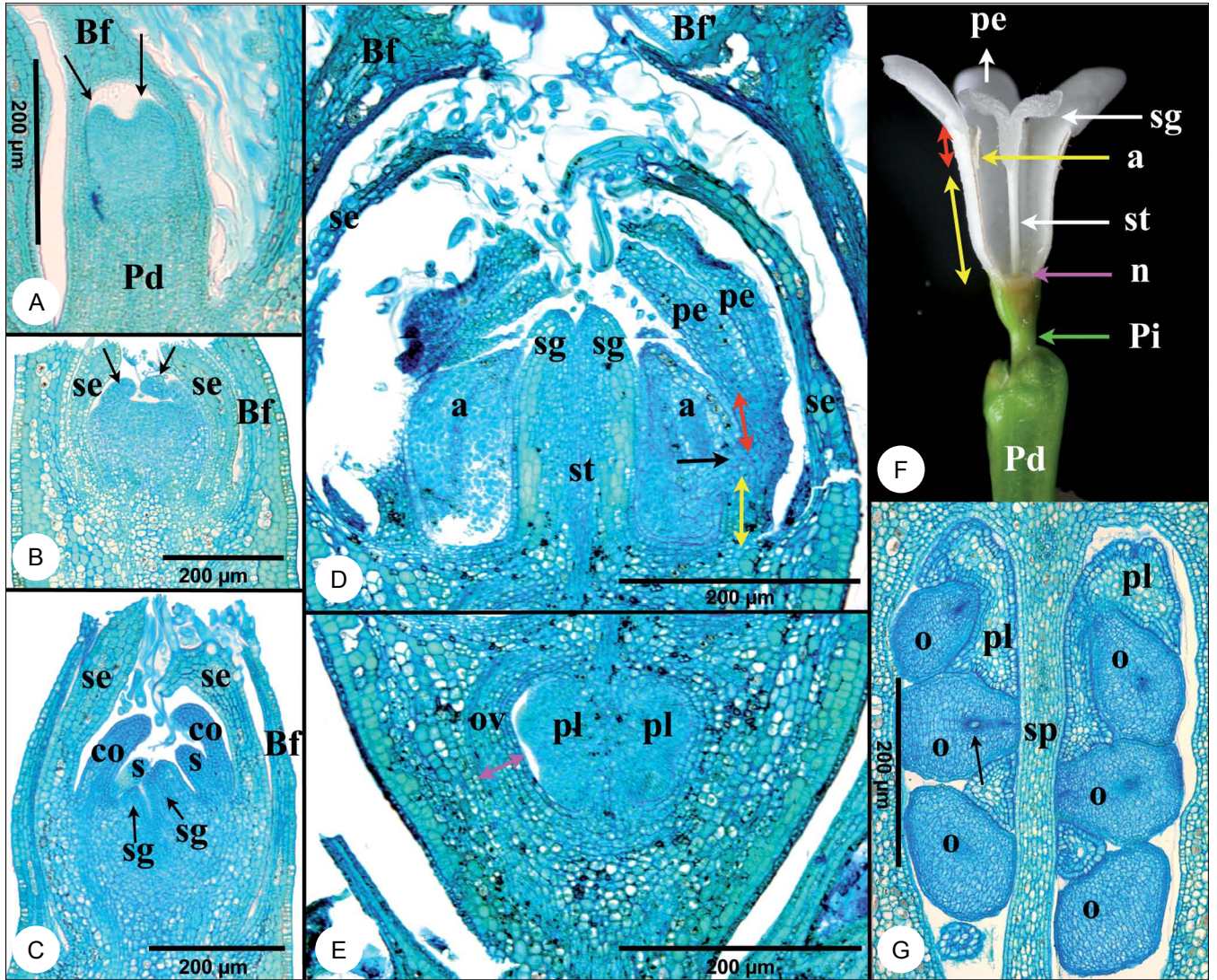


Figure 11 – LM (A–E, G) and macrophotographic (F) images of developing and mature flowers in *C. madagascariensis*. A–E, longitudinal median sections through developing flowers. A, very young inflorescence with terminal flower primordium already initiating the calyx (arrowed); B, developing terminal flower. The calyx (se) is developing and the corolla is initiated (arrowed); C, developing terminal flower. Stamen primordia (s) appear at the base of the corolla tube. Centrally, stigma primordia (arrowed) are visible; D–E, developing terminal flower. D, longitudinal median section through the upper half of the flower. The young anthers are connected to the corolla tube by a very short filament (arrowed in black). The yellow double arrow indicates the stamen-corolla tube, the red double arrow indicates the corolla tube sensu stricto; E, longitudinal median section through the lower half of the flower. The ovary wall of the inferior ovary is distinct from the surrounding tissue (double arrow); F, inflorescence with one flower at anthesis, its corolla opened longitudinally. The corolla-stamen tube is indicated by a yellow arrow, the corolla tube by a red arrow; G, non-median, longitudinal section through mature ovary, perpendicular to the septum, showing unitegmic ovules. Micropyles are visible (arrowed). Abbreviations: a, anther; Bf, first-order bract; co, corolla; n, nectary; o, ovule; ov, ovary wall; Pd, peduncle; pe, petal lobe; Pi, pedicel; pl, placenta; s, stamen; se, sepal lobe; sg, stigma (primordium); sp, septum; st, style.

