

Different ways to obtain similar results: the development of the corolla and epipetaly in Rubieae (Rubioidae, Rubiaceae)

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Background and aims – Rubieae is a tribe in the subfamily Rubioideae characterised by herbaceous plants with verticillate leaves and flowers with a rudimentary or absent calyx and a short, cup-shaped corolla. This is in contrast to the flowers of most other Rubiaceae, in which the tubular corolla is longer than the corolla lobes. Also, the description by Payer, a French 19th century pioneer of floral ontogenetic research, of the floral development in *Asperula*, *Galium*, and *Rubia* deviates from recent insights about the development of tubular corollas, which are based on investigations of flowers of tropical Rubiaceae. Tubular corollas are currently considered as resulting from the development of underlying annular intercalary meristems, whereas Payer explained the tubular corollas in the three taxa by postgenital fusion. We therefore tested both hypotheses in six Rubieae genera, including the three taxa studied by Payer.

Methods – Floral ontogeny of ten species in six Rubieae genera based on scanning electron (SEM) and light microscopy (LM).

Conclusions – Our results suggest that, in all species studied, the mature phenotype of the corolla as well as the epipetaly of the stamens is caused by a combination of three developmental processes (the development of a stamen-corolla tube, the development of a corolla tube *sensu stricto*, and postgenital fusion), and the relative moment of activation of each of these processes during floral development (plastochron variation or heterochrony).

Keywords – Corolla; corolla tube *sensu stricto*; epipetaly; floral ontogeny; herbaceous Rubiaceae; heterochrony; plastochron; stamen-corolla tube.

INTRODUCTION

Rubiaceae, also known as coffee or madder family, is the fourth largest angiosperm family and consists of approximately 13 200 species in 614 genera. It has a cosmopolitan distribution and centres of diversity in the (sub)tropics (Heywood et al. 2007; Davis et al. 2009; Stevens 2001 onwards). Rubiaceae are easily recognizable by the simple opposite leaves with entire margins, inter- or intrapetiolar stipules, and sympetalous flowers with inferior ovary. Tropical Rubiaceae are usually woody and occur in the understory of humid and dry forests. However, some Rubiaceae lineages

are herbaceous, predominantly occurring in subfamily Rubioideae, which is characterized by the presence of raphides, valvate petal aestivation, and many representatives are heterostylous (Robbrecht 1988). Among these herbaceous lineages, some are tropical (e.g., tribe Spermacoceae), while the cosmopolitan tribe Rubieae occurs in regions with Mediterranean and temperate climates (Robbrecht 1988). Certain members of these herbaceous lineages can exhibit secondary woodiness (Lens et al. 2009). This study focuses on temperate representatives of tribe Rubieae, belonging to subfamily Rubioideae.

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Plant Ecology and Evolution is published by Meise Botanic Garden and Royal Botanical Society of Belgium

ISSN: 2032-3913 (print) – 2032-3921 (online)

Tribe Rubieae

Rubieae are characterized by an herbaceous habit (rarely subwoody) and by verticillate leaves (except *Didymaea* Hook.f.), which are in fact pseudowhorls of opposite leaves and leaf-like stipules or stipule segments (Rutishauser 1999). Other characters are the often rudimentary calyx, the bilocular ovary with a single ovule per locule, and the pluricolpate pollen (Robbrecht 1988). The Rubieae comprise c. 1000 species in 11 (Bremer & Eriksson 2009: 789), 12 (Ehrendorfer et al. 2018: 1; including *Kelloggia* Torr. ex Benth. & Hook.f.), or 13 (Robbrecht & Manen 2006; including *Kelloggia* and *Theligonum* L.) genera. For this study, we follow the tribal delimitation of Bremer & Eriksson (2009), excluding the genera *Kelloggia* and *Theligonum*. Recently, the monospecific genus *Pseudogalium* L.-E. Yang, Z.-L. Nie & H. Sun was described to accommodate an early-diverging species of *Galium* L., bringing the number of genera in the Bremer & Eriksson (2009) delimitation of the tribe up to 12 (Yang et al. 2018). Two monospecific genera, *Microphysa* Schrenk and *Galiasperula* Ronniger, are controversial and are not taken into account here (Ronniger 1931; Tao & Ehrendorfer 2011).

Most of the Rubieae species belong to the cosmopolitan *Galium* (c. 635 species), the Old World *Asperula* L. (c. 195 species), and *Rubia* L. (c. 90 species) with a Eurasian-African distribution. The remaining genera are smaller (Govaerts et al. 2020): *Callipeltis* Steven (3 species), *Crucianella* L. (30 species), *Cruciata* Gilib. (8 species), *Didymaea* (5 species), *Mericalpaea* Boiss. (1 species), *Phuopsis* (Griseb.) Hook.f. (1 species), *Pseudogalium* (1 species), *Sherardia* L. (1 species), and *Valantia* L. (7 species). The monophyly of the Rubieae was confirmed by molecular evidence, but the delimitation of the two largest genera within the tribe remains problematic: *Galium* is paraphyletic and *Asperula* is polyphyletic (Ehrendorfer et al. 2018).

Typical Rubiaceae flowers are tetracyclic, possessing calyx, corolla, androecium, and gynoecium. Flowers are tetramerous or pentamerous. In most species, the calyx and corolla are tubular and topped by calyx and corolla lobes. The corolla tube is usually as long as or longer than the corolla lobes (Robbrecht 1988). The inferior ovaries are often bi- to multilocular with a single to numerous ovules per locule. In contrast, in the tribe Rubieae, the calyx is usually rudimentary or absent. Most species have a short corolla tube that is cup-shaped rather than tubular, although there are exceptions (*Phuopsis*, Puff et al. 1996; *Crucianella*, Schönbeck-Temesy & Ehrendorfer 1989). Ovaries in Rubieae are bilocular with a single ovule per locule (Robbrecht 1988).

Floral ontogeny

Floral ontogenetic studies of Rubiaceae mostly deal with tropical, herbaceous or woody species (e.g., Von Faber 1912; Van der Meulen 1939, both on *Coffea* L.; Rutishauser et al. 1998 on *Theligonum*; Ronse De Craene & Smets 2000 on *Galopina* Thunb.; Groeninckx et al. 2007 on *Mitrasacmopsis* Jovet; De Block & Vrijdaghs 2013 on *Canephora* Juss.; Vrijdaghs et al. 2015 on *Paederia* L., *Pentas* Benth., *Pentodon* Hochst., *Sacosperma* G. Taylor and *Spermacoce* L.).

To our knowledge, Payer (1857) was the first to describe all the developmental stages in flowers of the temperate gen-

era *Asperula*, *Galium*, and *Rubia*, with a single figure showing the floral developmental stages in *Rubia tinctorum* L. (as *Rubia tinctoria* Salisb.). The species studied by Payer share a late development of a rudimentary calyx, appearing only as a rim without calyx lobes after the development of the corolla and stamens. Payer even questioned whether the rim without calyx lobes actually is a calyx. Erbar & Leins (1996) also considered *Asperula tinctoria* L., *Cruciata laevipes* Opiz., *Galium verum* L., and *Phuopsis stylosa* (Trin.) G. Nicholson as taxa without a calyx. This is in contrast to the woody *Canephora*, *Paederia*, and *Sacosperma* and the herbaceous *Pentas*, *Pentodon*, and *Spermacoce*, where a tubular calyx with calyx lobes originates before or more or less simultaneously with the corolla (De Block & Vrijdaghs 2013; Vrijdaghs et al. 2015). Payer (1857) reported that in *Asperula*, *Galium*, and *Rubia* the corolla originates from four free-standing petal primordia that simultaneously appear. Later in the development, the bases of the petals fuse. About the development of the androecium, Payer (1857: 633) wrote: “Complètement indépendantes de la corolle d’abord, elles deviennent ensuite connées à leur base et sont insérées à sa gorge” [“Initially totally independent from the corolla, they [the stamens] subsequently become connate at their bases and are inserted at its [the corolla] throat”]. As for the development of the gynoecium, the description of Payer for *Asperula*, *Galium*, and *Rubia* corresponds with the description of the development of the gynoecium in other Rubiaceae. Payer stressed that the gynoecium consists of an inferior part, the ovary, which becomes inferior by the rising of parts of the receptacle, whereby locules are formed. The single style is the result of a fusion of two “bourrelets semi-circulaires” [“two semi-circular bulges”] (Payer 1857: 634), which are “les rudiments du style” [“the rudiments of the style”] and which “deviennent promptement connées” [“become immediately connate”].

Pötter & Klopfer (1987) stated that sepal primordia were not detected and that petal and stamen primordia appear simultaneously in *Galium aparine*. According to these authors, sympetaly is the result of the development of an underlying meristem. Naghiloo & Classen-Bockhoff (2016) performed a floral developmental analysis in *Cruciata glabra* (L.) Ehrend., *C. laevipes*, and *Rubia tinctorum*. Apart from the absence of a calyx, they focused on the more or less simultaneous appearance of petal and stamen primordia and on the “late and very weak sympetaly” (Naghiloo & Classen-Bockhoff 2016: 57). According to them, the functionally male flowers in *Cruciata laevipes* result from a delayed initiation of the gynoecium development.

De Block & Vrijdaghs (2013) and Vrijdaghs et al. (2015) hypothesised that the tubular corolla, with or without epipetalous stamens, is the result of three developmental processes that act in different proportions depending on the species, thus causing the variability that occurs in tubular corollas. These developmental processes are: 1) the development of a stamen-corolla tube from an annular common primordium, 2) the development of a corolla tube *sensu stricto* from an annular primordium or intercalary meristem, and 3) postgenital fusion of petals or corolla lobes. The development of a stamen-corolla tube concurs with the floral cup hypothesis of Leins & Erbar (2010), which states that floral cups can be

Table 1 – Species studied and voucher data.

Species	Accession number Meise Botanic Garden
<i>Crucianella macrostachya</i> Boiss.	20171259-12
<i>Cruciata laevipes</i> Opiz.	20171106-53
<i>Galium aparine</i> L.	90000072
<i>Galium glaucum</i> L.	19630367
<i>Galium odoratum</i> (L.) Scop.	20050039-2
<i>Galium pumilum</i> Murray	19921263-82
<i>Galium verum</i> L.	19921431-56
<i>Phuopsis stylosa</i> (Trin.) G.Nicholson	19681293
<i>Rubia tinctorum</i> L.	10001292
<i>Sherardia arvensis</i> L.	10001471

formed by annular intercalary meristems underlying one or more floral whorls, in the case of a stamen-corolla tube below petals and stamens. In Rubiaceae, the stamens are mostly epipetalous. If only a stamen-corolla tube is formed, the stamens emerge from the corolla tube in between the bases of the corolla lobes (fig. 12D). If a stamen-corolla tube and a corolla tube *sensu stricto* are formed and/or if postgenital fusion of the corolla lobes occurs, the stamens are inserted somewhere inside the tubular corolla (fig. 12B, C, F). Sometimes, a corolla tube *sensu stricto* of only a few cell layers thick is formed, in which case the stamens seem to emerge from in between the bases of the corolla lobes (Vrijdaghs et al. 2015). If no stamen-corolla tube develops, the stamens remain free from the corolla, inserted on the hypanthium.

Aims

Intrigued by Payer's (1857) description of the development of corolla and androecium in *Asperula*, *Galium*, and *Rubia*, we test the developmental hypothesis of Vrijdaghs et al. (2015) for the corolla and androecium in these three and other temperate herbaceous genera. In addition, aspects of the development of the gynoecium are illustrated.

MATERIAL AND METHODS

Plant material

Inflorescences of ten Rubieae species were collected from the living collection of Meise Botanic Garden (table 1) and preserved in 70% ethanol. The five *Galium* species belong to the monophyletic clade X according to Ehrendorfer et al. (2018), which justifies combining observations of different *Galium* species in a single figure (figs 3, 4).

Dissection was performed in 70% ethanol under a Wild M3 (Leica Microsystems AG, Wetzlar, Germany) stereo-microscope equipped with a cold-light source (Schott KL1500; Schott-Fostec LLC, Auburn, NY, USA). The dissected samples were treated for 30 minutes in a Branson 2210 Ultrasonic Cleaner (Branson Ultrasonics Corporation, Danbury, USA).

Scanning electron microscopy (SEM)

The samples were transferred twice to 70% ethanol for 5 minutes and then to a mixture (1/1) of 70% ethanol and DMM (dimethoxymethane) for 5 minutes, followed by 20 minutes pure DMM. Next, CO₂ critical point drying was performed with a CPD 030 critical point dryer (BAL-TEC AG, Balzers, Liechtenstein). The dehydrated samples were mounted on aluminium stubs using double sided adhesive 12 mm carbon tabs (Agar Scientific Ltd. Stansted, UK). Gold coating was done with a SPI-Module™ Sputter Coater (SPI Supplies, West-Chester, PA, USA). SEM micrographs were obtained with a Jeol JSM-6360 (Jeol, Tokyo, Japan) scanning electron microscope.

Light microscopy (LM)

Dehydration of previously dissected material was performed through a graded ethanol series. Subsequently, the samples were embedded in KULZER's Technovit 7100 (based on HEMA, hydroxyethyl-methacrylate). Seven µm thick sections were obtained using a rotation microtome Leica RM2135 with Leica DB80 disposable blades (Leica Biosystems, Amsterdam, The Netherlands). Next, the sections were stained with 0.05% toluidine blue in distilled water. LM images were obtained using an Olympus BX51 (Olympus Corporation Belgium, Antwerp, Belgium) microscope equipped with a Color View Soft Imaging System camera.

