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CELLULAR AND MOLECULAR BIOLOGY

Anther structure and pollen development in species of Rubiaceae and anatomical evidence of pathway to morphological dioecy

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Abstract: we conducted anatomical analysis of anthers with the aim to establish the differences in the development pattern of microsporophytes and microgametophytes between perfect and imperfect flowers in the tribe Gardenieae (Rubiaceae). The species studied were: *Tocoyena formosa* (monoecious with perfect flowers), *Cordiera concolor, Genipa americana, Randia calycina*, and *Randia heteromera* (dioecious with imperfect flowers). Flowers in successive stages of development were collected and fixed. The material was processed and examined using light microscopy and scanning electron microscopy. The present study revealed the stage when pollen is arrested in the functionally pistillate flowers of the dioecious taxa. Based on these observations an evolutionary sequence of changes towards the reduction of non-functional anthers in Rubiaceae is proposed. In addition, we describe and discuss characters that might be of importance in future phylogenetic studies in Rubiaceae (e.g., pollen morphology and its dispersal unit, the presence of orbicules, and a new type of placentoid).

Key words: Arrested development, permanent tetrads, placentoid, Rubiaceae, secondary pollen presentation.

INTRODUCTION

Dioecy is a breeding system in which different plants of the same species produce staminate and pistillate flowers. It is associated with 7% of Angiosperms and seems to evolve independently 800 to 5000 times (Renner & Ricklefs 1995, Vamosi et al. 2003, Renner 2014). In flowering plants, Mitchell & Diggle (2005) defined imperfect flowers as Type I if abortion of the gynoecium or androecium occurs; and Type II as a flower that is imperfect from the beginning of its formation.

Rubiaceae members are characterized by a diverse range of breeding systems among which are hermaphroditism, monoecy, dioecy, heterodistyly, and others (Verdcourt 1958). In this family, the tribe Gardenieae *s.l.* includes almost 100 genera of trees, treelets, shrubs and lianas (Mouly et al. 2014, Persson & Delprete 2017, Robbrecht 1988). The tribe has monoecious and dioecious members, even within the same genus as in *Randia* L. (Hallé 1967, Lorence & Dwyer 1987). In the neotropics, the tribe comprises 18 genera; more than half are dioecious. The dioecy in Gardenieae *s.l.* was described as having functionally pistillate flowers with staminodes (stamens with empty anthers), and functionally staminate flowers with rudimentary ovaries and non-functional stigma (Robbrecht & Puff 1986). These flowers would correspond to the Type I of Mitchell & Diggle (2005).

In Rubiaceae the anatomical descriptions of the stamens have been mainly focused on species with perfect flowers (Dedecca 1957, Galati 1991, Hansson & El-Ghazaly 2000, Vinckier & Smets 2005, Magalhães Souza et al. 2008,

Romero et al. 2017, Yue et al. 2017). The only known embryological study on imperfect flowers was conducted in *Mussaenda pubescens* (Li et al. 2010). In this species the anatomy showed that the apparent style dimorphism masked the dioecy. However, in the dioecious species of the tribe Gardenieae, the anatomical mechanisms that lead to the loss of the functionality of the androecium are not known.

Among the characters associated with androecium that present taxonomic and reproductive relevance in this family the morphology and the dispersion unit of pollen, the presence of orbicules, and the existence of a mechanism of secondary pollen presentation are distinguished (Robbrecht 1988). In the family, different pollen dispersal units have been described: monads, tetrads and polyads (Hallé 1967, Persson 1993). These dispersal units can be used as a source of information for phylogenetic analysis (Dessein et al. 2005) or analyzed in combination with molecular data (Gustafsson & Persson 2002). The orbicules (or Ubisch bodies) also represent a very interesting taxonomic feature in Rubiaceae because they vary in abundance, size, shape and ornamentation (Huysmans et al. 1997, 1998, Vinckier et al. 2000, Dessein et al. 2005, Verellen et al. 2007, Verstraete et al. 2011, Romero et al. 2017).

Among the most common breeding strategies known for Rubiaceae are: heterostyly, secondary presentation of pollen, and imperfect flowers (Robbrecht 1988). Also, Hallé (1967) mentions the protandry in Gardenieae, this mechanism has already been described for *Tocoyena formosa* (Silberbauer-Gottsberger 1972). In the secondary pollen presentation, the pollen is presented on a structure different from the anther, such as the gynoecium, the perianth or the bracts (Howell et al. 1993). This mechanism is common in the family (Hallé 1967, Robbrecht 1988, Howell et al. 1993, Yeo 1993) and is generally mentioned as a pollen presenter on the style or stigma (Howell et al. 1993, Yeo 1993, De Block & Igersheim 2001).

This study focused on the anatomy of staminate flowers to establish the differences in the development pattern of microsporophytes and microgametophytes between monoecious and dioecious species of the tribe Gardenieae.

MATERIALS AND METHODS

Studied taxa

The five species of Gardenieae *s.l.* analyzed in this paper are arborescent and shrub species from the Neotropical region and studies related to their embryology and reproduction system are unknown. Considering taxonomic antecedents, *Tocoyena formosa* (Cham. & Schltdl.) K. is a monoecious species with perfect flowers, while *Cordiera concolor* (Cham.) Kuntze, *Genipa americana* L., *Randia calycina* Cham., and *Randia heteromera* M.D. Judkevich & R.M. Salas are dioecious species with imperfect flowers. Voucher specimens were deposited in the herbarium "Carmen Cristóbal" (CTES) of the Instituto de Botánica del Nordeste, Corrientes, Argentina (voucher numbers in the appendix). Flowers at successive developmental stages were collected in the field and preserved in formalin-acetic acid-alcohol (5 mL formalin, 5 mL acetic acid, and 90 mL 70% ethanol). From each species, 10 flowers belonging to the different stage were dissected for anatomical analysis.

In the analyzed species and for comparative purposes, six developmental stages of the pollen were categorized from the microspore mother cell stage. At each stage, the structure and layers that form the anther wall and connective are described for all species and also those particular to each taxon and flower type. Stage 1 corresponds to the sequence of events and characteristics of the anthers in all types of flowers, stages 2 to 5 belong to perfect flower

(PF) and functional staminate flower (FSF), and the stage named "X" describes the point in time of the arrested pollen development in functional pistillate flower (FPF).