many intercellular air spaces, which, in our opinion, is similar to spongy parenchyma (leaf character, fig. 7). According to Wernham (1912), fibrous strands occur in the wings. However, compared with the unambiguous fibrous strands found in the stem-like central part, we interpret some of the larger ‘fibrous strands’ in the wings as vascular bundles (fig. 7). Wernham (1912) stated that there are no stomata but our LM sections give evidence of the presence of stomata on both surfaces of the phylloclade (fig. 7). Within the peduncle of *C. madagascariensis*, the characters ‘stomata’, ‘spongy parenchyma’, and ‘small vascular bundles’ are leaf-like. Stem-like are the position in the axil of a bract, the presence of massive xylem and phloem and the presence of nodes.

To conclude, older descriptions of the inflorescences in *C. madagascariensis* infer terms such as ‘phylloclade’, ‘cladode’, and ‘phyllode’ to indicate the peduncle. In our opinion, the flattened, ‘phylloclade-like’ structure does not only comprise the peduncle but also the first-order bracts and the reduced first-order branches of the inflorescence (fig. 14A). Despite the leaf-like aspects of this flattened structure, there are no doubts as to its morphological origin. The ‘phylloclade-like’ flattened structure grows from a primordium in the axil of a bract and, consequently, is a stem-derived structure in which a combination of developmental processes, including leaf and stem morphogenetic processes, takes place. This specific morphogenesis results in a double-organ