Colour code in figs 2–11

Amber, raphides; green, bract(eoles), hypanthium (inferior ovary), calyx rim; purple, (upper part of the) gynoecium and ovules; red, corolla; yellow, androecium; and blue or dark purple, other structures.

RESULTS

Our observations are arranged according to the species floral morphology, starting with the predominant morphology in the tribe of flowers with cup-shaped corolla tubes found in most species of *Cruciata*, *Galium*, and *Rubia*, and followed by three species from the genera *Crucianella*, *Phuopsis*, and *Sherardia* with tubular corolla tubes. The inflorescence in the ten species studied is essentially a thyrses, but species-specific variation occurs.

Cruciata laevipes

Inflorescence – The inflorescence has an indeterminate main axis with pairs of oppositely positioned bracts (fig. 1A). Within each of these bracts, two cymosely branched partial inflorescences are formed, each consisting of a terminal flower with two lateral flowers, which each may have two higher order lateral flowers. The result is a complex compound inflorescence (fig. 2A, C).

Floral development – On the slightly concave floral primordium, four petal primordia and, alternating with them, four stamen primordia differentiate quasi simultaneously (fig. 2B). Petals and stamens develop, enclosing the floral apex (fig. 2C). Surrounding the floral apex, two semi-circular carpellary bulges originate, of which the abaxial zones will sub-

sequently develop into two semi-circular nectaries at the top of the gynoecium. First, the carpellary bulges together with the outer whorls develop a perigynous hypanthium, creating a cavity that encloses the floral apex. This becomes an inferior bilocular ovary. Simultaneously, the central part of the adaxial zone of each of the two carpellary bulges develops into a style and stigma (fig. 2D, E, L–N). Meanwhile, the bases of the filaments and the petals adhere, forming a tube. At the top of this tube, the filaments and distal parts of the petals, now called corolla lobes, continue elongating (fig. 2E–G, J, O). The tube remains small compared to the corolla lobes and the free parts of the filaments (fig. 2I, J). On the adaxial surface of the tube, four glands are formed in between the bases of the corolla lobes (fig. 2H, I). The calyx does not develop and is only visible as a rim (fig. 2H) below the tube formed by the fusion of the bases of the petals and filaments. A relatively small inferior, bilocular, didymous ovary becomes visible below the rim of the calyx. In each locule, a single, anatropous, unitegmic ovule is formed (fig. 2K–M). At maturity, a stamen-corolla tube, only a few cell layers thick, is visible below the part of the corolla formed by the fusion of filaments and corolla lobes (fig. 2N, O).

Galium

Inflorescence – The inflorescence consists of an indeterminate main axis with spirally placed bracts that subtend cymosely branched partial inflorescences (fig. 1B). Terminal flowers have two bracteoles, which may subtend a first order lateral flower. The bracteoles of such a lateral flower may subtend higher order lateral flowers (fig. 3A–C).

Floral development – The flower primordium is relatively flattened and protected by two bracteoles (fig. 3D). Simultaneously, four free petal primordia and, alternating with them, four free stamen primordia appear (fig. 3E). The initial development of the corolla and androecium occurs at more or less the same rate (fig. 3C, E–H). While the stamens differentiate into filament and anther, and with both stamens and petals covering/enclosing the floral apex, two semi-circular bulges develop (fig. 3I). The central parts of the two semi-circular bulges develop into styles (fig. 4A, B) and, simultaneously, the rest of the bulges develop into two nectaries, surrounding the bases of the two styles (fig. 4C, D, F, H). At this stage, the filaments are free-standing on the hypanthium in between the semi-circular bulges and the corolla (fig. 4A–C).

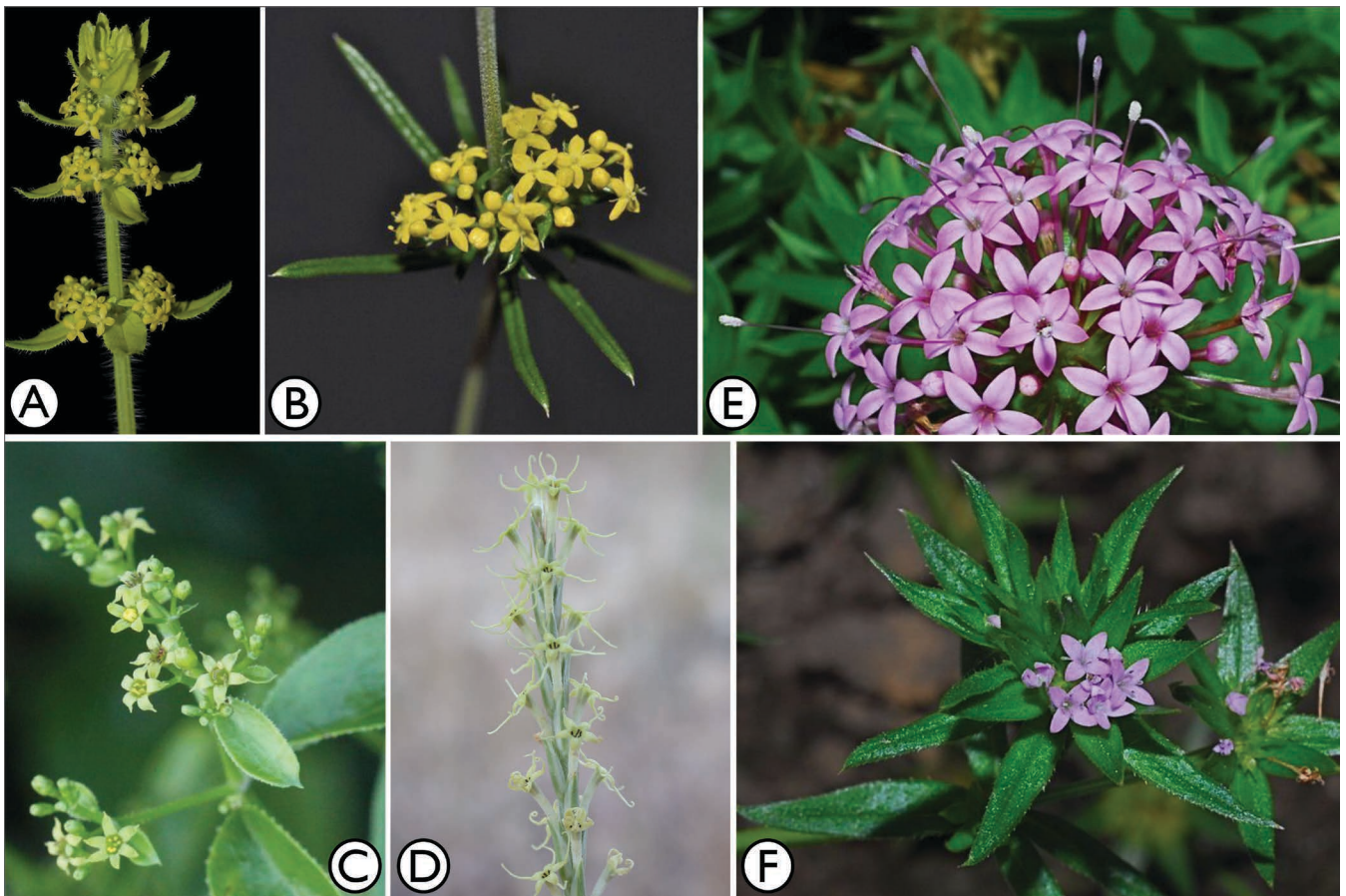


Figure 1 – Macrographs of inflorescences of species studied. A. *Cruciana laevipes*. B. *Galium verum*. C. *Rubia tinctorum*. D. *Cruciana macrostachya* with long tubular appendices at tips of corolla lobes. E. *Phuopsis stylosa* with protruding pollen presenters. F. *Sherardia arvensis*. Photographs: A, B, E, and F, photo library of Meise Botanic Garden; C, Peter L. Meininger; D, Gideon Pisanty (Gidip), https://commons.wikimedia.org/wiki/File:Cruciana_macrostachya_1.JPG, under the Creative Commons Attribution 3.0 Unported license.

Tetrasporangiate, introrse, and dorsifixed anthers are formed. At early developmental stages, the petals appear to be bladder-shaped (fig. 4C). In transverse sections, the abaxial epidermis of the petals appears to be separated from the inner tissue and adaxial epidermis (fig. 4A, C). Later in the floral development, the basal parts of the filaments and the petals appear fused, forming a tube (fig. 4F, G, J, K). The free parts of the petals are now called corolla lobes. Meanwhile, an inferior, bilocular, didymous ovary is formed, with a single ovule per locule (fig. 4D, E). At (semi-)maturity, each of the two short styles (conspicuously shorter than the filaments) has a spherical, papillose stigma (fig. 4E, F). Throughout the floral development, the styles remain free (fig. 4F, H–J). At (semi-)maturity, a short stamen-corolla tube, only a few cell layers thick, is visible below the tube formed by the fusion of petals and filaments (fig. 4H, I). Above the point where the filaments are free from the corolla, a short tube occurs formed by fusion of the margins of the corolla lobes (fig. 4G). In the stamen-corolla tube, no distinction can be made between the corolla tube and the ‘filaments’, which are only visible as vascular bundles (fig. 4H). More distally, the filaments adhere to the corolla tube but can be distinguished from it (fig. 4F, G, I–K). The total tubular corolla is short in comparison to the corolla lobes. At anthesis, the tubular part of the corolla forms a plate-like cup perpendicular to the longitudinal axis of the ovary, while the corolla lobes as well as the free parts of the stamens fold downwards (fig. 2J).

Rubia tinctorum

Inflorescence – The inflorescence units consist of cymosely branched flower clusters in the axil of a bract (fig. 1C). These subtending bracts are arranged in pairs in the distal part of the plant. Each flower cluster has a terminal flower and two reduced bracteoles. Each bracteole subtends a lateral flower with two bracteoles, which can subtend higher order lateral flowers (fig. 5A).

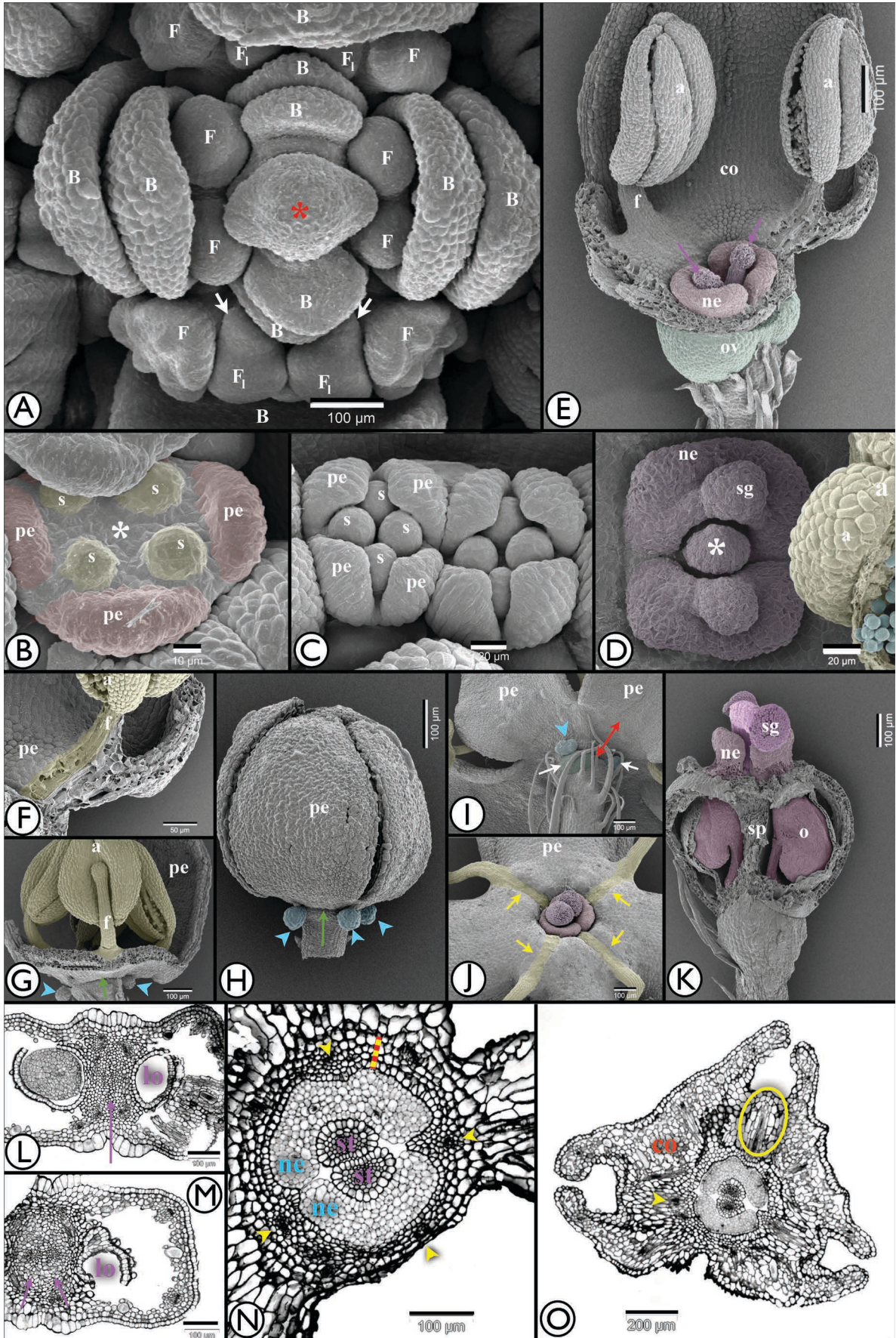
Floral development – With the appearance of (four) five petal primordia, the floral apex becomes slightly concave (fig. 5B, C). Subsequently, the petals develop and, alternating with them, an equal number of stamen primordia appears adaxially (fig. 5C–E). With the petals and stamens developing, two bulges, surrounding an elliptic cavity, arise in the centre of the flower (fig. 5F) and develop into semi-circular nectaries. The central parts of the bulges develop into two styles, covering and closing the central elliptic cavity (figs 5G, H; 6A, B), which corresponds to the early inferior ovary. Simultaneously, each stamen differentiates into a filament and a tetrasporangiate, introrse, dorsifixed anther (figs 5G, H; 6A, B, E). At this stage, the filaments are free-standing on the hypanthium in between the corolla and the developing nectaries (figs 5G, H; 6A, B). The fixation points of the filaments and the petals (now corolla lobes) are raised by the formation of a stamen-corolla tube below them (fig. 6C, F), in which no distinction can be made between corolla and filament tissue. More distally, just below the corolla lobes, the filaments adhere more loosely to the tubular corolla and become free at the height of the corolla lobes (fig. 6E, G, H).