For general observations, fixed flower buds were dehydrated and embedded in paraffin (Johansen 1940; modified by Gonzalez & Cristóbal 1997) and then cut into 12 µm sections using a Microm HM350 rotary microtome (Microm International, Walldorf, Germany). Cross sections were stained with safranin and astra blue (Luque et al. 1996) and mounted in synthetic Canada balsam. The presence of lignin and crystals was confirmed by observation with polarized filters (PLM). Observations and digital images were acquired using a Leica DM LB2 (Leica Microsystems) light microscope (LM) equipped with a Leica DATA digital camera. To detect callose, microspore mother cells and tetrads were treated with 0.1% aniline blue and observed with a Leica DM 1000 fluoresce microscope, photographs were taken with a Canon EOS Rebel TDi digital camera.

For Scanning Electron Microscopy (SEM), fixed anthers were dehydrated in an increasing acetone series and then critical point dried using liquid CO_2 (Denton Vacuum, DCP-1, Pleasanton, NJ) and sputter-coated with gold-palladium (Denton Vacuum, Desk II, Pleasanton, NJ). The samples were analyzed with a Jeol LV 5800 (JEOL, Tokyo, Japan) at 10 kV in the Service of Electron Microscopy facility at the Universidad Nacional del Nordeste. To determine the presence of orbicules the critically point dried mature anthers were opened, and pollen was removed prior to metallization.

Samples of pollen were taken from mature anthers of fixed material (FAA) and herbarium material, which were acetolized according to Erdtman (1966) and analysed by LM and SEM. In each species an average of 25 pollen grains was measured. The terminology used follows Punt et al. (2007).

Histochemical analysis

Samples of cross-sectioned anthers were subjected to the following reagents: Lugol (Johansen 1940) for detection of starch; ferric chloride (Johansen 1940) for tannins; and Aniline blue 0,1 % (Zarlavsky 2014) for callose. The samples stained with Aniline blue were observed under Fluorescence Microscope Leica DM1000 equipped with an EOS T2i digital camera.

Also, to identify pollenkitt on the pollen surface, fresh anthers were emptied onto a slide in a drop of Sudan III (Johansen 1940).

RESULTS

Floral morphology

Of the species analyzed (Figure 1), *T. formosa* formosa has perfect flowers arranged in a multi-flowered inflorescence (Figure 1a). The remaining four species have functional staminate flowers (FSF) arranged in multi-flowered inflorescences (Figure 1b, d, f, h) and solitary functional pistillate flowers (FPF) (Figure 1c, e, g, i).

Both the perfect and imperfect (FSF and FPF) flowers have an androecium formed by stamens alternating with the corolla lobes. The anthers are bithecal, dorsi-medifixed and fused to the corolla tube by a short filament. In the anthetic flowers, the stamens of the PF (Figure 1j) and FSF (Figure 1k) have open anthers with exposed pollen, while in the FPF the anthers remain closed and do not produce pollen (Figure 1l).

The gynoecium of PF (Figure 1m) and FPF (Figure 1n) consists of an inferior ovary, a style, and a bilobed or bifid stigma. In the FSF a pistillode with a rudimentary ovary is present (Figure 1o). In all species there is a ring-shaped nectary surrounding the base of the style (Figure 1m-o).