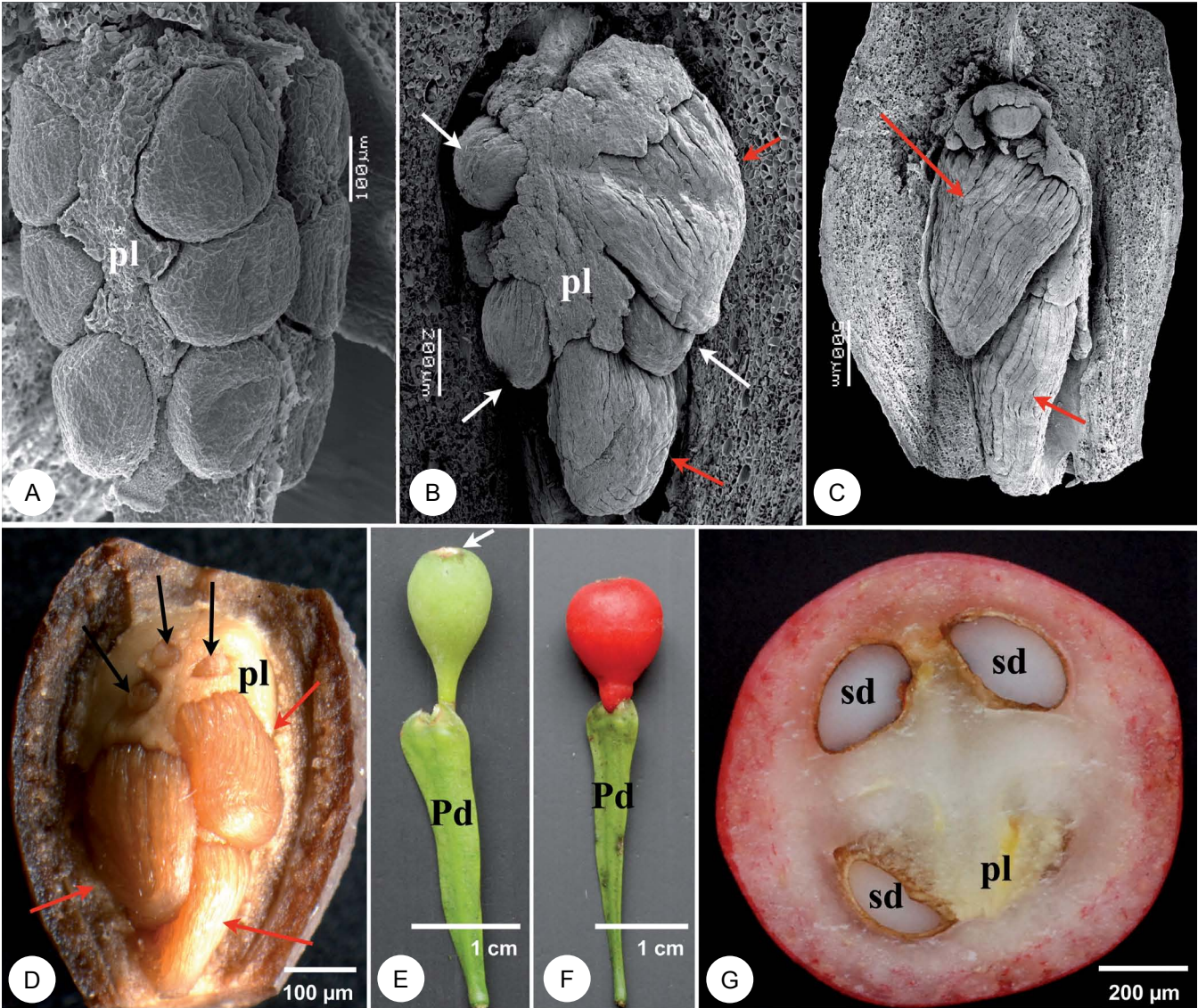


Figure 12 – SEM micrographs (A–C) and macrophotographic images (D–G) of developing seeds and fruits in *C. madagascariensis*. A–D, development of ovules into seeds. A, abaxial view of placenta with ovules in two longitudinal rows in flower at anthesis; B, abaxial view of placenta with ovules in postmature flower. Some ovules develop into seeds (red arrows) while others abort (white arrows); C, idem as ‘B’, but older stage. Developing seeds are arrowed in red; D, idem as ‘C’, but older stage. Developing seeds are arrowed in red and aborted ovules in black; E, infructescence bearing a single, immature fruit. The fruit is crowned by the small sepal lobes (arrowed); F, infructescence bearing a single, mature fruit; G, transverse section through mature berry. Abbreviations: Pd, peduncle; pl, placenta; sd, seed.

identity (leaf-stem) of the flattened structure and, hence, in its ‘phylloclade-like’ nature. In our opinion, this has no consequence for its morphological or structural origin; the leafy aspect is an example of analogy to foliage leaves.