Simultaneously, the ovary below the floral whorls increases in volume (fig. 6A–D). At the top of each style, a papillose, more or less spherical stigma develops, while the nectaries increase in size (fig. 6D). The tubular part of the corolla elongates, becoming proportionally much longer than the free part of the filaments (fig. 6E). At anthesis, the tubular part of the corolla is c. 3.5 mm long. The stamens are inserted in the throat and the free parts of the filaments are c. 1 mm long. The stigmas protrude c. 1 mm above the throat.

Crucianella macrostachya

Inflorescence – The compound inflorescence is an open spike of cymosely branched inflorescence units (fig. 1D). Each inflorescence unit, consisting of a single terminal flower and two bracteoles (fig. 7A, B), is subtended by a bract.

► **Figure 2** – SEM (A–K) of the floral development and LM (L–O) of transverse sections through a semi-mature flower in *Cruciata laevipes*. **A**. Apical view of inflorescence with, centrally, apex of main axis (red asterisk) and, below apex, pairs of decussate bracts. The distalmost bracts subtend massive primary flower primordia, which in lower pairs develop into two flowers per bract. These flowers (F) occur as terminal flowers with two lateral flower primordia (F_l), with in their turn two higher order lateral flower primordia (arrowed). **B–C**. Successive stages. **B**. Apical view of flat floral apex (white asterisk) with four petal primordia and, alternating with them, four stamens. **C**. Two flowers. **D**. Apical view of two semi-circular bulges surrounding floral apex (white asterisk), their central parts rising to form a style/stigma, the rest developing into semi-circular nectaries; with semi-mature anther on the right. **E–H**. Semi-mature flower. **E**. Longitudinally opened flower, showing stamens inserted on tubular corolla and, centrally, two short styles with spherical papillose stigmas (purple arrows), each style surrounded by semi-circular nectary; below, a didymous inferior ovary. **F**. Lateral view of filament and, below it, the zone where filament and corolla form a tube. **G**. Longitudinally opened flower, showing the nearly horizontal tubular part of corolla on which filaments are fixed; anthers dorsifixed, introrse and tetrasporangiate. **H**. Lateral view of corolla and calyx rim (green arrow). In between rim and corolla, four glands (three visible, blue arrowheads) occur alternating with corolla lobes. **I–K**. Flower at anthesis. **I**. View from below (tubular part of corolla indicated with red double arrow, calyx rim indicated with white arrows). **J**. Apical view of tubular part of corolla, surrounding nectaries and styles; filaments distinguishable in tubular part of corolla (yellow arrows). **K**. Longitudinally opened ovary, showing two locules separated by massive septum, each with single anatropous ovule. **L**. Section through middle part of ovary with septum showing original separation between carpels (purple arrow). **M**. Idem, somewhat higher, showing bases of styles (purple arrows). **N**. Section through semi-circular nectaries and four/five cell layers thick stamen-corolla tube (red-yellow dotted double arrow), with several vascular bundles, of which four are staminal (yellow arrowheads). **O**. Idem, somewhat higher, through extension of basal stamen-corolla tube, showing fusion zones of filament and petal (one encircled in yellow) and, on the left, staminal vascular bundle (yellow arrowhead). Abbreviations: a, anther; B, bract; co, corolla (lobe); f, filament; F, terminal flower (primordium); F_l, lateral flower (primordium); lo, locule; ne, nectary; o, ovule; ov, ovary; pe, petal; s, stamen; sg, stigma; sp, septum; st, style; red *, apex.



The bracts occur in decussate pairs and are fused at their bases (figs 7A; 8E, H). New bract pairs are continuously produced just below the apex of the spike (fig. 7A). The two flowers subtended by a pair of opposite bracts develop at the same rate and, consequently, have the same height (fig. 8H).

Floral development – Initially, the floral apex is convex and the two bracteoles are formed first (fig. 7B). Soon, the floral apex becomes slightly concave and five initially unequally developing petals appear on its rim (fig. 7C). Alternating with the petals and at the adaxial side of the rim of the concave floral apex, five stamen primordia appear (fig. 7C). Meanwhile, the petals develop, enclosing the centre of the flower (fig. 7C). Subsequently, the stamens differentiate into filaments and anthers. Simultaneously, the petals (now

corolla lobes) and stamens are raised by the development of a stamen-corolla tube below them (fig. 7D–F) and two styles develop from two semi-circular bulges surrounding the centre of the flower (fig. 7D). The stamen-corolla tube and corolla lobes elongate (fig. 7E, F). A small inferior bilocular ovary develops, separated from the corolla by a rim (calyx) that becomes more conspicuous during the further development of the flower (fig. 7E–G). The developing ovary becomes didymous. At the base of each of the two styles, the two semi-circular bulges (except for the central parts that become styles) develop into nectaries (fig. 7G), surrounding the bases of the styles (figs 7G; 8C). At the top of each of the styles, a spherical, papillose stigma appears. Both stigmas are in contact with the long cylindrical tips of the corolla lobes, which are bent inwards (fig. 8A, N). At this stage, the

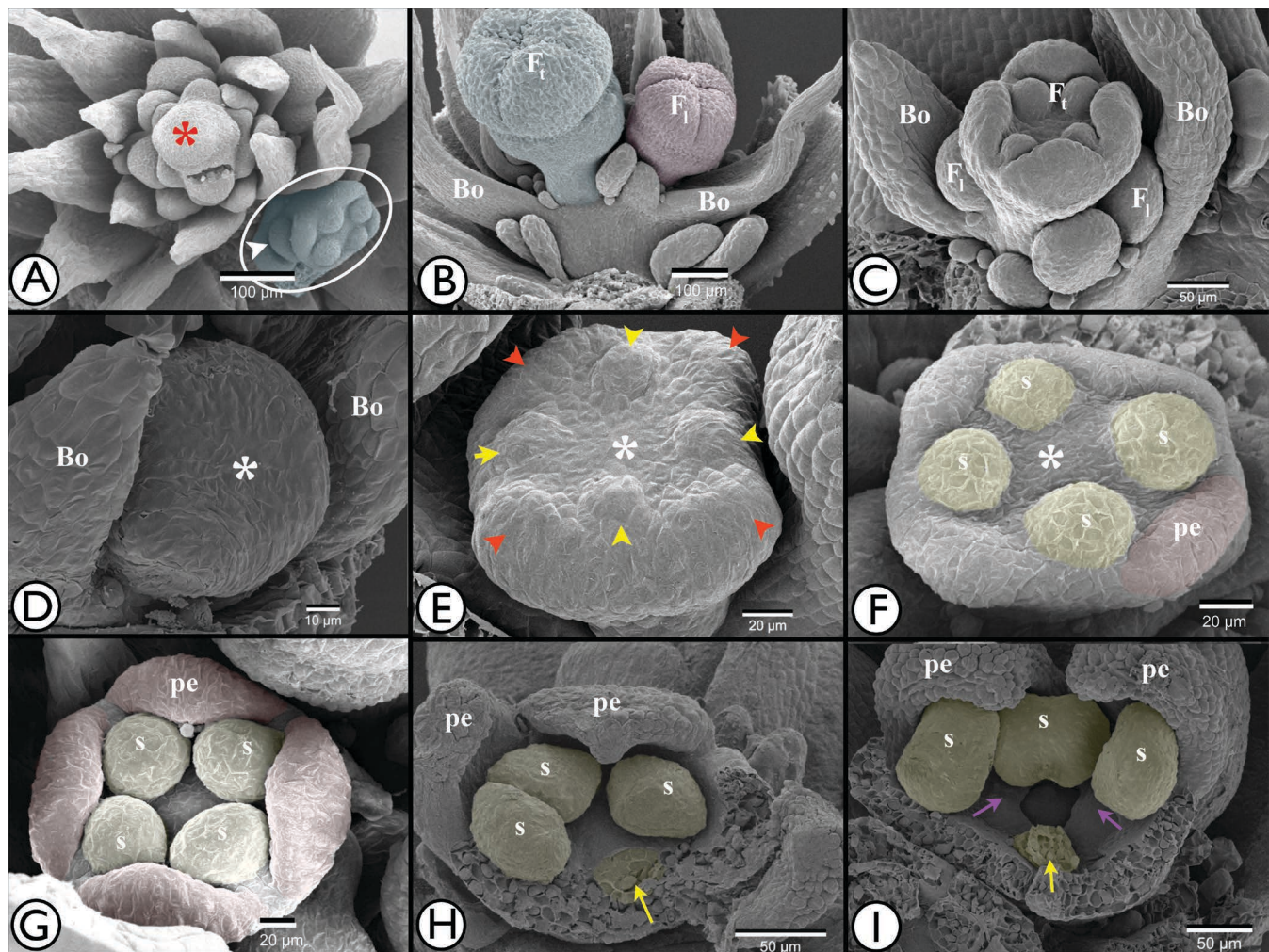


Figure 3 – SEM of the early floral development in *Galium aparine* (A, B, F, G), *G. glaucum* (C), and *G. verum* (D, E, H, I). **A.** Apical view of inflorescence with, centrally, apex of main axis (red asterisk) and, below apex, spirally placed bracts with inflorescence/flower primordia in their axils. Below right, inflorescence unit (encircled) consisting of developing terminal flower with bracteole subtending lateral flower primordium (white arrowhead). **B.** Terminal flower with two bracteoles, one subtending lateral flower. **C.** Lateral-apical view of developing terminal flower and cluster of flower primordia below it. **D–I.** Apical view of successive stages. **D.** Flower primordium with flat floral apex (white asterisk) and bracteoles. **E–F.** Floral apex (white asterisk) surrounded by four, nearly simultaneously appearing, petal primordia (red arrows) and, alternating with them, four stamen primordia (yellow arrows). In F, one petal is coloured. **G.** Petals and stamens developing. **H–I.** Scar of removed stamen (yellow arrow) on hypanthium. **H.** Floral apex flat. **I.** Stamens differentiate into anther and filament; floral apex situated in depression, surrounded by two semi-circular bulges (purple arrows). Abbreviations: Bo, bracteole; F_t, terminal flower; F_l, lateral flower; pe, petal; s, stamen; red *, axis apex; white *, floral apex.

filaments are inserted in between the bases of adjacent corolla lobes (fig. 8D). Each of the two locules, separated by a massive septum, contains a single anatropous, unitegmatic ovule (fig. 8C, N–P). One of the two styles grows faster than the other (fig. 8C, N). The stamens have short filaments and long dorsifixed, introrse anthers (fig. 8B–D, I–M). Below the insertion points of the filaments, no distinction can be made between filaments and tubular corolla; where filaments are expected, vascular bundles leading to the filaments are present (fig. 8I). At maturity, the tubular part of the corolla is approximately twice as long as the bracteoles (fig. 8E, F, H) and also continues somewhat above the insertion points of the filaments (fig. 8J, K), forming a short corolla tube *sensu stricto*. The corolla lobes are conspicuously shorter than the tubular part of the corolla (fig. 8G). The calyx consists only of a rim (fig. 8E) between the corolla and the ovary.

Phuopsis stylosa

Inflorescence – The inflorescence consists of a terminal spherical head of cymosely branched inflorescence units (fig. 1E). Each unit consists of a terminal flower with two bracteoles, each subtending a lateral flower, in their turn having two bracteoles (fig. 9D, K).

Floral development – Initially, the floral apex is slightly concave and more or less simultaneously, five free-standing petal primordia and, alternating with them, five free-standing stamen primordia originate (fig. 9A). Subsequently, petals (now corolla lobes) and stamens are raised by the development of an underlying stamen-corolla tube (fig. 9B–D, F, H).

Centrally in the flower, two semi-circular bulges originate, whose central parts develop into two styles that cover the underlying depression with the floral apex (fig. 9C–E), thus closing the early inferior ovary. The bases of the two styles fuse at an early stage (fig. 9F). The fused part subsequently elongates and develops into a long single style, topped by two very short free stigmatic lobes (fig. 9H, I). Below these, the upper part of the style becomes fusiform (c. 1 mm long at anthesis) with large and dome-shaped epidermal cells (fig. 9I). Meanwhile, the remaining parts of the two semi-circular bulges develop into two nectaries (fig. 9C, E, F, H). A bilocular, inferior ovary develops, each locule containing a single anatropous, unitegmatic ovule (fig. 9D–H).