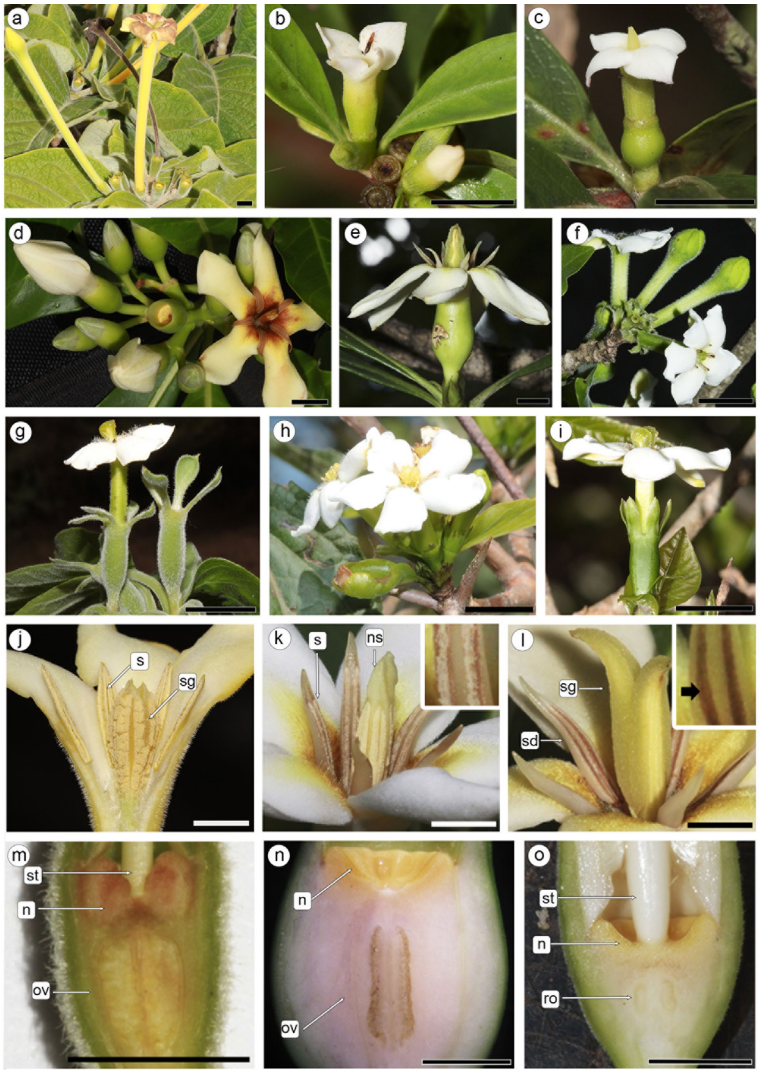

Figure 1. Species of Gardenieae and floral characteristics. **a, j, m.** Tocoyena formosa. **b-c.** Cordiera concolor. **d-e, k-o.** Genipa americana. **f-g.** Randia calycina. **h-i.** R. heteromera **a, j, m.** PF. **b, d, f, h, k, n.** FSF. **c, e, g, i, l, o.** FPF. **j,** Detail of a PF showing the pollen deposited on the stigma. **k,,** Detail of a FSF showing the pollen deposited on the non-functional stigma; above, portion of a dehiscent anther with pollen still present. **l,** Detail of a FPF; above, portion of a staminode, note the aborted tissue inside the thecae (arrow). **m,** Longitudinal section (LS) of PF showing a nectary around the style base. **n,** LS of inferior ovary of a FPF (the style has been removed). **o,** LS of the rudimentary ovary of a FSF. Abbreviations: n= nectary; ns= non-functional stigma, ov= ovary, ro= rudimentary ovary, s= stamen, sd= staminode, sg= stigma, st= style. Scales: a, d-i= 1 cm; b-c, k-l, n-o= 5 mm; m= 4 mm.

Anther structure and pollen development

The characteristics of each stage are summarized below, and details are presented in Table I.

Stage 1. Microspore mother cells

In the young anther (Figure 2a) the microspore mother cells (mmc) are derived by mitotic divisions from sporogenous tissue. The mmc have dense cytoplasm and a voluminous central nucleus and are surrounded by a layer of callose (Figure 2b-c). The tapetum completely surrounds each locule. This is of the secretory type with uninucleated cells and dense cytoplasm (Figure 2a-b, g-k). The rest of the wall of the young anther has middle layers (1-3 stratified), endothecium without any fibrous thickening at this stage (1-3 stratified) and a uni-stratified epidermis. The septum has elongated cells (Figure 2d). The stomium region has 2-3 cell layers (Figure 2d). In the connective tissue, a circular concentric periphloematic vascular bundle is recognized (Figure 2a, e). The epidermis has stomata (Figure 2f), especially on the abaxial side of the connective, the epidermis only has tannins in *C. concolor*. There may be druses in the cells of connective and septum.

Table I. Summary of the anatomical characteristics of the androecium, stages of development and flower type of the study species of Rubiaceae.

Species	Flower sexuality	st1. mmc	st2a. meiosis	st2b. tetrads	st3. microspores	st4. microametogenesis	st5. anther dehiscence	Unit of dispersion	Placentoid	Pollenkitt	mature wall tissue			
											Endothecium with thickenings	Middle layers	Aborted tissues	Orbicules
Tocoyena formosa (monoecious plant)	PF	→	→	→	→	→	yes	monads	yes, parenchyma + tapetum	yes	yes + plt	no	none	no
Cordiera concolor (dioecious plant)	FSF	→	→	→	→	→	yes	monads	no	yes	yes	no	none	yes
	FPF	→	→	stX	-	-	no	none	no	no	yes	yes	tp	no
Genipa americana (dioecious plant)	FSF	→	→	→	→	→	yes	monads	no	yes	yes	no	none	no
	FPF	→	stX	-	-	-	no	none	no	no	yes	yes	tp	no
Randia calycina (dioecious plant)	FSF	→	→	→	→	→	yes	permanent tetrads	yes, tapetal	yes	yes	no	none	no
	FPF	→	→	stX	-	-	no	none	yes, tapetal	no	yes	yes	tp	no
Randia heteromera (dioecious plant)	FSF	→	→	→	→	→	yes	permanent tetrads	yes, tapetal	yes	yes	no	none	no
	FPF	stX	-	-	-	-	no	none	yes, tapetal	no	no	yes	tp	no

Abbreviations: PF: perfect flower; FPF: functional pistillate flower; FSF: functional staminate flower; ml: middle layers; mmc: microspore mother cells; plt: placentoid with thickening; st: stage; stX: stage X or arrested development; tp: tapetum. The arrows indicate that this state occurs normally during the development of pollen.

In *Tocoyena formosa* (Figure 2g) and both species of *Randia* (Figure 2j-k) the pollen sacs are C-shaped due to the presence of a placentoid.

Stage 2. Meiosis and tetrads

The mmc undergo meiosis (Figure 2l). The cytokinesis following meiosis is simultaneous, producing decussate (Figure 2m) and tetrahedral (Figure 2n) tetrads. In this stage the tetrads are still surrounded by callose. The pollen sacs increase in volume. The middle layers collapse and the cells of the tapetum increase in volume (Figure 2o).

Stage 3. Microspores

In *Tocoyena formosa*, *Cordiera concolor*, and *Genipa americana*, the callose surrounding the tetrads disintegrates, releasing the microspores into the pollen sacs (Figure 2p). In *Randia* species, microspores are attached to each other forming permanent tetrads (Figure 2q). In both cases - free microspores and permanent tetrads - each microspore has a central nucleus that is then displaced to the periphery by a vacuole (Figure 2p-q). The tapetum and the cells of the epidermis begin to lose their shape in this stage (Figure 2r).