Floral whorls and annular intercalary meristems, early sympetaly

In most Rubiaceae, including *Canephora*, the calyx and corolla consist of a tube plus distinct distal lobes, and the ovary is inferior (Robbrecht 1988). Because of the presence of a hypanthium, it can be difficult to determine the structural origin of the calyx. In *C. madagascariensis*, we did not observe an

annular primordium from which the calyx tube originates. At the earliest developmental stages, individual sepal primordia give rise to sepal lobes. They arise before the appearance of the petal primordia and develop at different rates in an irregular sequence (fig. 8A & B). Payer (1857: 633), however, observed a different floral ontogenetic sequence in *Asperula* L., *Galium* L., and *Rubia*, notably, that the calyx originates only after the appearance of corolla and stamens. Payer’s (1857) observation was confirmed in *Galopina* (Ronse Decraene & Smets 2000) and *Galium* (Pötter & Klopfer 1987). This is in contrast to our observations in *Canephora* and those in other Rubiaceae genera, such as *Spermacoce* (Vaes et al. 2006), *Mitrasacmopsis* (Groeninckx et al. 2007), *Gaertnera* Lam.,

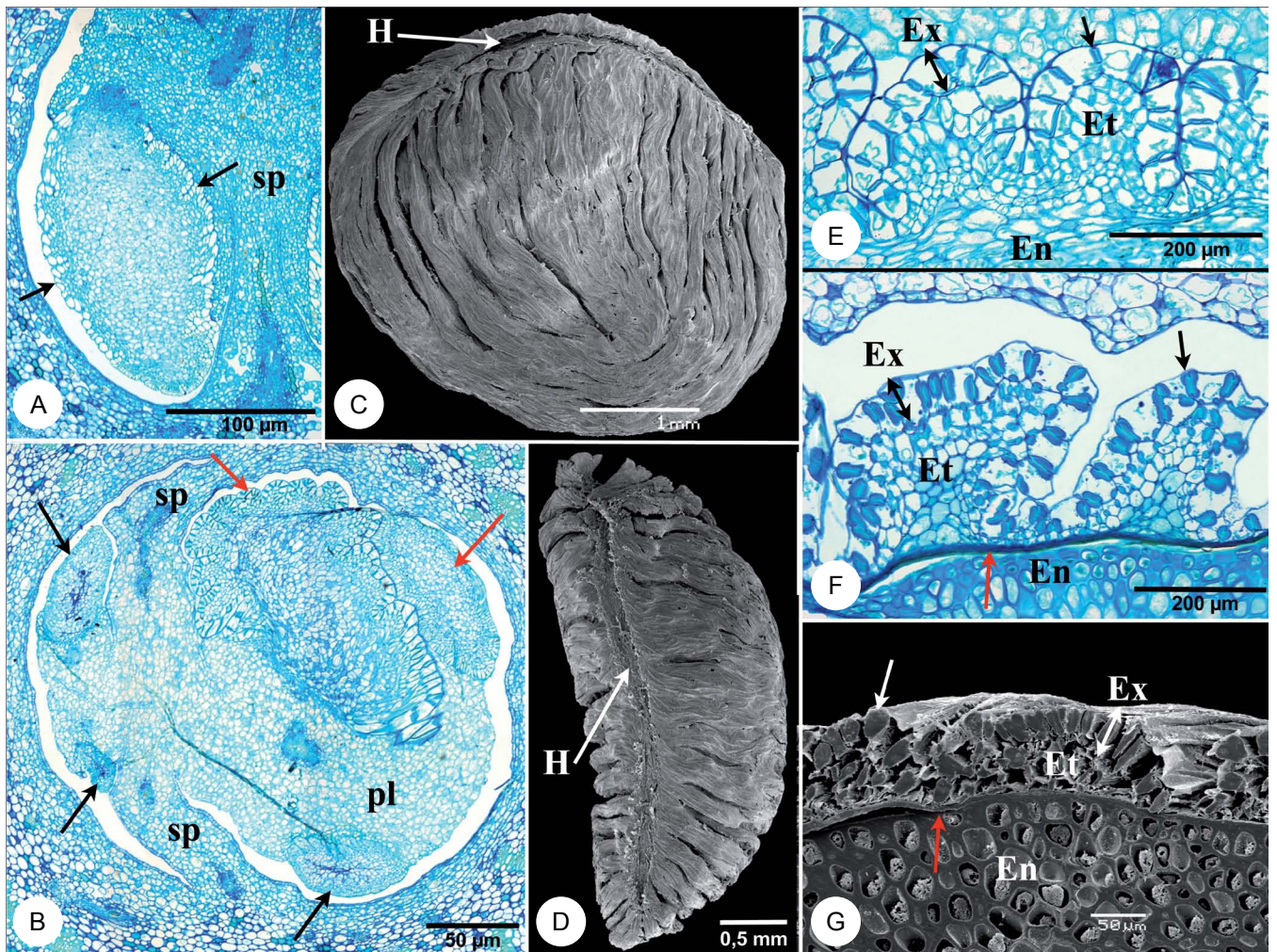


Figure 13 – LM images (A–B, E–F) and SEM micrographs (C–D, G) of seed and fruit development in *C. madagascariensis*. A, longitudinal section perpendicular to the septum through a very young developing fruit (only one locule visible). The exotesta shows invaginations (arrowed) into the inner tissues of the developing seed; B, transverse section through a young developing fruit. The septum is pushed to one side by the growth of the developing seeds (red arrows) in the right locule. Aborted ovules (black arrows) are visible in both locules; C, lateral view of mature seed showing the fibrous and folded exotesta; D, adaxial view of mature seed, showing the narrow, superficial, linear hilum. E–G, successive stages of the development of the seed coat, showing endosperm, endotesta and exotesta (exotesta indicated by double arrow); E, exotesta, folded with regard to the endotesta. The radial walls of the exotestal cells are hardly thickened (black arrow); F, the thickenings along the radial walls of the exotestal cells have increased (black arrow). The