The tubular part of the corolla (fig. 9H–L) and the style (fig. 9H, I) elongate at the same rate. At (semi-)maturity, the tubular part of the corolla consists of a long stamen-corolla tube below the insertion points of the filaments. Additionally, a short corolla tube *sensu stricto* develops above the insertion points of the filaments. Consequently, the stamens are inserted somewhat below, and not in between, the bases of the corolla lobes (fig. 9H, L). During the development of the corolla, the proportion of the tubular part above the insertion points diminishes relative to the part below (fig. 9H, L). At anthesis, the tubular part of the corolla is 9–10 mm long, with the portion above the insertion points of the filaments being approximately 1/10th of the total length. The filaments are inserted c. 1 mm below the level of the throat; the anthers are nearly sessile and almost completely included in the tubular part of the corolla. Until just before anthesis, the fusiform part of the style is situated at the same height as

the anthers (fig. 9H, I). At anthesis, the style has elongated and protrudes for approximately half of its length above the throat (fig. 1E).

Sherardia arvensis

Inflorescence – The inflorescence axes are terminated by a cymosely branched flower cluster consisting of a terminal flower with two bracteoles that each subtend a lateral flower (fig. 1F). Below the terminal flower, nodes occur with two opposite bracts that are shifted 90° with respect to the upper (and lower) node, each subtending a cluster of three flowers, a terminal flower and two lateral flowers (fig. 10A, B).

Floral development – Initially, the floral apex is flat (fig. 10C) and more or less simultaneously four free-standing petal primordia and, alternating with them, four free-standing stamen primordia appear (fig. 10D). The growing petals cover the stamens and the floral apex (fig. 10E). The stamens differentiate into filaments and tetrasporangiate, dorsifixed anthers (fig. 10F–H). Basally, a stamen-corolla tube is formed, raising the filaments to a higher position (fig. 10F, H).

At this stage, two semi-circular bulges originate, surrounding the floral apex, which now is situated in a cavity (fig. 10F, G). The central parts of the two bulges develop into two styles (figs 10G; 11A–C), the rest into semi-circular nectaries (fig. 11C, G), surrounding the base of the style. Simultaneously, a bilocular inferior ovary is formed (figs 10H; 11A, B). In each locule, a single ovule develops (fig. 11A). At the top of each style, a spherical, papillose stigma is formed (fig. 11A–C, E).

Meanwhile, irregular calyx lobes appear (fig. 11A) with large trichomes at their margins (fig. 11B, D, F). The stamen-corolla tube elongates, lifting up stamens and petals (now corolla lobes; fig. 10H; 11A–F). The filaments are inserted on top of the tubular part of the corolla and in between the bases of the corolla lobes (fig. 10H). This relative position of the insertion points of the filaments with respect to the corolla lobes does not change in the course of the further development. The proximal parts of the two styles fuse into a single style (fig. 11G, H), with their distal parts forming two style branches terminated by spherical stigmas (fig. 11E, I). At (semi-) maturity, the calyx lobes have large spine-like trichomes, the portion of the corolla corresponding to the stamen-corolla tube is longer than the corolla lobes, and the stamens protrude above the throat. The two style branches and stigmas protrude above all other flower parts (fig. 11F). The ovary is conspicuously didymous (fig. 11F). In the stamen-corolla tube, the filaments cannot be distinguished from the corolla; only vascular bundles leading to the filaments are present (fig. 11G, H).

DISCUSSION

After our earlier studies of tropical woody and herbaceous Rubiaceae (De Block & Vrijdaghs 2013; Vrijdaghs et al. 2015), we now focused on temperate species of the herbaceous tribe Rubieae (subfamily Rubioideae). We studied representatives of six (*Cruciata*, *Crucianella*, *Galium*, *Phuopsis*, *Rubia*, and *Sherardia*) out of the 12 genera in Rubieae *sensu* Bremer & Eriksson (2009), comparing our results with

those from the floral ontogenetic literature of Rubiaceae. We did not include *Theligonum cynocrambe* L. (Rutishauser et al. 1998), the flowers of which have particular but, to our study, irrelevant characteristics (e.g., unisexuality, polyandry). Because of the derived nature of the flowers of *Theligonum* (Robbrecht 1993; Rutishauser et al. 1998), which raises floral ontogenetic questions that are far from the focus of the present study, we preferred to exclude comparison with the literature about *Theligonum*.

We test the hypothesis of Vrijdaghs et al. (2015) about the development of a tubular corolla and epipetaly in Rubiaceae. Because of the epigynous nature of the flowers in Rubiaceae, the development of the calyx is also considered.

Development of the calyx

The usual floral developmental sequence is: calyx – corolla – androecium – gynoecium. However, in *Crucianella*, *Cruciata*, *Galium*, *Phuopsis*, and *Rubia*, no calyx tube or calyx lobes develop: only a calyx rim appears between the inferior ovary and the corolla late in the floral development. Payer (1857) even questioned whether the rim without calyx lobes is actually a calyx. In contrast, *Sherardia* does have a calyx, consisting of a short tube and conspicuous lobes (fig. 11A–D, F). The four calyx lobes only develop after all inner floral whorls and the inferior gynoecium have been formed (fig. 10H), which suggests a developmental delay of the calyx. This is in contrast to *Canephora* Juss. (subfamily Ixoroideae) and *Paederia*, *Pentas*, *Pentodon*, *Sacosperma*, and *Spermacoce* (subfamily Rubioideae), where a tubular calyx with calyx lobes originates before or more or less simultaneously with the corolla (De Block & Vrijdaghs 2013; Vrijdaghs et al. 2015).

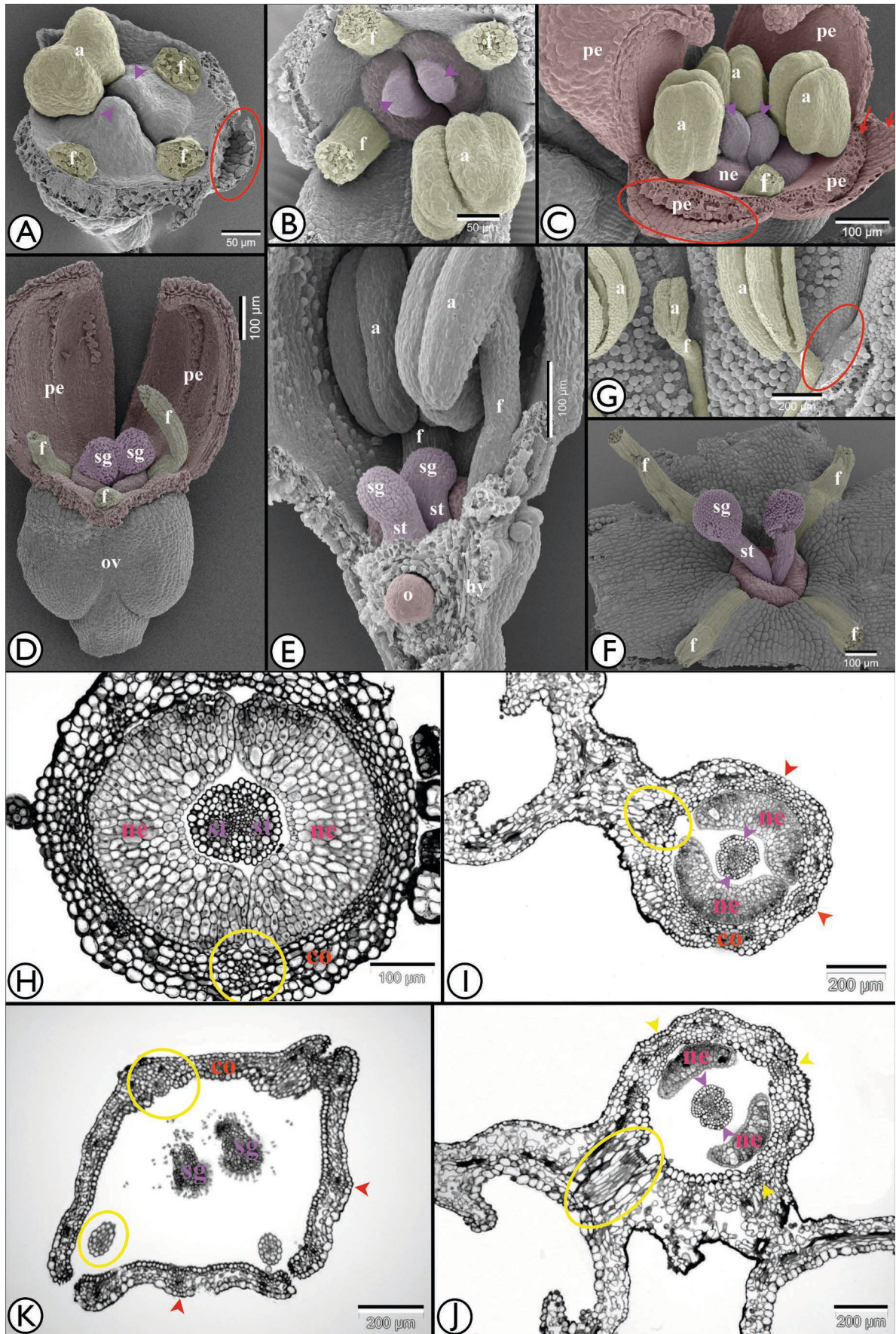
Postgenital fusion or annular underlying meristems?

Payer (1857) explained the development of a tubular corolla and epipetaly in *Asperula*, *Galium*, and *Rubia* as the result of postgenital fusion, whereas De Block & Vrijdaghs (2013) and Vrijdaghs et al. (2015) considered underlying annular intercalary meristems *sensu* Leins & Erbar (2010) as the cause of the development of floral cups. We discuss the contribution of the three processes suggested by Vrijdaghs et al. (2015) and the influence of plastochrons towards the development of the corolla and androecium.

Development of a stamen-corolla tube – A stamen-corolla tube is an example of a floral cup *sensu* Leins & Erbar (2010), originating from a circular intercalary meristem situated below one or more floral whorls. During the development of the floral cup from the underlying intercalary meristem, the whorl(s) in question are raised. In the case of a stamen-corolla tube, there is an underlying annular intercalary meristem below corolla and androecium. With the development of the stamen-corolla tube, petals and stamens are raised to a higher position. The correct interpretation of a stamen-corolla tube may be blurred by a temporal delay of the development of the tube. In some cases, the stamen-corolla tube starts developing at very early floral developmental stages, resulting in an initially conspicuously concave floral apex. Next, the petal primordia (corolla lobes) appear on the rim of the concave floral apex, followed by the stamen primordia, which appear on the adaxial side of the rim of the concave floral apex and alternate with the corolla lobes (e.g., *Ixora coccinea* L. in Erbar & Leins 1996; *Galopina tomentosa* Hochst. in Ronse Decraene & Smets 2000; *Spermacoce occultiseta* Harwood in Vrijdaghs et al. 2015). This corresponds to Erbar's (1991) "early sympetaly".

Among the species studied here, only the floral development of *Crucianella macrostachya* and *Phuopsis stylosa* (figs 7; 9; 12) concurs with early sympetaly. In *Rubia tinctorum* and *Sherardia arvensis*, our observations suggest that a stamen-corolla tube is formed with some delay, which explains why initially free-standing petal and stamen primordia appear on a rather flat floral apex. However, fig. 5B and Naghiloo & Classen-Bockhoff (2016: 54, figure 3A–C) suggest that the petals are standing on an annular ring, which we think is the undeveloped stamen-corolla primordium. Erbar (1991) recognised that there are transition patterns between early and late sympetaly and, in our opinion, these correspond to cases where the development of a stamen-corolla tube is delayed. Payer (1857) explained the development of the corolla tube in *Rubia* by postgenital fusion, as, in his time, the idea of floral structures originating from underlying intercalary meristems was unknown. However, we have no indication of such fusion (figs 6F–H; 11G–I). Therefore, we prefer to explain the tubular part of the corolla and the epipetaly in *Rubia* and *Sherardia* by a delayed development of a stamen-corolla tube (figs 5; 10; 12).

► **Figure 4** – SEM (A–G) of the floral development and LM (H–K) of transverse sections through a flower at anthesis at different heights in *Galium glaucum* (A, C), *G. odoratum* (B, E, G), *G. verum* (D, F, K), and *G. pumilum* (H–J). **A–D**. Early successive floral developmental stages. **A–B**. Apical view, three out of four stamens removed; stamens free-standing between corolla and carpels (corolla lobe encircled in red); anthers tetrasporangiate and introrse; centrally, two carpels (purple arrowheads) covering floral apex, each developing into semi-circular nectary and independent style. **C–D**. Lateral view. **C**. Petals bladder-shaped (encircled and red arrows); anthers dorsifixed; spherical papillose stigmas (purple arrows) developing. **D**. Short tube below corolla lobes and free parts of filaments; inferior, bilocular ovary. **E**. Longitudinally opened flower, revealing single ovule per locule. **F–G**. Tubular zone of adherence between bases of distinguishable filaments and corolla lobes. **H**. Short stamen-corolla tube of six to seven cell layers thick; four staminal vascular bundles (one encircled in yellow) and tubular corolla forming continuous circular tissue; semi-circular nectaries surrounding two free styles. **I–J**. Inclined sections at successively higher levels. **I**. Filaments distinguishable from adaxial surface of tubular corolla (encircled in yellow). **J**. Fusion zone between basal parts of two petals and one filament (encircled in yellow); staminal vascular bundles indicated with yellow arrowheads. **K**. Inclined section in transition zone between tubular corolla and corolla lobes (red arrowheads); filament tissue distinguishable from corolla (encircled in upper part of picture); at level of corolla lobes, filaments free (one encircled left below). Abbreviations: a, anther; co, corolla (lobe); f, filament; hy, hypanthium; o, ovule; ov, ovary; ne, nectary; pe, petal; sg, stigma; st, style.