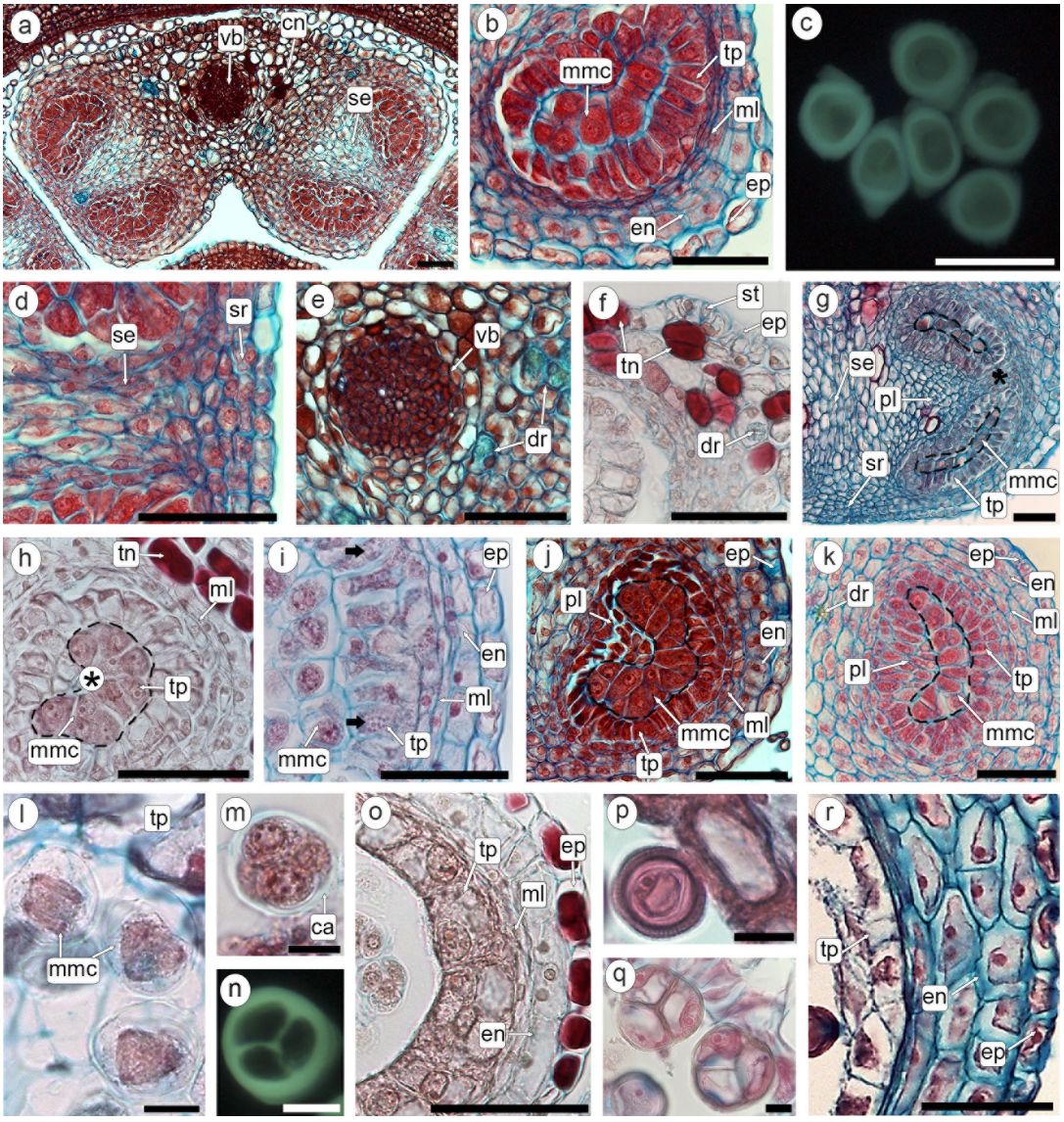

Figure 2. Cross section of anthers from the microspore mother cell stage (mmc) to microspore stages. **a-e j**, Randia calycina. **f, h, l, o, p**, Cordiera concolor. **g**, Tocoyena formosa. **I, m, r,** Genipa americana. **k, n, q,** Randia heteromera **a**, Anther (FSF). **b**, Portion of the anther where the mmc and several parietal strata are distinguished (FPF). **c**, mmc stained with aniline blue and observed under a fluorescence microscope (FSF). **d**, Septum and stomium region (FPF). **e**, Detail of the vascular bundle and cells with druses in the connective tissue. **f**, Portion of the connective tissue showing stomata, druses, cells with tannin in the epidermis and parenchyma (FPF). **g**, Detail of the placentoid (parenchyma + tapetum), the pollen sac is divided into two regions (*) due to the invasion of the placentoid. **h**, Detail of the anther showing: epidermis with tannin; tapetal cells with a large vacuole and the central mmc (*) in meiosis (FSF). **i**, Detail of an anther wall (FPF), the nuclei of the tapetal cells have several nucleoli (arrows). **j**, Tapetal placentoid in FSF. **k**, Tapetal placentoid in FPF. **l,o-p,,** Cordiera concolor. **n, q,** Randia heteromera **m, r**, Genipa americana. **l**, Detail of the mmc in meiosis surrounded by uninucleated tapetum. **m**, Decused tetrad. **n**, Tetrahedral tetrad stained with aniline blue and observed with fluorescence microscope. **o**, Cross section of anther showing wall layers during stage 3. **p**, Free microspore; the nucleus is displaced by a large vacuole. **q**, Microspores joined together to form permanent tetrads. **r**, Anther wall with the cytoplasm of the tapetum cells contracted. Abbreviations: ca= calose; cn= connective tissue; dr= druse; en= endothecium; ep= epidermis, ml= middle layers; mmc= microspore mother cell; pl= placentoide; se= septum; sr= stomiun region; st= stomata; tn= tannin; tp= tapetum; vb= vascular bundle. Scales: a-k, o, r= 50 µm,l-n, p-q= 10 µm.

Stage 4. Microgametogenesis

Whether the microspores are free or in permanent tetrads, a mitotic division of the microspore forms the vegetative and generative cells (Figure 3a). Each bicellular pollen grain has three apertures, of which one oncus formed by intine and protoplast is projected (Figure 3b). The tapetum collapses. The endothecium develops U-shaped thickening and the epidermis collapses (Figure 3c-f, h-k).

Tocoyena formosa: the endothecium is 5-stratified in the connective region, decreasing to 2-stratified in the stomium. The parenchymatous tissue of the placentoid also develops fibrous thickening (Figure 3c-d). Subsequently the innermost cells of the stomium disintegrate so there is fusion of the pollen sacs in each theca (Figure 3d).

Cordiera concolor (Figure 3e-f) and *Genipa americana* (Figure 3h): the septum develops fibrous thickening. Stomium cells loose volume. There is no fusion between the pollen sac of each theca. *Cordiera concolor* is the only species where orbicules are observed on the inner tangential face of the tapetal membrane in anthers of the FSF (Figure 3f). In the connective tissue, the parenchymatic cells accumulate simple grains of starch (Figure 3g).