cell-layers of the endotesta have been crushed by the expansion of the endosperm, except in the folds of the exotesta. The densely stained, amorphous layer (red arrow) consists of crushed endotestal cells; G, mature seed. The exotestal cells are now strongly thickened along their radial walls (white arrow). Even at this stage, some cell-layers of the endotesta remain intact within the folds of the exotesta. The red arrow indicates the layer of crushed endotestal cells. Abbreviations: En, endosperm; Et, endotesta; Ex, exotesta; H, hilum; pl, placenta; sp, septum.

Psychotria L., *Chassalia* Comm. ex Poir., *Palicourea* Aubl. (Igersheim et al. 1994) and *Coffea* (Marchand 1864), as well as those in *Paederia* L., *Pentas* Benth., *Pentodon* Hochst., and *Sacosperma* G. Taylor (Vrijdaghs et al. in prep.). The differences in development cannot be attributed to systematic position within the family, since, except for *Canephora* and *Coffea*, all these genera belong to the subfamily Rubioideae (fig. 1). Rather, they are related to the fact that, in *Asperula*, *Galium*, *Rubia* and *Galopina*, calyx lobes do not develop (except sporadically in *Galopina*, where they occur after the initiation of the petals and corolla tube: Ronse Decraene & Smets 2000), whereas calyx lobes are generally present in the other genera mentioned above. In *C. madagascariensis* and in all other studied genera with calyx lobes, the calyx

tube grows out at a later, nearly semi-mature stage in the floral development (fig. 11D), long after the development of the calyx lobes (Marchand 1864, Igersheim et al. 1994, Vaes et al. 2006, Groeninckx et al. 2007). Often, as in *C. madagascariensis*, the calyx tube remains small. In *Asperula*, *Galium*, *Rubia* and *Galopina*, only a short calyx rim is developed after the formation of petals and stamens (Payer 1857, Pötter & Klopfer 1987, Ronse Decraene & Smets 2000). Therefore, our observations of the development of the calyx and those of Payer (1857) do not differ in the sense that the calyx tube grows out at the same relative moment in the developmental sequence. The variation in the size of calyx tube and lobes is an example of differentiation based on the principle of variable proportions: “Aus der Konsequenz der Organisation und