In *Cruciata* and *Galium*, our results suggest that a stamen-corolla tube of a few cell layers thick is formed quite late in the floral development (figs 2N, O; 4G–I). Naghiloo & Classen-Bockhoff (2016: 55, 56) presented slightly younger floral developmental stages in *Cruciata laevipes* and *C. glabra* in their figures 4A and 5A, B. In both stages, as well as in the slightly later stage shown in fig. 2B, no annular rim is visible. Our observations partially concur with those of Pötter & Klopfer (1987: 312) in *Galium aparine*, who reported that “Im weiteren Verlauf differenziert sich unter den Petalprimordien ein Meristemwulst und verbindet sie miteinander...” [“In the further course of the floral development, an annular meristem develops below the petal primordia and links them...”]. These authors interpreted this as an example of “extremely early sympetaly of the corolla”. However, since individual petal and stamen primordia are present at the early

developmental stages, we think that the annular meristem not only underlies the corolla but also the androecium, resulting in a stamen-corolla tube later in the floral development. Erbar & Leins (1996: 105) compared the initial floral developmental stages in species without a calyx (*Cruciata*, *Galium*, *Phuopsis*, and *Asperula*), stating that the flower primordium changes its shape from “initially hemispherical via plug-like to funnel-like”, which they considered to be the first steps in the formation of the corolla tube. Based on our results, we interpret it as a stamen-corolla tube. In *Cruciata* and *Galium*, however, a large tubular part of the corolla is present at the top of the rudimentary stamen-corolla tube. This part of the tubular corolla results from a postgenital fusion/coherence of the bases of the filaments and the corolla lobes (figs 2F, G, J, O; 4F, G, I; 12E, F). The coherence of the filaments to the

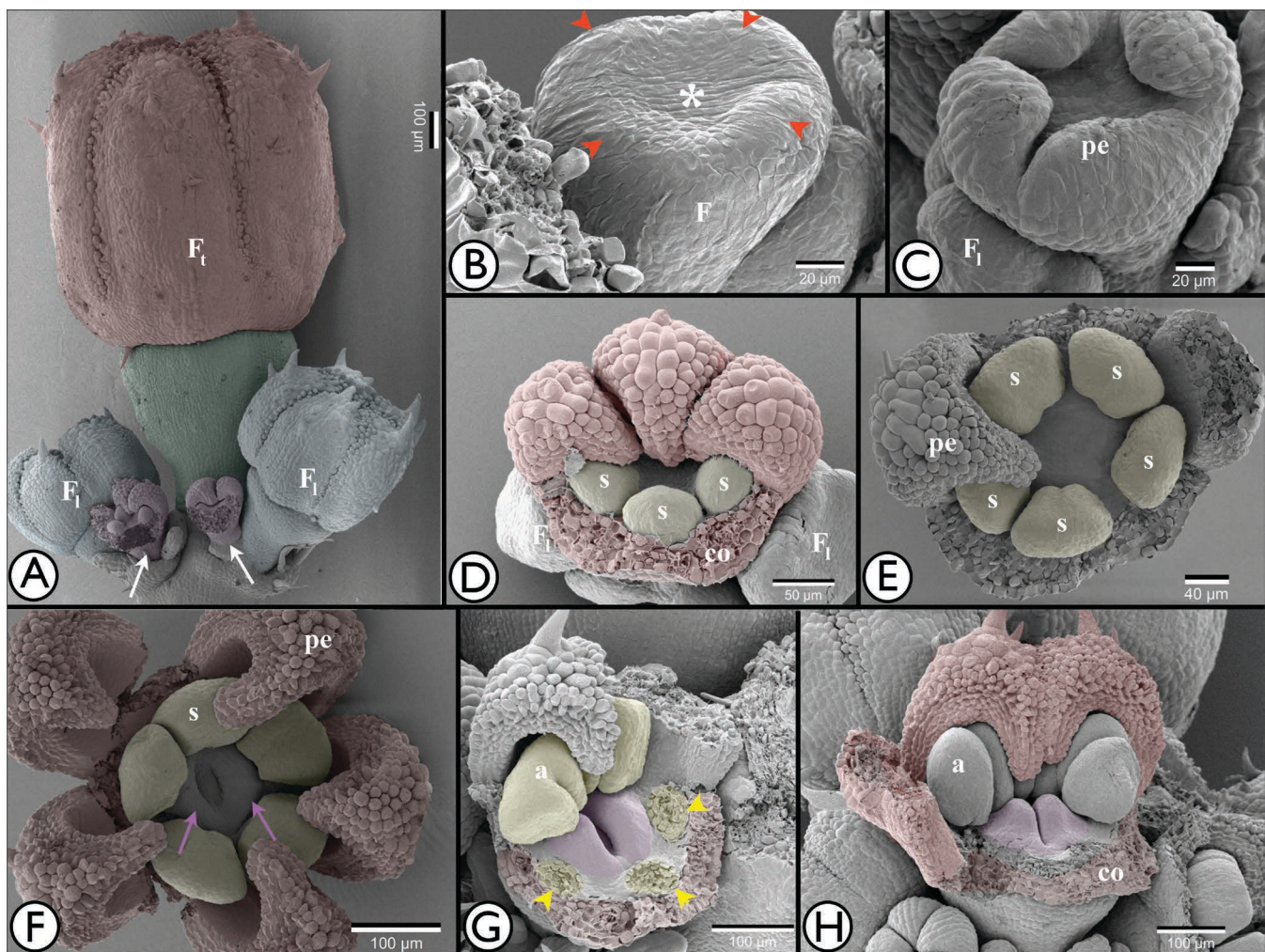


Figure 5 – SEM of the early floral development in *Rubia tinctorum*. **A**. Lateral view of cymosely branched flower cluster; reduced bracteoles of terminal flower (F_t) subtending first order lateral flowers (F₁) with two second order lateral flowers (arrowed). **B–G**. Apical view of successive stages. **B**. Slightly concave floral apex (white asterisk) and four petal primordia (red arrowheads). **C**. Four developing petals. **D–G**. Five developing petals and, alternating with them, five stamens differentiating into filament and anther. **D**. Petals covering floral apex. **E**. Floral apex flat. **F**. Floral apex situated in depression, surrounded by two semi-circular bulges (purple arrows). **G**. Three out of five corolla lobes and stamens removed; filament scars (yellow arrowheads) situated in between basal parts of petals and developing nectaries; anthers tetrasporangiate and introrse. **H**. Longitudinally opened flower (somewhat older than G) with semi-circular bulges developing into styles and nectaries. Abbreviations: a, anther; co, corolla (lobe); F, flower primordium; F₁, lateral flower; F_t, terminal flower; pe, petal; s, stamen; white *, floral apex.

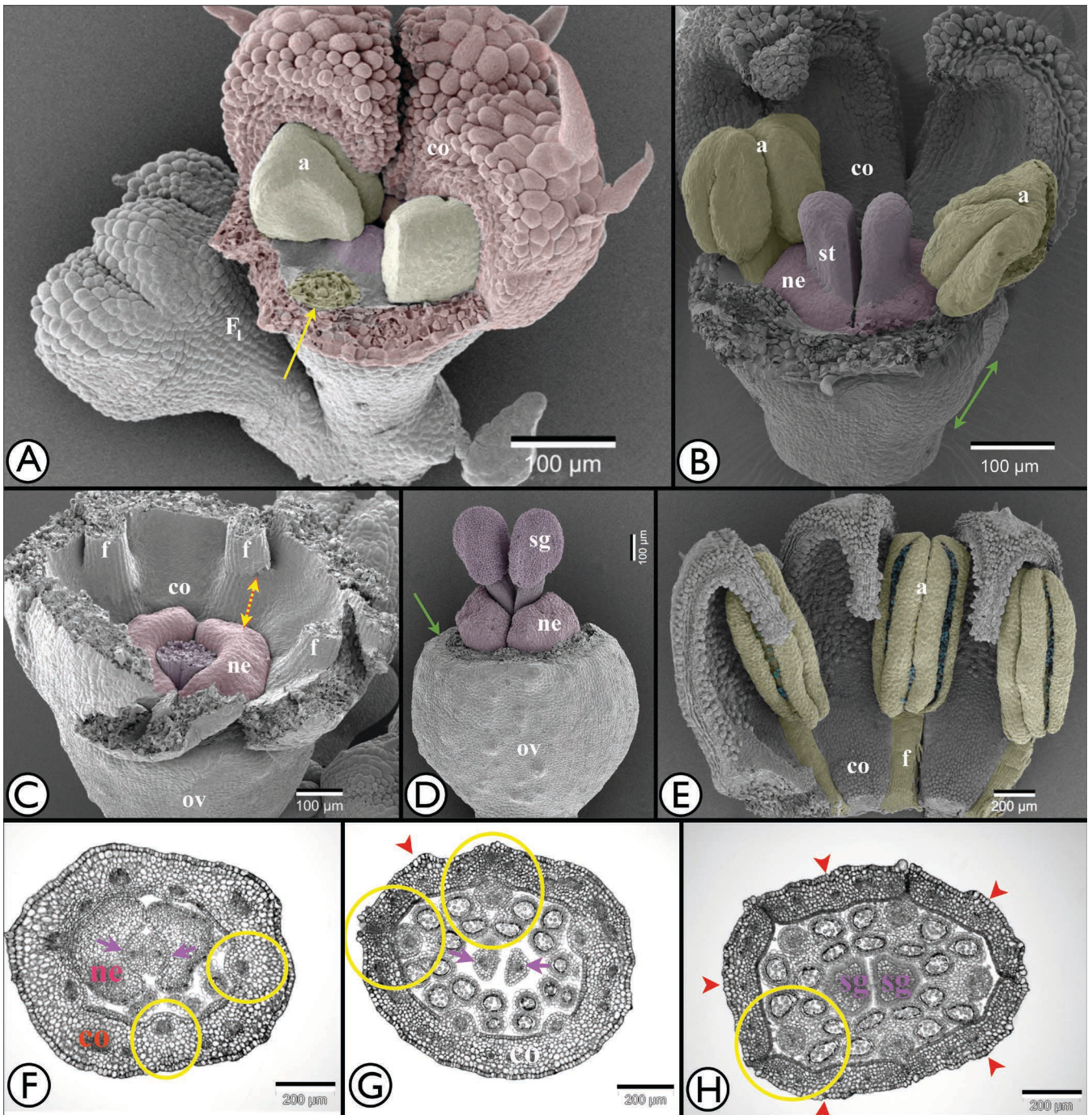


Figure 6 – SEM (A–E) of developing flowers and LM (F–H) of transverse sections through (semi-)mature flowers of *Rubia tinctorum*. A–C. Lateral-adaxial view of developing pentamerous flower with part of corolla and stamens removed. A. Scar (coloured, yellow arrow) of filament in between corolla and early gynoecium. B. Two developing styles, each surrounded by semi-circular nectary; below, developing hypanthium (green double arrow). C. Tubular corolla (red-yellow dotted double arrow) developing below petals and insertion points of filaments, lifting them up. D. Lateral view of gynoecium; calyx rim (green arrow) in between inferior ovary and scar of corolla; two semi-circular nectaries surrounding bases of styles, each with spherical, papillose stigma. E. Longitudinally opened semi-mature corolla; filaments distinguishable in tubular part of corolla; anthers tetrasporangiate and introrse. F. Stamen-corolla tube; staminal vascular bundles (two encircled in yellow) and tubular corolla forming continuous circular tissue; styles arrowed in purple. G. Slightly inclined section at height of throat; two free filaments (encircled) adjacent to only visible corolla lobe (red arrowhead); three staminal vascular bundles; styles arrowed in purple. H. Corolla lobes (red arrowheads); filaments all free (one encircled in yellow). Abbreviations: a, anther; co, corolla (lobe); f, filament; F₁, lateral flower; ne, nectary; ov, ovary; sg, stigma; st, style.