Randia species (Figure 3i,k): stomium cells have decreased in volume. The cells of the septum collapse and disintegrate which causes the fusion of the anther pollen sacs.

Stage 5. Anther dehiscence

As a consequence of the degeneration of the stomium cells, the anthers of the PF and FSF open by longitudinal dehiscence (Figure 3j, k). The generative cell of the pollen acquires a lenticular shape (Figure 3l) and divides to form two spermatic cells. The pollen is released at a 3-cellular stage (Figure 3m).

Stage X. Arrested development

The sequence of arrested development of the pollen occurs exclusively in the FPF (Figure 3n-x). The stage at which development stops varies among species (Table I). The mmc or tetrads become dark, and the cytoplasm has collapsed. While the developing pollen grains are aborted, in the anther wall the tapetum loses its shape and the cytoplasm is seen to retract, the endothecium increases in volume and develops thickening. At the end, there is a collapse of the sterile tissues, except in the anther middle layers whose cells keep their shape and are not degraded. The pollen sacs decrease in volume until they become linear. The anthers are indehiscent, and the aborted tissue remains inside.

The following are the particularities of each species: in *Cordiera concolor* meiosis and cytokinesis occur and tetrads are formed, but they abort, and the pollen sacs lose their shape (Figure 3n-n'). The cells in the anther middle layer increase in volume and they look elongated. The stomium collapses and the pollen sacs of each theca become closer together (Figure 3o). *Genipa americana*: the mmc abort at different stages of meiosis (Figure 3p-p'). The pollen sacs are narrowing (Figure 3q-r). *Randia calycina*: the tetrads abort (Figure 3s-s'). The pollen sacs become linear due to the increased volume of the septum cells (Figure 3t-u). The loss in volume in the stomium cells brings the pollen sacs closer together (Figure 3u). *Randia heteromera:* the mmc abort (Figure 3v-x). The endothecium increases in volume, which together with the loss in volume of the stomium cells causes the pollen sacs to approach each other. The pollen locules become linear and the cells of the septum increase in size. This is the only species in which the endothecium does not develop any thickening. The tissues of the anther wall collapse (Figure 3x).

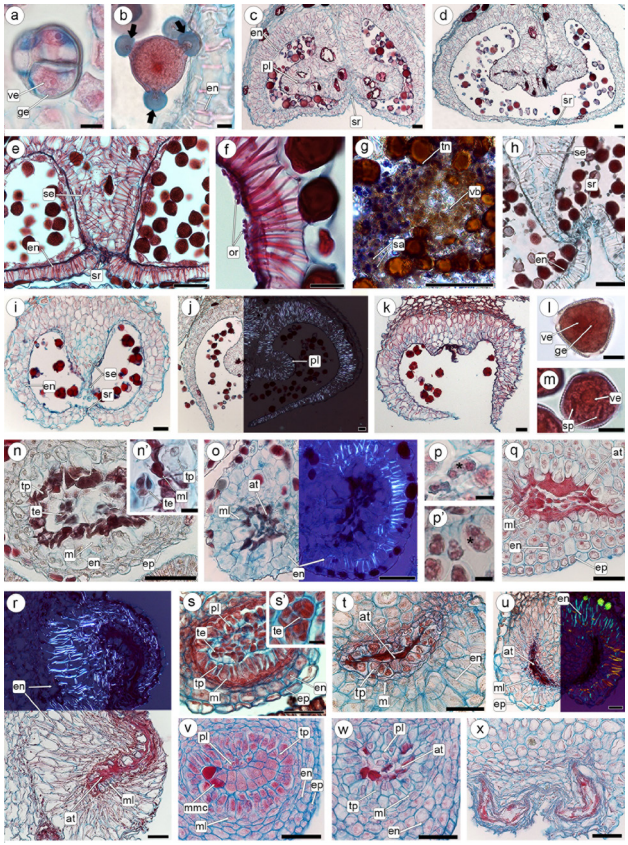

Figure 3. Cross section of anthers with pollen grains (a-i) and in a dehiscence stage (j-m), and cross section of anthers of FPF in developmental arrest stages. **a**, Randia heteromera (FSF). **b-d, j,** Tocoyena formosa (PF). **e-g, l-m,** Cordiera concolor (FSF). **h**, Genipa Americana (FSF). **i, k,** Randia calycina (FSF). **a**, Permanent tetrads, in one pollen grain the vegetative and generative cells can be observed. **b,** Pollen grain with oncus (arrows) that protrudes from each aperture. **c**, Multilayered endothecium and placentoid, both with fibrous thickenings. **d**, Fusion of pollen sacs by the disintegration of the innermost cells of the stomium region. **e**, Septum with fibrous thickenings, no fusion of locules. The cells of the stomium have collapsed. **f**, Anther wall showing orbicules in the tapetal membrane. **g**, Connective cells with starch grains (stained with Lugol) and tanniferous idioblastos (stained with ferric chloride). **h**, Septum with fibrous thickenings, no fusion of locules. **i**, The cells of the septum collapse and disintegrate; the pollen sacs fuse in a common locule. **j-k**, Dehiscent anthers, note in j the placentoid with fibrous thickenings (right portion with polarized light). **l**, Pollen grain released at the 2-cell stage. **m**, Pollen grain released at the 3-cell stage. **n-o**, Cordiera concolor. **p-r**, Genipa americana. **s-u**, Randia calycina. **v-x**, Randia heteromera n, One pollen sac with tetrads and collapsed tapetum, the middle layers have enlarged cells. n', Detail of a collapsed tetrad. 0, Pollen sacs of one theca with aborted tissues, endothecium with thickening (right portion with polarized light: PL). p-p', mmc at different stages of meiosis (*). q, One pollen sac with tapetum and mmc cells aborted, middle layers of greater volume. r, Endothecium with thickening, collapsed stomium and linear and closed pollen sacs (image on the right with PL). s, The tetrads formed in the pollen sac begin to collapse. s', Tetrads surrounded by callose. t, Linear pollen sac with aborted tetrads, the tapetum and placentoid cells have contracted cytoplasm, the middle layers are still present and the endothecium cells are voluminous. u, Endothecium with fibrous thickenings, collapsed tapetum, and pollen sacs of one theca nearby (image on the right with PL). v, the mmc in the pollen sac begin to collapse, the cytoplasm of the tapetum begins to contract. w, The cells of the tapetum and the placentoid with completely contracted cytoplasm, compressed pollen sac, and aborted mmc. x, Completely collapsed theca; linear pollen sacs with aborted cells inside. Abbreviations: at= aborted tissue; en= endothecium; ge= generative cell; ml= middle layers; mmc= microspore mother cell; or= orbicules; pl= placentoid; sa= starch grains; se= septum; sp= sperm cell; sr= stomium region; te= tetrad; tp= tapetum. vb= vascular bundle; ve= vegetative cell. Scales: a-b, l-m, n', p-p', s'= 10 µm; c-k, n, o, q-s, t-x= 50 µm.