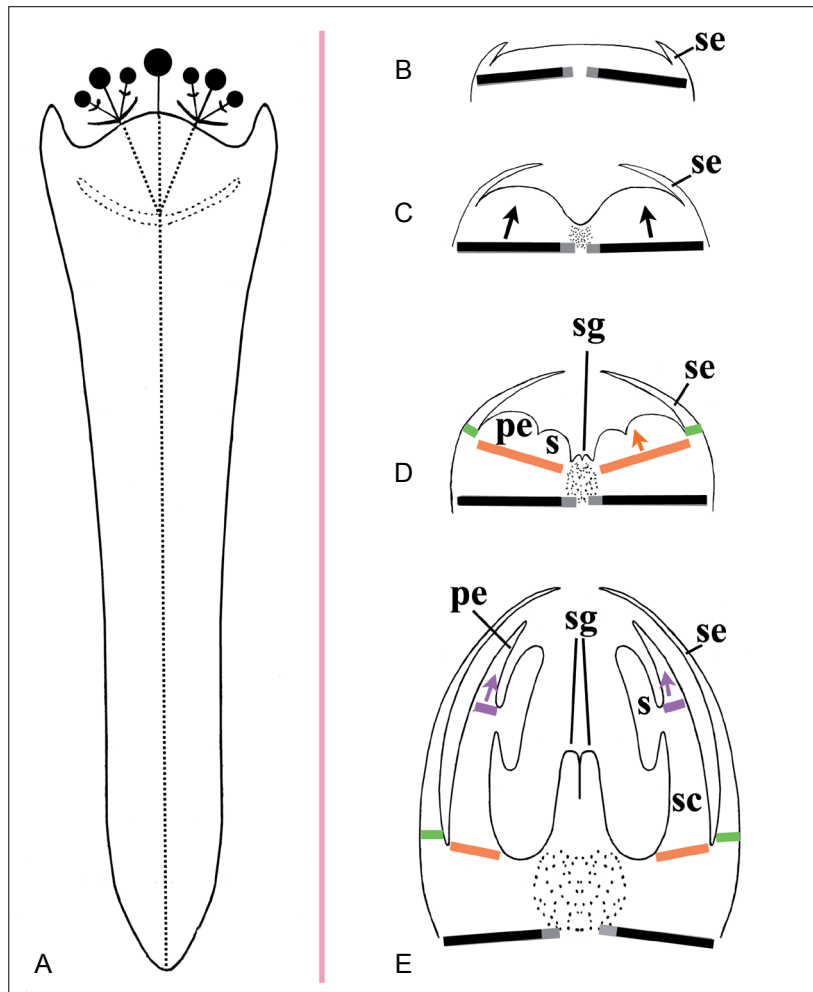


Figure 14 – Theoretical outline of the ‘phylloclade-like’ inflorescence (A), and of the development of floral tubes (B–E) in *C. madagascariensis*. A, peduncle, first-order bracts and first-order branches fuse into a flattened, green, ‘phylloclade-like’ structure (fused structures indicated by dotted lines); B, longitudinal section through the floral apex at disciform stage, with sepals already present. A hypanthium is formed by an annular intercalary meristem comprising either calyx, corolla and androecium (perigynous hypanthium: black lines) or calyx, corolla, androecium and the dorsal parts of the carpels (gynoecial hypanthium: black + grey lines); C, longitudinal section through the floral apex at concave stage, with an early hypanthium surrounding a zone determined to become gynoecium (dotted); D, longitudinal section through the developing flower, with primordia of petals and stamens appearing on the early hypanthium. Below, an annular intercalary meristem starts developing a stamen-corolla tube (orange lines). Below the sepals, an annular intercalary meristem (green line) initiates the formation of a calyx tube; E, longitudinal section through developing flower. Above the insertion point of the stamens, an annular intercalary meristem (purple lines) initiates the formation of a corolla tube sensu stricto. Abbreviations: pe, petal; s, stamen (primordium); sc, stamen-corolla tube; se, sepal; sg, stigma (primordium); dotted zone, gynoecium.

aus der Möglichkeit, dass ein Teil eine andere Proportion annehmen kann, entsteht die Mannigfaltigkeit der Gestalt” (Goethe in Troll 1949: 493), or, the diversity in structures of the same type can be explained by quantitative variation in the growth of its parts (Claßen-Bockhoff 2001, Kaplan 2001).