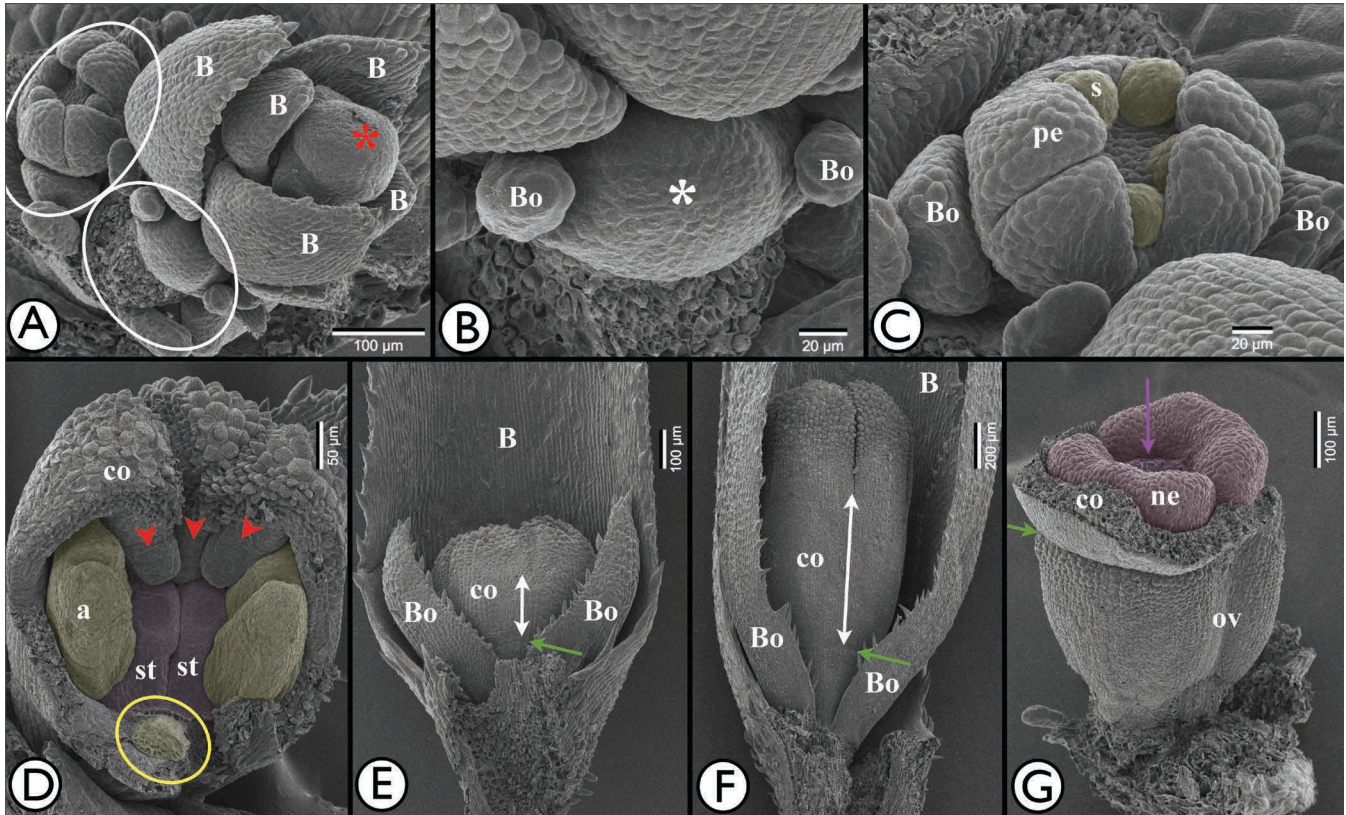
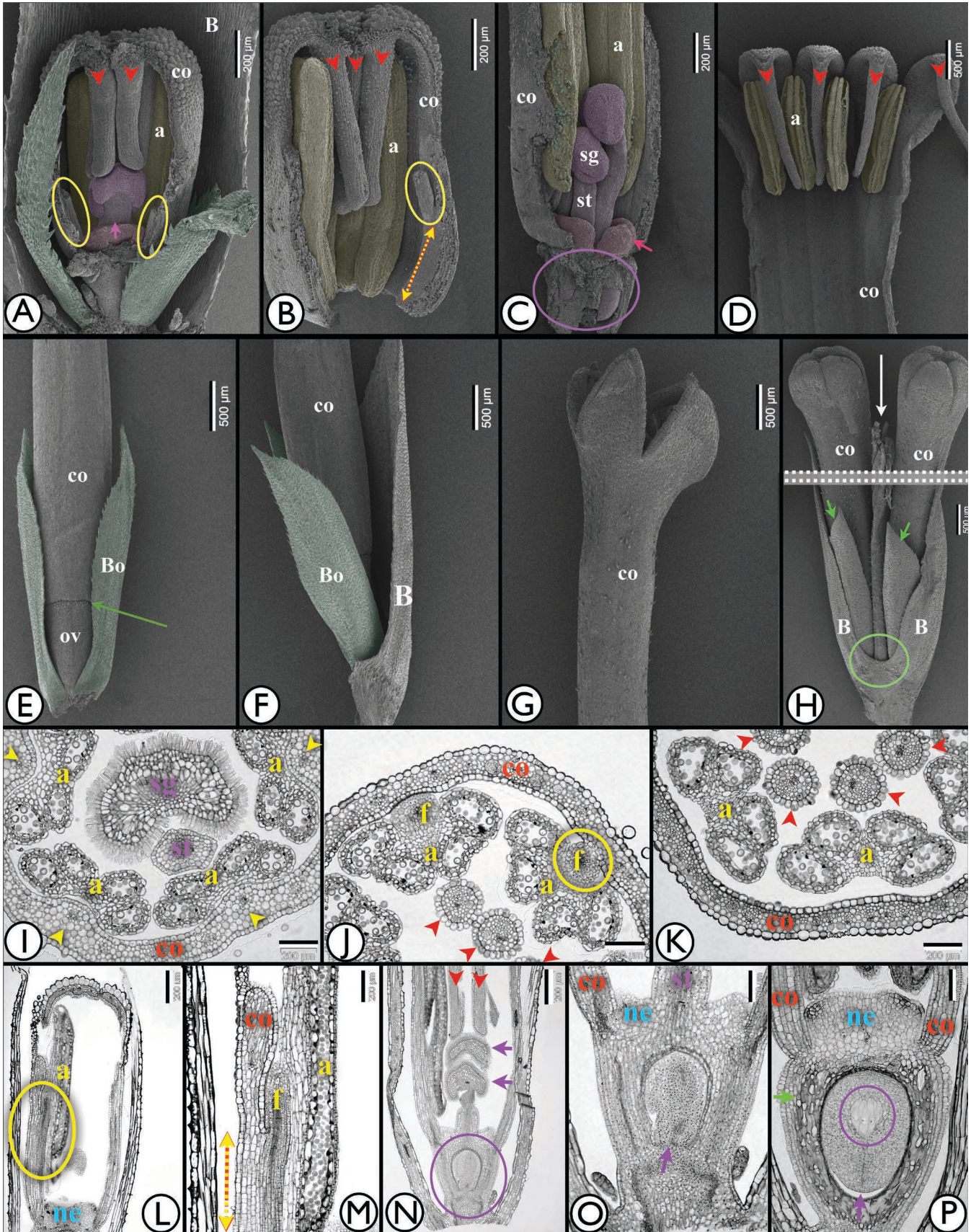


Figure 7 – SEM of the floral development in *Crucianella macrostachya*. **A**. Developing inflorescence axis with indeterminate apex (red asterisk) and several pairs of decussate bracts at different developmental stages; proximally, (removed) bracts subtend developing inflorescence units (two encircled). **B–C**. Apical view. **B**. Inflorescence unit consisting of flower primordium and two bracteoles; floral apex convex (white asterisk). **C**. Five slightly unequally developing petals and, alternating with them, four out of five stamen primordia, surrounding the now somewhat concave floral apex. **D–G**. Lateral view. **D**. Two stamens with developing tetrasporangiate, introrse anthers; scar of filament (encircled) situated basally on tubular part of corolla; tips of corolla lobes (red arrowheads) bent inwards touching the two stigmas. **E–F**. Successive developmental stages; elongating tubular corolla (white double arrow) and lobes; calyx rim (green arrow) in between inferior ovary and corolla. **G**. Bilocular, inferior ovary; two semi-circular nectaries surrounding scar of removed styles (purple arrow); calyx rim (green arrow) more conspicuous. Abbreviations: a, anther; B, bract; Bo, bracteole; co, corolla (lobe); ne, nectary; ov, ovary; pe, petal; s, stamen; st, style; red *, apex of the inflorescence axis; white *, floral apex.

► **Figure 8** – SEM (A–H) of the later floral development and LM of transverse (I–K) and longitudinal (L–P) sections of (semi-)mature flowers in *Crucianella macrostachya*. **A–H**. Lateral view. **A**. Longitudinally opened flower; tips of corolla lobes developing into long cylindrical structures (red arrowheads), curved inwards and touching upper part of stigmas; two insertion points of filaments on basal tubular part of corolla encircled in yellow; one of two styles with papillose stigma (purple arrow). **B**. Longitudinally opened corolla; filament (encircled in yellow) inserted at top of stamen-corolla tube (red-yellow dotted double arrow). **C**. Successive stage; two styles differing in length, each surrounded by semi-circular nectary (purple arrow); below, two partially opened locules separated by massive septum (encircled in purple), each with single ovule. **D**. Upper part of elongated stamen-corolla tube, showing equally long anthers and cylindrical tips of corolla lobes (red arrowheads); staminal vascular bundles forming longitudinal thickenings in stamen-corolla tube. **E**. Flower with two bracteoles; calyx rim (green arrow) in between inferior ovary and corolla. **F**. Idem as E, perpendicular orientation, showing bracteoles and bract subtending flower. **G**. Upper half of semi-mature flower. **H**. Node and part of inflorescence axis (white arrow) with two opposite bracts, fused at base (encircled in green); each bract subtending semi-mature flower with two bracteoles (green arrows). **I**. Stamen-corolla tube; staminal vascular bundles (yellow arrowheads) and tubular corolla forming continuous circular tissue; anthers dorsifixed, tetrasporangiate and introrse; stigmatic lobes covered by large papillae. **J**. Corolla tube *sensu stricto* at the height of insertion points (encircled in yellow) of filaments; filament at the left free from corolla; centrally, circular sections of tips of corolla lobes (red arrowheads). **K**. Corolla tube *sensu stricto* above insertion points of filaments. **L**. Upper part of developing flower; filament inserted at top of stamen-corolla tube (encircled in yellow). **M**. Detail of encircled part of L; stamen-corolla tube indicated by red-yellow dotted double arrow. **N**. Developing flower; centrally, styles/stigmas with different lengths (purple arrows); cylindrical tips of corolla lobes (red arrowheads) touching highest stigma; below, locule with developing anatropous unitegmic ovule (encircled in purple). **O**. Detail of encircled part of N, with micropyle (arrow). **P**. Micropyle (purple arrow) and embryo sac (encircled); hypanthium consisting of outer and inner layer (green arrow). Abbreviations: a, anther; B, bract; Bo, bracteole; co, corolla (lobe); f, filament; ne, nectary; ov, ovary; sg, stigma; st, style.



corolla gradually becomes looser towards the distal part of the tube, eventually resulting in free filaments.

Development of a corolla tube *sensu stricto* – A corolla tube *sensu stricto* is the part above the insertion points of epipetalous stamens, or, in case the stamens are inserted on the hypanthium, is the tubular portion of the corolla. The latter case is not present in the species studied here. The presence of a corolla tube *sensu stricto* above a stamen-corolla tube results in the insertion of the epipetalous stamens below the level of the throat. This can be seen in *Phuopsis stylosa* (figs 9H; 12C) and, to a lesser degree, in *Crucianella macrostachya* (figs 8J, K; 12C). In the other species studied, no corolla tube *sensu stricto* was observed.

Postgenital fusion – We follow Endress (2006; 2019), who equated postgenital fusion to epidermal fusion after the independent origin of the fusing structures. In contrast, postgenital coherence may also result from a fusion of parts of floral organs through the secretion of mucus (Endress 2006). Postgenital fusion/coherence between the proximal parts of the petals and the filaments was only observed in *Cruciata* and *Galium*. It occurs in combination with a late appearance of a rudimentary stamen-corolla tube (figs 2F, G, J, N, O; 4G, F, I–K; 12E, F), which is only visible using LM because the stamen-corolla tube is only a few cell layers thick. In contrast, the part of the tubular corolla resulting from postgenital coherence is considerable, which explains that the insertion of the free parts of the filaments in the tubular corolla is high above the stamen-corolla tube. This suggests a kind of pseudo-epipetaly (figs 2E–G, J; 4F, G, J; 12E, F) corresponding to ‘false sympetaly’ *sensu* Weberling (1992), albeit that the fusion occurs simultaneously between members of the same whorl, such as petals, and members of another, adaxially situated whorl, for example the filaments. In *Galium*, above the insertion points of the filaments, the tubular fusion zone continues with a short tube formed by the fused margins of the corolla lobes (fig. 4G), which suggests a pseudo-corolla tube *sensu stricto*.