Placentoid

Both species of *Randia* (Figure 4a-d) and *Tocoyena formosa* (Figure 4a'-d') have anthers with a placentoid. The tissue of the placentoid extends into the locule, which thereby becomes C-shaped in cross section (Figure 4a-a).

In *Randia* the placentoid is formed only by the tapetum. This type of placentoid has a low protrusion into the locule and begins to retract along with the tapetum as the anther matures (Figure 4b-c), disappearing before anther dehiscence (Figure 4d).

In *T. formosa* the placentoid is formed by parenchyma of the septum and tapetum (Figure 4a). The parenchymatic cells have druses and tanniferous idioblasts. The placentoid is massive and its development is such that it can subdivide the locule (Figure 4b). In the microspore stage the tapetal portion retracts and collapses along with the rest of the tapetum (Figure 4c) while the parenchymal portion continues to grow and develops fibrous thickening along with endothecium (Figure 4d'). This placentoid persists in the dehiscent anthers.

Orbicules

They are only present in anthers of the FSF of *C. concolor*. They are abundant and are randomly scattered on the inner tangential side of the cells of the tapetum and on the tapetal membrane (Figures 3f, 4e-f). The orbicules are spherical to slightly elongated in shape, have a diameter

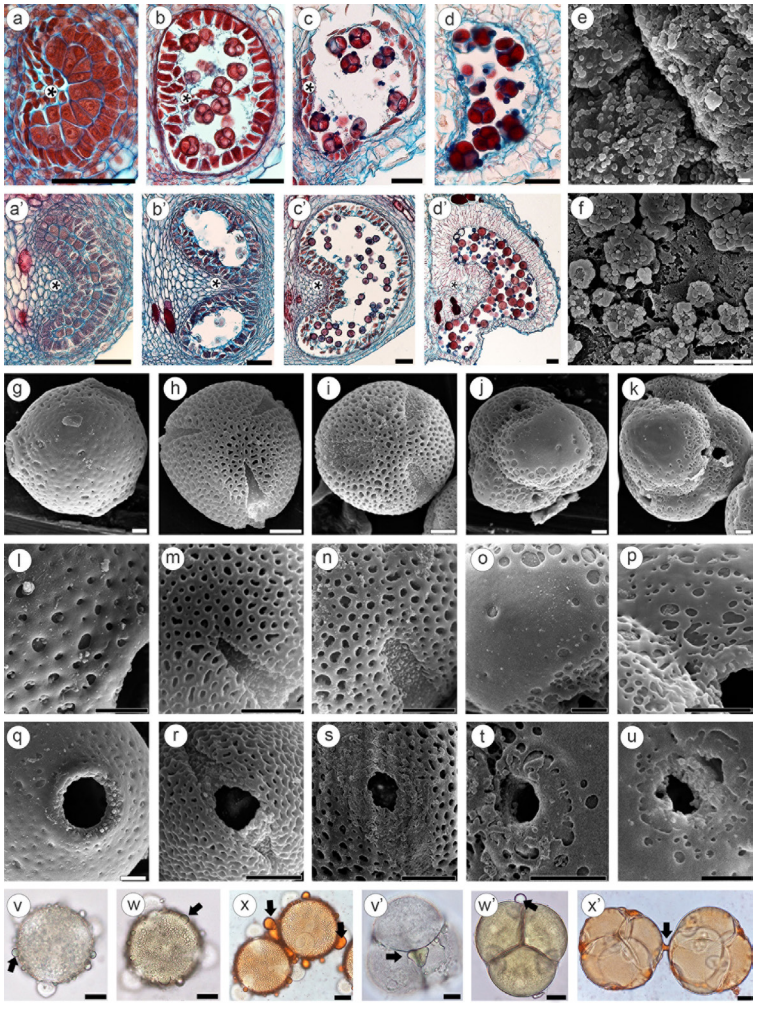

Figure 4. Detail of pollen sacs with placentoid, orbicules (of Cordiera concolor), and pollen grains. a-d', v-x', LM. e-u, SEM. a-d, j, o, t , Randia calycina. a'-d', g, l, q, Tocoyena formosa. e-f, h, m, r, Cordiera concolor. i, n, s, Genipa americana. k, p, u, Randia heteromera a-d, asterisks points to the placentoid (tapetal). a'-d', asterisks points to the placentoid (tapetal and parenchymatic). a, Young placentoid b, mature placentoid invading the pollen sac. c, Collapsed placentoid. d, Pollen sac of a mature anther without placentoid. a', Young placentoid, both the tapetal and parenchymal portions are well developed. b', Mature placentoid dividing the pollen sac in two. c', The tapetal portion of placentoid is collapsed. d'. The parenchymatic portion of placentoid develops thickenings along with the endothecium. e, General view of the orbicules. f, Detail of orbicules forming aggregates. g-k, General view of pollen grains. l-p, Detail of the exine. q-u, Detail of the aperture. v-x' Non-acetolized pollen grains with pollenkitt, v-x, Genipa americana, monads. v'-x', Randia heteromera, permanent tetrads. v, v', Fresh pollen grain with pollenkitt (arrow). w, w', Pollen grain stained with Lugol; negative reaction of pollenkitt (arrow). x, x' Pollen grains stained with Sudan III; positive reaction in pollenkitt (arrow). Heteromera Scales: a-d'=50 µm; e-f= 2 µm; g-u= 5 µm; v-x'=10 µm.

of 0.1-0.3 µm and the surface is smooth. They can form globular aggregates of 1.0-1.7 µm in diameter (Figure 4f).