The formation of corolla tubes in Rubiaceae was studied by Erbar (1991) and Erbar & Leins (1996, 2011), who distinguished between early and late sympetaly and transitional states. In early sympetaly, petals arise on a ring primordium or are connected already at initiation, whereas, in late sympetaly, petals arise separately and become connected at a later stage. According to Ronse Decraene & Smets (2000), early sympetaly is expressed by the initial formation of a concave floral apex, the annular collar of which corresponds to the corolla ring primordium of Erbar & Leins (1996). In *C. madagascariensis*, the petal primordia originate on the annular rim of a concave floral apex (figs 9A, 14C & D), and can therefore be assigned to ‘early sympetaly’.

Ronse Decraene & Smets (2000: 165) questioned the nature of the ring primordium, originally considered by Erbar & Leins (1996) as the primordium of the corolla tube and lobes. Noting that Rubiaceae with epipetalous stamens develop a stamen-corolla tube (a tubular section consisting of a fusion of corolla and androecium) below the corolla tube sensu stricto, Ronse Decraene & Smets (2000) stressed that, in those cases, the ring primordium cannot be restricted to just the corolla. In later publications, Leins (2000) and Leins & Erbar (2010) concluded that not just the corolla tube but floral tubes in general grow from intercalary meristematic rings below the primordia of the floral whorls, whereby one or several floral whorls may be raised by the development of these floral tubes.

From the earliest stages of the development of the ovary in *Canephora madagascariensis*, there is a conspicuous distinction between the innermost layers of the ovary wall and the surrounding tissue (figs 9G, 10B, 11E). This can be explained in two ways, (1) the formation of a perigynous hypanthium, or, (2) the formation of a gynoeceal hypanthium (fig. 14B–E). In the first case, the cup-shaped form of the floral apex is due to the development of a hypanthium from an annular intercalary meristem below the primordia of all the floral whorls from androecium to calyx [fig. 14 B–E (black line); Leins & Erbar 2010: 102, figure 99, 6]. In the second case, the hypanthium is formed from an annular intercalary meristem below the primordia of all the floral whorls from the dorsal parts of the carpels to the calyx [fig. 14 B–E (black + grey lines); Leins & Erbar 2010: 102, figure 99, 7], followed by a subsequent, secondary differentiation of the ovary wall with respect to the hypanthium. This probably corresponds to Robbrecht’s (1988: 74) view that rubiaceous hypanthia “consist of only gynoeceal and axial tissue” as suggested by Leinfellner (1954). In either case, a hypanthium is formed surrounding the zone determined to differentiate into a gynoeceum. Consequently, the gynoeceum becomes inferior (fig. 14C–E). Based on our results, we cannot determine which hypanthium type is formed in *C. madagascariensis*. Conspicuous differences between ovary wall and peripheral tissue portions surrounding the inferior ovary were also

reported from e.g. *Paederia* (Svoma 1991) and *Gaertnera* (Igersheim et al. 1994: 403, figure 1A & B).

Upon the early hypanthium sepal lobes originate, followed by the appearance of individual petal primordia (figs 8B, 9A, 11B), which are raised together with the later appearing stamen primordia by an underlying intercalary meristem (fig. 14D), forming a stamen-corolla tube (figs 9C & H, 11D & F, 14D & E). Simultaneously, a very small (approximately 0.2 mm at maturity of the flower) calyx tube develops. Eventually, above the insertion of the stamens, a small corolla tube sensu stricto (approximately 0.5 mm at maturity of the flower) protrudes (figs 9H, 14E).

In the asterids, the corolla tube consists of different parts (e.g. Erbar 1991, Erbar & Leins 1996, Erbar & Leins 2011, Leins 2000, von Hagen & Kadereit 2002): a stamen-corolla tube, a corolla tube sensu stricto, and, possibly, a corolla tube resulting from petal fusion. Depending on the development of each part, the tubular corolla will have a particular, though not always visibly different, phenotype. This can, again, be explained by the principle of variable proportions. In the Rubiaceae, a well-developed stamen-corolla tube and a short corolla tube sensu stricto occur in e.g. *Ixora* L. (Erbar & Leins 1996) and in *Canephora*. A corolla tube consisting of a short stamen-corolla tube and a well-developed corolla tube sensu stricto occurs in e.g. *Paederia* (Puff & Igersheim 1991). Obviously, the position of the insertion of the stamens in the corolla depends on the degree of development of the stamen-corolla tube and the corolla tube sensu stricto.