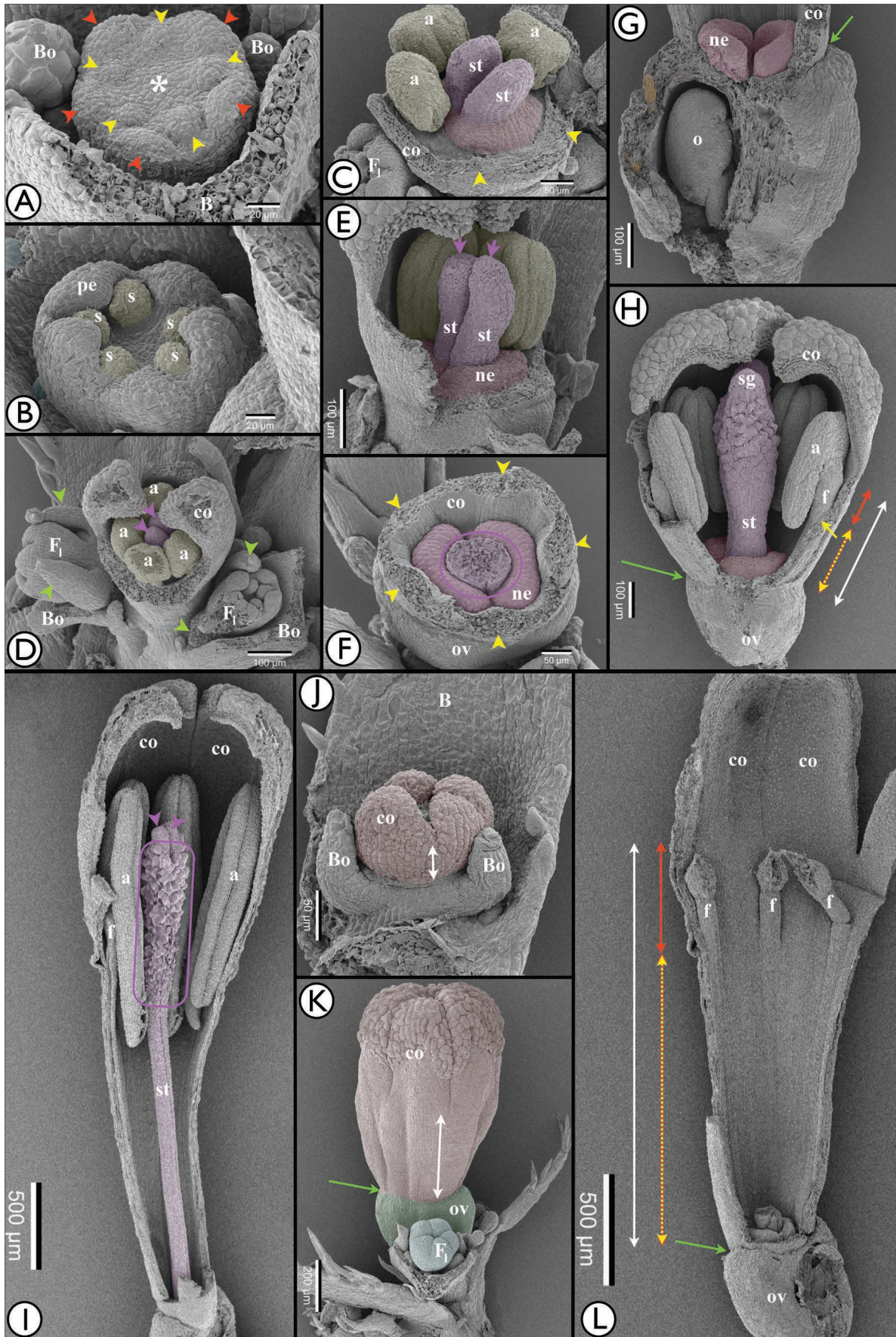
Plastochron variation

Extending the definition of the term plastochron “the time interval between the successive similar developmental events” of Henderson’s dictionary of biological terms (Lawrence 1996: 446) to floral apices, we define a plastochron as the time interval between the successive appearances of two floral organs or of two floral whorls (assuming that the floral organs in a whorl originate simultaneously).

Several examples of the influence of plastochrons in the floral development can be given: 1) Payer (1857) already interpreted the quasi absence of a calyx in *Asperula*, *Galium*, and *Rubia* as a delay of the calyx development (however, also questioning whether there is a calyx at all). The appearance of a rudimentary calyx very late in the floral development can be considered as a negative plastochron with respect to the corolla/androecium (sequence switch of appearance of corolla/androecium and calyx). The delayed development of the calyx in *Sherardia* can also be considered as an example of a negative plastochron. 2) The more or less simultaneous appearance of petal and stamen primordia in *Cruciata*, *Galium*, *Phuopsis*, and *Sherardia* suggests a reduction of the plastochron between corolla and androecium. This was also observed for *Cruciata laevipes* and *C. glabra* (Naghiloo & Classen-Bockhoff 2016). The simultaneous initiation of the corolla and androecium may be a first step towards the development of a stamen-corolla tube or may be a remnant of a stamen-corolla tube that was lost in the course of evolution, unless postgenital fusion of initially free filaments and petals occurs as in *Cruciata laevipes* and *Galium*. 3) We interpret the late development of a stamen-corolla tube in *Cruciata* and *Galium* as the result of a large plastochron between the more or less simultaneous appearance of free petals and stamens (figs 2; 3) and the initiation of the stamen-corolla tube (figs 2N; 4H, I).

Another example of the importance of plastochrons is the andromonoecy in *Cruciata glabra*, explained by a larger than usual plastochron between the initiation of the corolla/androecium and the gynoecium (Naghiloo & Classen-Bockhoff 2016).

► **Figure 9** – SEM of the floral development in *Phuopsis stylosa*. **A–D**. Apical view of successive developmental stages. **A**. Young flower with two bracteoles; five free-standing petal primordia (red arrowheads) and, alternating with them, five stamen primordia (yellow arrowheads), surrounding slightly concave floral apex (white asterisk). **B**. Developing petals and stamens; floral apex concave. **C**. Scar of stamen-corolla tube with two staminal vascular bundles (yellow arrowheads); centrally, two semi-circular bulges developing into nectaries, their central parts forming two styles. **D**. Terminal flower with two bracteoles (Bo), each subtending lateral flower (F_l); each lateral flower with two second order bracteoles (green arrowheads); stamens inserted on adaxial surface of stamen-corolla tube, differentiating into filament and anther; centrally, two developing styles (purple arrowheads). **E**. Successive stage; longitudinally opened flower; stigmas appearing at top of styles (purple arrowheads). **F**. Apical view of base of stamen-corolla tube with five staminal vascular bundles (yellow arrowheads); centrally, semi-circular nectaries surrounding scar of common base of fused styles (encircled in purple). **G–L**. Lateral view. **G**. Bilocular, inferior ovary with one locule opened, showing single anatropous ovule; calyx rim (green arrow) in between inferior ovary and corolla. **H**. Longitudinally opened flower; tubular part of corolla (white double arrow) consisting of stamen-corolla tube (red-yellow dotted double arrow), and, above insertion points of filaments (yellow arrow), corolla tube *sensu stricto* (red double arrow); anthers dorsifixed, tetrasporangiate and introrse; pollen presenter starting to differentiate. **I**. Longitudinally opened flower at later stage; pollen presenter (encircled in purple) positioned in between anthers; stigmas indicated by purple arrowheads. **J–L**. Elongation of tubular part of corolla (white double arrow) and corolla lobes. **J–K**. Abaxial view, successive stages; calyx rim (green arrow) appearing after development of inferior ovary (coloured green). **L**. Longitudinally opened flower; tubular part of corolla (white double arrow) consisting of long stamen-corolla tube (red-yellow dotted double arrow), and, above insertion points of filaments, shorter corolla tube *sensu stricto* (red double arrow). Abbreviations: a, anther; Bo, bracteole; co, corolla (lobe); f, filament; F_l, lateral flower; ne, nectary; o, ovule; ov, ovary; pe, petal; s, stamen; sg, stigma; st, style; white *, floral apex.



Gynoecial development

In all species studied, the development of the gynoecium concurs with the description of Payer (1857), although we interpret the carpels *sensu* Endress (2019). Moreover, with regard to the development of the styles, we need to nuance Payer's (1857: 634) statement that the initial, semi-circular bulges "immediately become connate". This is not the case in *Crucianella macrostachya*, *Cruciata laevipes*, and *Galium*, where the two styles remain separate throughout the floral development (figs 2J, M–O; 3I; 4A–I, F; 7D; 8C, I, J). In flowers of *Rubia tinctorum*, the two styles become loosely fused at the base (fig. 6F), and, in *Phuopsis stylosa* and *Sherardia arvensis*, the bases of the two styles fuse postgeni-

tally early in the development to further develop into a single style, with distally two style branches (figs 9F, I; 11F–H).

The style of *Phuopsis stylosa* differs from that of all other Rubieae studied in that the distal part, just below the small stigma lobes, develops into a pollen presenter (figs 1E; 9H, I). *Phuopsis stylosa* is the only species in the tribe Rubieae and even in the subfamily Rubioideae showing secondary pollen presentation (Puff et al. 1996), a reproductive strategy promoting outbreeding that involves protandry. Protandry is a form of dichogamy (the separation in time of gender expression in a bisexual flower) in which the anthers release the pollen before the stigma becomes receptive. Pollen are relocated from the anthers onto a pollen presenter, in *Phuopsis stylosa* in the form of the swollen distal part of the style with

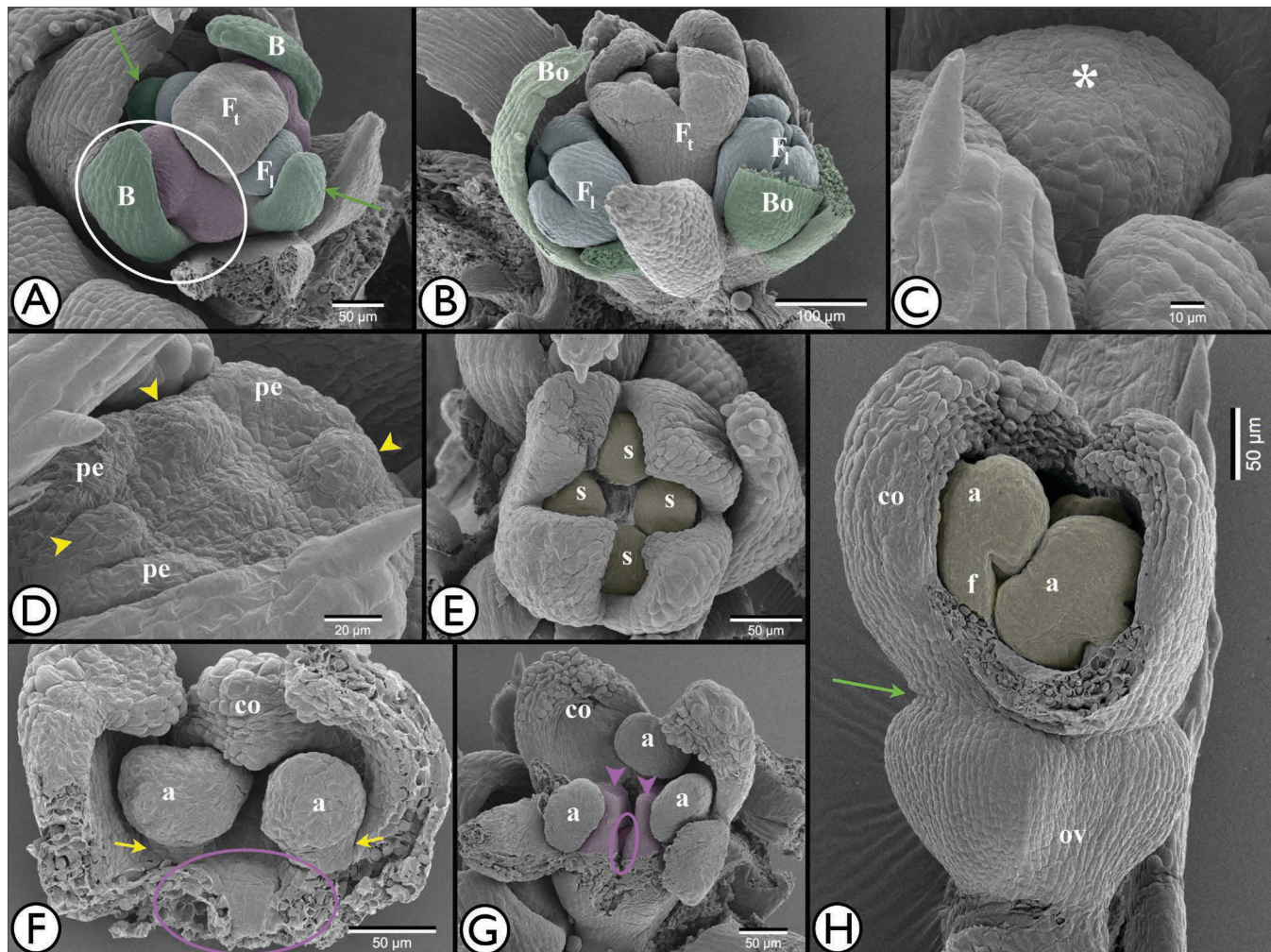


Figure 10 – SEM of the early floral development in *Sherardia arvensis*. **A**. Cymosely branched inflorescence with decussate bracts, consisting of terminal flower (F_t) with two bracteoles (green arrows), each subtending lateral flower (F_l); proximally, bracts subtending cymosely branched, primordial inflorescence units (one encircled in white). **B**. Idem as A, lateral view, later stage. **C–H**. Successive stages. **C**. Flower primordium with flat floral apex (white asterisk). **D**. Four free-standing petal primordia and, alternating with them, four free-standing stamen primordia (yellow arrowheads). **E**. Developing petals and stamens. **F**. Longitudinally opened flower, perpendicular orientation to G and corresponding to section in between semi-circular bulges, but at younger stage; bulges just appearing, only one visible and encircled; floral apex situated in depression in between bulges; filaments inserted on early stamen-corolla tube (yellow arrows). **G**. Floral apex enclosed by semi-circular bulges (at 90° with respect to F; purple arrowheads), differentiating into nectaries and styles. **H**. Lateral view of subsequent stage; filaments inserted on stamen-corolla tube; stamens differentiating into filaments and anthers; calyx rim (green arrow) in between inferior ovary and corolla. Abbreviations: a, anther; B, bract; Bo, bracteole; co, corolla (lobe); f, filament; F_l , lateral flower; F_t , terminal flower; ov, ovary; pe, petal; s, stamen; white *, floral apex.