Morphology and dispersal units of the pollen

The differences found in the pollen grains are the type of dispersal units, size of individual grains, shape of the apertures and exine ornamentation (Table II; Figure 4g-x'). The pollen of *R. calycina* has been described in previous research by the author and the data are used to compare it with the species in the current study (Judkevich et al. 2015, marked with * in Table II).

In the tetrads of both species of *Randia* the exine is seen to be discontinuous in the union between the monads (Figure 4j-k), so tetrads are of the acalymmate type.

Pollenkitt

In all the PF and FSF of the species analysed it was observed that pollen grain presents pollenkitt, in both monads (Figure 4v-x) and tetrads (Figure 4v'-x'). The pollen grain of FPF does not form pollenkitt. Pollenkitt is found in the form of small droplets distributed over the entire pollen surface (Figure 4v,v'). Tests with Lugol indicate the absence of carbohydrates in this substance (Figure 4w,w'). In positive tests with Sudan III, the pollenkitt is coloured dark orange indicating its lipidic nature (Figure 4x,x').

Secondary pollen presentation

This reproductive strategy is present in the PF of *T. formosa* and the FSF of dioecious species (Figure 5a-o). In all species the mechanism can be summarized as follows:

Table II. Morphology and dispersal units of the pollen in the species analysed of Gardenieae. Abbreviations: EDG= equatorial diameter of pollen grain, LPG= length of the polar axis of the pollen grain, TD: tetrad diameter. *: data from Judkevich et al. (2015).

Species	Unit of dispersion	Pollen grain (µm)	Pollen grain shape	Exine	Apertures
Tocoyena formosa (Figure 4g, l, q)	monads	LPG: 47.9–55.1 EDG: 47.3–54.8	oblate-spheroidal	foveolate thick: 1.2–2.2 µm	3-porate
Cordiera concolor (Figure 4h, m, r)	monads	LPG: 27.7–30.7 EDG: 31.1–33.9	suboblate	reticulate, heterobrocate thick: 1.1–1.7 µm	3-colpore; colpus with acute ends,; endoaperture lolongate
Genipa americana (Figure 4i, n, s)	monads	LPG: 29.7–34.1 EDG: 31.5–36.5	oblate-spheroidal	reticulate, heterobrocate thick: 0.8–1.6 µm	3-colpore; colpus with acute ends; endoaperture lolongate
Randia calycina * (Figure 4j, o, t)	tetrahedric tetrad TD: 43.0–63.0 µm	LPG: 27.3–29.8 EDG: 32.7–33.7	oblate-spheroidal	psilate (to foveolate, present paper) thick: 1.2–1.5 µm	3-porate
	decussate tetrad TD: 55.1–56.4 µm	LPG: 27.6–28.8 EDG: 33.0–34.2			
Randia heteromera (Figure 4k, p, u)	tetrahedric tetrad TD: 53.5–65.6 µm	LPG: 26.0–36.7 EDG: 37.2–45.1	oblate-spheroidal	psilate to foveolate thick: 1.0–1.7 µm	3-porate
	decussate tetrad TD: 57.3–62.9	LPG: 27.6–32.9 DEG: 36.4–44.1			

Phase 1 (young bud; Figure 5a): the corolla lobes are folded, this pressure causes the anthers, style and stigma to be in direct contact. The pressure generated by the increase in volume of the anthers during microsporogenesis modifies the surface of the stigma and style, generating depressions (Figure 5i-j).

Phase 2 (pre-anthesis at beginning of the floral anthesis; Figure 5b): the corolla lobes begin to unfold and the pressure towards the inside of the bud decreases. The microgametogenesis is finalized and anther dehiscence occurs. The pollen load of each theca is deposited in the grooves of the surface of the pollen presenter. In *T. formosa*, *G. americana* (Figure 5d), and *Randia spp.* (Figure 5e-f) the pollen presenter is the stigma, whereas in *C. concolor* it is the style (Figure 5g-k). In both cases the surface of the pollen presenter has ridges in which pollen is deposited (Figure 5i-k). In addition, in *C. concolor* the style may present some simple trichomes that contribute to retaining pollen (Figure 5j).

Phase 3 (anthesis; Figure 5c): corolla lobes are fully deployed and anthers are away from the style and stigma. The pollen deposited on the pollen presenter is exposed to the pollinators.

In addition, in *T. formosa* there is a mechanism of protandry, the flower presents first a staminate period and then a pistillate one. The secondary presentation of pollen occurs simultaneously in the staminate period of the protandry, when the pollen is deposited in the closed stigma (Figure 5l-m). During the anthesis of the flower the pollinators collect the pollen (Figure 5n) and then the stigmatic branches unfold exposing the receptive surface (pistillate period of the protandry; Figure 5o).

DISCUSSION

Floral morphology

The flowers of *Tocoyena formosa* are perfect and their general morphology is similar to that of other Rubiaceous with perfect flowers (Robbrecht 1988). However, considering the floral structure of *Cordiera concolor, Genipa americana, Randia calycina*, and *R. heteromera* with their functional or cryptic dioecy, it might be possible that this type of dioecy has been overlooked in other representatives of the family.

In Rubiaceae species presented a cryptic dioecy masked by a morphological heterostyly (floral dimorphism with a long-styled morph and a short-styled morph), as is the case in *Chassalia corallioides* (Tribe Psychotrieae, Pailler et al. 1998), *Morinda villosa* (Tribe Morindeae, Sugawara et al. 2011), *Mussaenda parviflora* (Tribe Mussaendeae, Naiki & Kato 1999), *Mussaenda pubescens* (Li et al. 2010), *Pagamea duckei* (Tribe Gaertnereae, Terra-Araujo et al. 2012) and *Psychotria rubra* (Tribe Psychotrieae, Watanabe et al. 2014). Terra-Araujo et al. (2012) suggest that as in the case of *Pagamea duckei* with observations based only on floral morphology heterostyly may have been incorrectly inferred in other Rubiaceae. In the above species it is proposed that the combination of cryptic dioecy and morphological heterostyly suggest that the dioecy may have evolved from heterostyly.