Placentation

Canephora madagascariensis has U-shaped pauci-ovulate placentas, a common placentation type in the angiosperms (Leinfellner 1951). In Rubiaceae, U-shaped placentas are reported in tribes closely related to Octotropideae, notably Pavetteae and Gardenieae, and in tribes of other subfamilies such as Pauridiantheae, Isertieae, Hedyotideae, Hippotideae (Hallé 1967, De Block & Robbrecht 1997). Furthermore, U-shaped placentas are found in the African Octotropideae genera *Feretia* Delile (Utzschneider 1947) and *Petitiododon* Robbr. (Tosh et al. 2008: 557, figure 3E).

The genera of the Octotropideae are described as having pendulous ovules arranged in ‘two series’ (longitudinal rows) (fig. 12A; Robbrecht 1980: 75, Ruhsam & Davis 2007: 559). In *C. madagascariensis*, two longitudinal rows of ovules occur, each row on one leg of the U-shaped placenta. Given the similarity in placentation between *C. madagascariensis* and the other genera of the Octotropideae (ovules in two longitudinal rows), we hypothesize that U-shaped placentas are common in the tribe. *Canephora madagascariensis* differs somewhat in that the ovules are partly embedded in the placenta. However, embedded ovules are also present in *Petitiododon* (Tosh et al. 2008).

Fruits and seeds

The fruits of *Canephora madagascariensis* are bilocular drupes, with in each locule several seeds imbricately arranged and pendulous from an apical placenta; the embryo radicle is directed upwards or laterally and the seed-coat is

fibrous and folded. The exotesta consists of elongated cells, thickened along their radial walls (see Robbrecht & Puff 1986, type e1, figure 20). Different from most Rubiaceae, the parenchyma layers of the endotesta are not completely crushed by the growing endosperm. Several cell layers of the endotesta remain intact in the folds of the exotesta. In the Octotropideae, the seeds have ruminant (e.g. *Pubistylus* Thoth.: Deb & Rout 1993) or entire endosperm (e.g. *Flagenium* Baill.: Ruhsam & Davis 2007). In *C. madagascariensis*, the invaginations of the exotestal layer into the inner tissues of the developing seed give the impression of a ruminant seed (fig. 13A, B & E). However, the exotesta does not actually invaginate the endosperm but only the cell-layers of the endotesta (fig. 13F & G). Consequently, in *C. madagascariensis*, the endosperm is not ruminant.

CONCLUSION

The inflorescence of *Canephora madagascariensis* is a condensed dichasium. The peduncle, the first-order bracts and the first-order branches together form a flattened, green phylloclade-like structure. Its leaf-like character makes it an analogue of a foliage leaf. The peculiar inflorescence of *C. madagascariensis* constitutes one of the states for the character 'axillary inflorescence' in Rubiaceae, and is an example of both reduction and congestion, two trends typical in Rubiaceae inflorescences.

The floral developmental pattern in *C. madagascariensis* does not differ significantly from that in other, already studied, Rubiaceae. *Canephora*'s floral developmental pattern can be explained by Leins and Erbar's theory on cup-shaped floral formations. In the floral development, underlying annular intercalary meristems determine the successive formation of cup-shaped or tubular floral whorls: initially a hypanthium bearing sepals is formed, from which next a stamen-corolla tube, and eventually a corolla tube *sensu stricto* grow out. After the initiation of corolla, androecium and gynoecium, a small calyx tube develops. In *Canephora*, the corolla tube is early sympetalous and consists of a long stamen-corolla tube and a short corolla tube *sensu stricto*, which are successively formed.

Canephora madagascariensis has U-shaped pauci-ovulate placentas with the ovules arranged in two series on the legs of the U. Its fruits and seeds show the typical characters of the tribe Octotropideae, e.g. seeds pendulous from an apical placenta, seed-coat fibrous and folded.

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