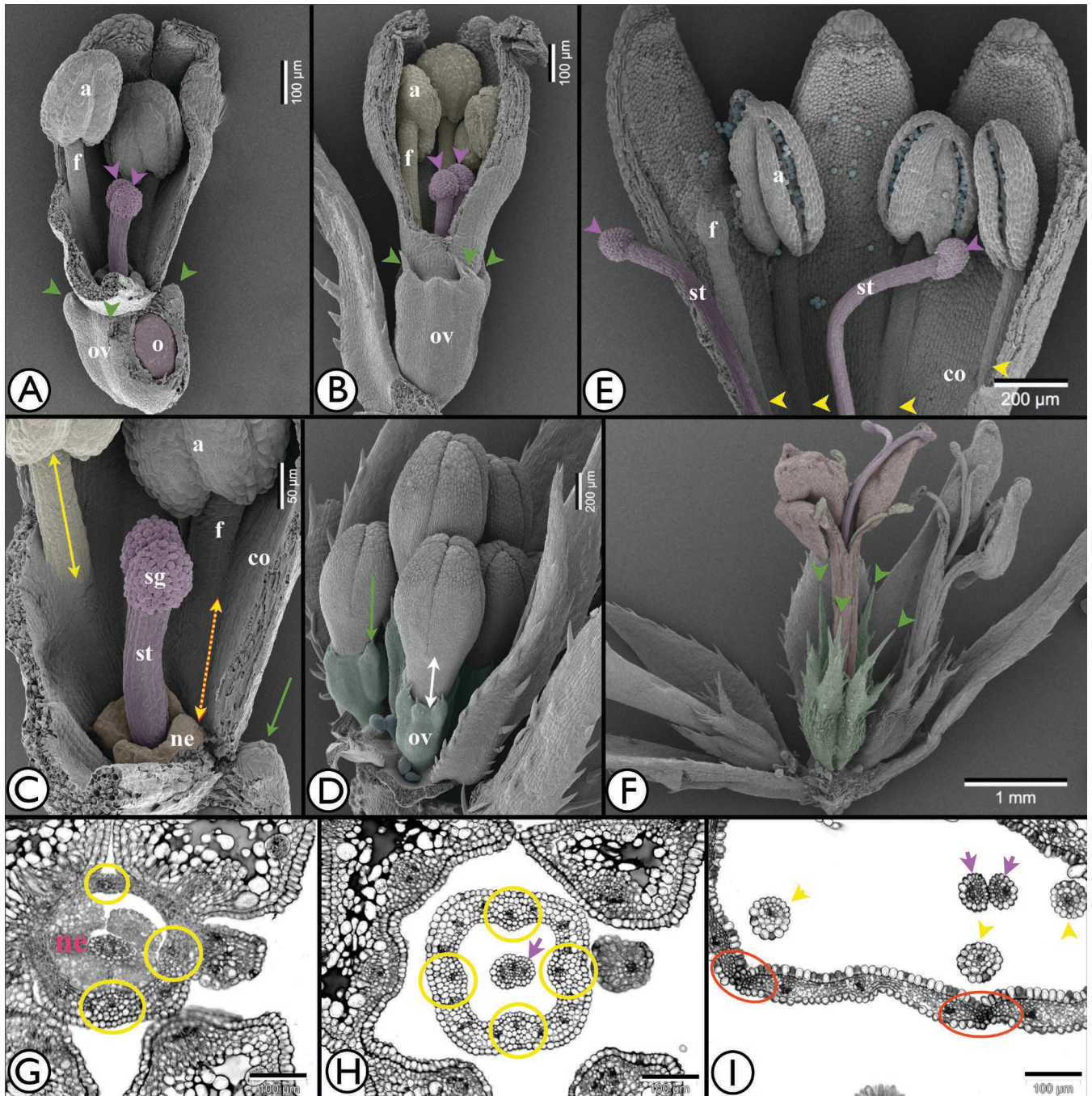


Figure 11 – SEM (A–F) of late floral development and LM (G–I) of transverse sections of (semi-)mature flowers of *Sherardia arvensis*. A–F. Lateral view from different angles. A–B. Calyx lobes developing from calyx rim (green arrowheads); stamen-corolla tube and corolla lobes elongating, lifting up stamens; bases of two styles postgenitally fusing, each topped by spherical, papillose stigma (purple arrowheads); bilocular, inferior ovary with single ovule per locule. C. Elongating stamen-corolla tube (red-yellow dotted double arrow); free parts of filaments elongating (yellow double arrow); common base of style surrounded by two semi-circular nectaries; calyx indicated with a green arrow. D. Abaxial view of flower cluster; inferior ovaries and developing calyx lobes (green arrow) coloured in green; elongating tubular part of corolla indicated by white double arrow. E. Distal part of longitudinally opened flower; longitudinal thickenings in stamen-corolla tube corresponding to staminal vascular bundles (yellow arrowheads); stigmas (purple arrowheads) and anthers reaching level of throat. F. Cluster of three flowers at anthesis with spiny calyx lobes (green arrowheads); stamens exerted from corolla tube; elongated styles now protruding far above anthers; inferior ovary didymous. G. Basal part of stamen-corolla tube, semi-circular nectaries, and common base of style; three out of four staminal vascular bundles encircled in yellow. H. Idem as in G, more distally; staminal vascular bundles (encircled in yellow) and tubular corolla forming continuous circular tissue; fused styles just below separation of style branches (purple arrow). I. Corolla lobes; edges of adjacent corolla lobes encircled in red; opposite them, three out of four free filaments (yellow arrowheads); free style branches (purple arrows). Abbreviations: a, anther; co, corolla (lobe); f, filament; ne, nectary; o, ovule; ov, ovary; sg, stigma; st, style.

large dome-shaped epidermal cells. At anthesis, the flower opens and the pollen presenter becomes exposed (functionally male stage). Later in the development, the stigmatic lobes become receptive for pollen from other plants (functionally female stage). The release of pollen occurs in the closed bud, with pollen being deposited on the pollen presenter.

Nectary – In all species studied, a nectary disk, consisting of several parts, develops on the top of the ovary, surrounding the style(s). This concurs with what is found in most representatives of the asterids where gynoeceal nectaries can be considered as a synapomorphy (Smets 1988; Smets & Cressens 1988; Bernardello 2007: 86). The development of the nectary occurs rather early in the species studied of *Cruciata*, *Galium*, *Phuopsis*, and *Rubia*. This, in combination with a reduced or delayed development of the calyx, suggests neoteny. Naghiloo & Classen-Bockhoff (2017) performed an extensive study on heterochronic changes, in particular sequence heterochrony *sensu* Smith (2001), in flowers of Dipsacoideae (Caprifoliaceae), concluding that the interaction between timing (initiation of floral organs) and space plays an important role in the diversification of flowers. Our previous and present studies of the perianth in Rubiaceae lead to a similar conclusion.

Homology

According to the conceptual framework of Ochoterena et al. (2019) for morphological homology assessments, the common structural origin of plant parts determines their nature, based on which a specific plant part can be assigned to a particular character such as stem, leaf, sepal, petal, etc. The developmental pathways leading to a specific mature plant part in different species may differ, resulting in different character states of the character to which the plant part has been assigned. In the case of a tubular corolla, the structural origin can be a meristem developing into a corolla tube *sensu stricto* or into a stamen-corolla tube. The latter actually consists of the so-called congenitally fused meristems of the petals and stamens. The fact that in a stamen-corolla tube, the corolla and androecium initially develop together, does not mean that the meristems from which the respective corolla and androecium parts of a stamen-corolla tube originate would be different from the meristems of a corolla and androecium that develop separately. Therefore, we consider meristems resulting in a corolla tube *sensu stricto* or in the corolla part of a stamen-corolla tube as essentially the same. In the same way, the meristems that give rise to independent stamens are the same as the meristems that develop the stamen parts of a stamen-corolla tube. Moreover, postgenital

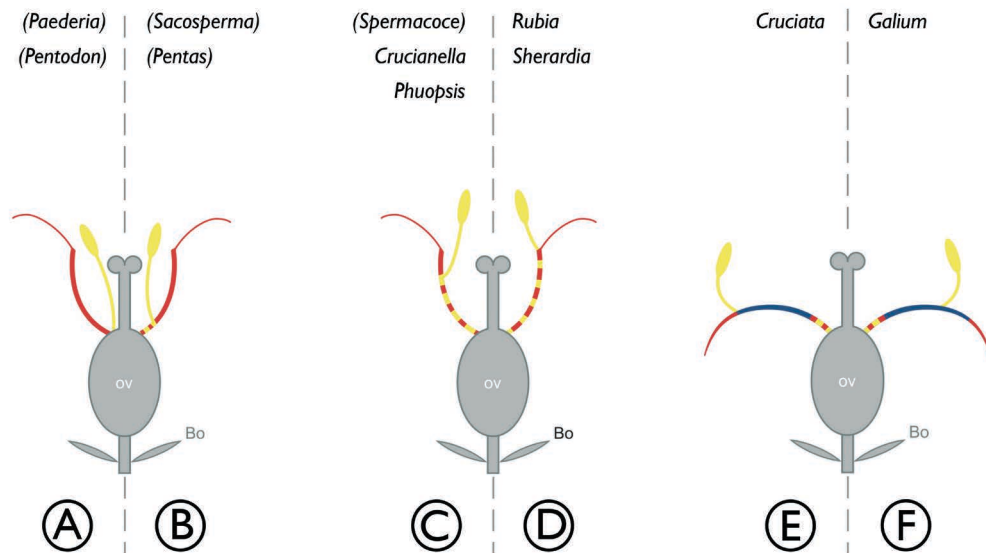


Figure 12 – Theoretical outline of the contribution of the different developmental processes to the corolla and the position of the stamens. Different morphs are separated by dotted lines. **A.** Flowers with corolla tube *sensu stricto* and corolla lobes; stamens positioned at top of inferior ovary. **B.** Flowers with rudimentary stamen-corolla tube below corolla tube *sensu stricto*; stamens inserted at top of stamen-corolla tube. **C.** Flowers with well-developed stamen-corolla tube and corolla tube *sensu stricto*; stamens inserted higher on tube as in B but below bases of corolla lobes. **D.** Flowers with well-developed stamen-corolla tube and no corolla tube *sensu stricto*; stamens inserted at top of stamen-corolla tube in between bases of corolla lobes. **E.** Flowers with rudimentary stamen-corolla tube below fusion zone of proximal parts of corolla lobes and filaments; filaments inserted at top of rudimentary stamen-corolla tube but adhering to fusion zone, becoming free between bases of free parts of corolla lobes. **F.** Idem as in E, but fusion zone of only corolla lobes occurring above insertion points of free parts of filaments. Colour code: blue, fusion zone; grey, schematic representation of flower parts including pedicel and bracteoles; bold red, corolla tube *sensu stricto*; red, (free parts of) corolla lobes; yellow, stamens; red-yellow, stamen-corolla tube. Abbreviations: Bo, bracteole; ov, ovary. Names in brackets were studied in Vrijdaghs et al. (2015).

fusion and plastochron variation do not affect the origin of the tubular corolla. Consequently, we think that all different morphs of tubular corollas in the species studied should be considered to be character states of the character ‘tubular corolla’. Similarly, whether the stamens are epipetalous or not does not affect their morphological origin. In fig. 12, a theoretical outline of all kinds of floral cups consisting of corolla and androecium, observed in Rubioideae species in this study and in Vrijdaghs et al. 2015, is presented. In subfamily Ixoroideae, De Block & Vrijdaghs (2013) observed that the development of the tubular corolla in *Canephora* concurs with fig. 12C. Erbar & Leins (1996) reported the development of a long tubular corolla in *Ixora*, consisting mainly of a stamen-corolla tube, which would also correspond to fig. 12C. We think that the “initial corolla ring primordium” observed by Erbar & Leins (1996: 105) in *Ixora coccinea* L. is rather an annular primordium of the stamen-corolla tube.

Additional observation

In *Galium aparine*, *G. glaucum*, *G. odoratum*, and *G. verum*, the petals are bladder-shaped in early developmental stages (*G. pumilum* not observed). Adaxially, there is a one-layered epidermis that appears to be free from the rest of the tissue of the petal (fig. 4A, C), suggesting a cavity. We have not observed such a phenomenon in any other species of the Rubiaceae. Later in the development, the petals/corolla lobes become more flattened.

CONCLUSION

The developmental hypothesis of Vrijdaghs et al. (2015) for the formation of the tubular corolla and the often related epipetaly in Rubiaceae is confirmed. In addition, the current study shows the importance of plastochron variation. A lengthening or shortening of the time interval between the successive appearance of floral whorls/floral developmental events or sequence heterochrony results in important variation: e.g., 1) Reduction of the time interval between the appearance of corolla and androecium, in extremis leading to a simultaneous appearance of both whorls (shortened plastochron); 2) Extension of the time interval between the appearance of corolla and stamen-corolla tube, resulting in a delayed development of the stamen-corolla tube (lengthened plastochron); and 3) Sequence switch of the appearance of corolla and calyx resulting in a negative plastochron with respect to the corolla, causing delay of the development of the calyx or, in extremis, inhibiting its development completely.

The development of a stamen-corolla tube, a corolla tube *sensu stricto*, postgenital fusion between petals/corolla lobes and filaments, and plastochron variation are the four processes that determine the shape of the corolla and the presence (or not) of epipetaly in all Rubiaceae examined in this and previous studies. The proportion in which each of these processes contributes to the final shape of the corolla causes the morphological variability of the corolla and the androecium among the species.

ACKNOWLEDGEMENTS

We thank Iris Van der Beeten for technical assistance with LM, Liliane Tytens for the graphics of fig. 12 (both Meise Botanic Garden). We thank Peter L. Meininger for kindly providing macrographs of *Rubia*. We are grateful to KU Leuven Master student Wouter Van Assche for his SEM contribution to figs 3 and 4. We thank Claudia Erbar and an anonymous reviewer for their constructive comments.

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Communicating Editor: Elmar Robbrecht.

Submission date: 3 Jun. 2020

Acceptance date: 3 Sep. 2020

Publication date: 23 Nov. 2020