However, in the tribe Gardenieae heterostyly is absent (Robbrecht 1988). In the species here analysed, it has been observed that there is a similarity in the structure of the anther and the development of pollen between the perfect flowers and the FSF of the dioecious species. However, in the FSF the gynoecium is underdeveloped (personal observation). This could be related to an origin from a perfect flower.

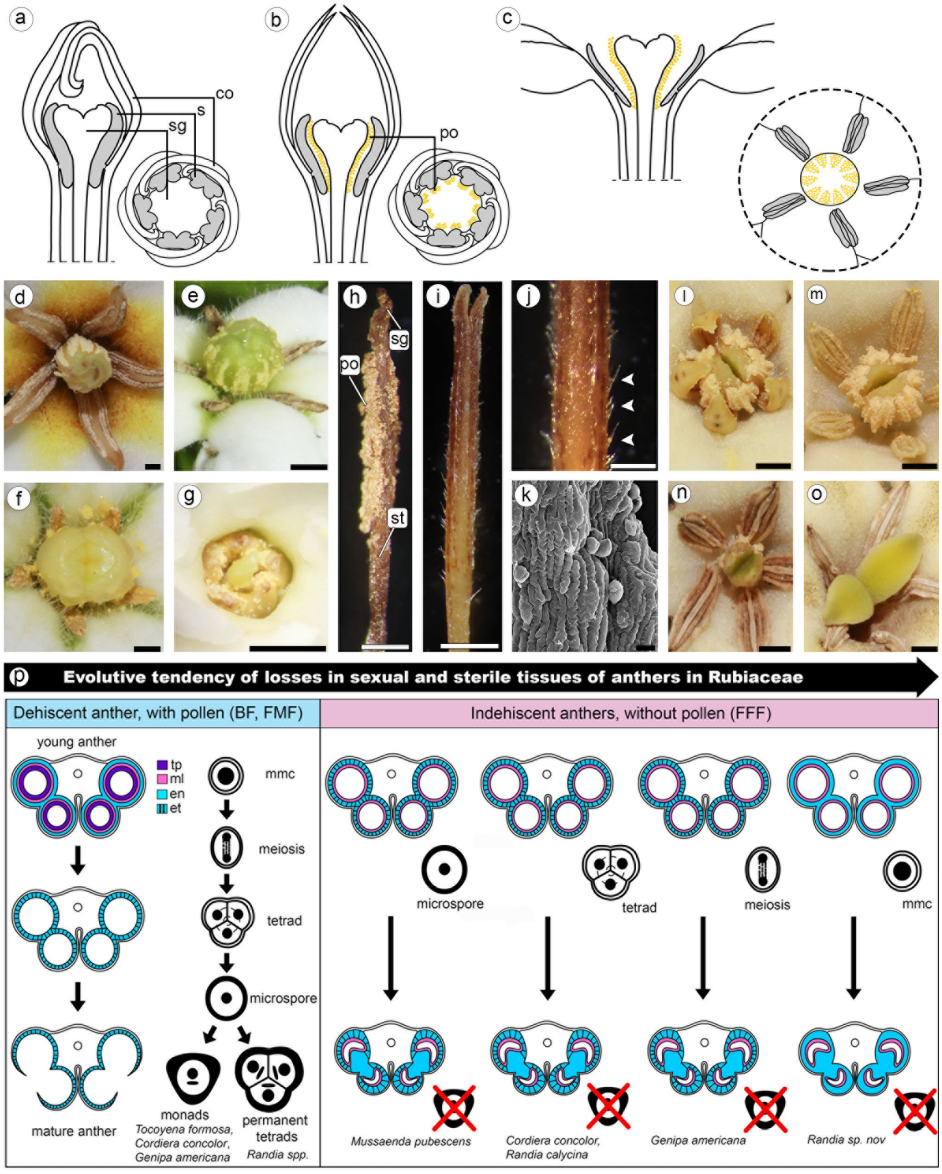

Figure 5. a-o, Mechanism of secondary pollen presentation. p, Hypothetical evolutionary tendency of losses of tissues of anthers in Rubiaceae taking into account an anther with normal development of its tissues versus abnormal anthers. a-c, Representative outlines of the stages of the presentation mechanism in lateral (left) and apical (right) views. a, Phase 1 (young bud). b, Phase 2 (pre-anthesis at beginning of the floral anthesis). c, Phase 3 (post anthesis). **d-f,** Pollen is presented on the stigma surface. d, Genipa americana, e, Randia calycina, f, Randia heteromera **g-k,** Cordiera concolor, pollen is presented on the style surface. h, Lateral view of the style with pollen (fixed material). i, Lateral view of the style without pollen, note the furrows on its surface (fixed material). j, Detail of the surface of the style with trichomes. k, Detail of the surface of style close to a furrow where the pollen grains are retained (SEM). l-o, Protandry in Tocoyena formosa. l-m, Phases 2 and 3 of secondary pollen presentation, stigma with pollen on the surface. n, The pollen was removed by the pollinators from the stigma surface. o, The branches of stigma unfold. p, At the left, the anthers of the FSF or PF with a normal development of their tissues and pollen are represented, while the abnormal anthers of the FPF are represented to the right. The top row corresponds to anthers when pollen development stops (stage X) and the bottom row corresponds to indehiscent anthers with collapsed pollen sacs and aborted tissue. Abbreviations: en= endothecium without thickenings; et= endothecium with thickenings; ml= middle layers; mmc= microspore mother cells; po= pollen; sg= stigma; st= style; tp= tapetum. Scales: d-g, 1 mm; h-i, 0.5 mm; j, 0.2 mm; k, 20 µm; l-o, 2 mm.

Pollen development

In the PF and FSF of the studied species, pollen follows a general pattern of development seen in most angiosperms, but the pollen grains of the *Randia* species are released in the form of permanent tetrads. A similar process of development has also been described in other Rubiaceae, such as *Mitriostigma axillare* (Hansson & El-Ghazaly 2000) and *Gardenia jasminoides* (Yue et al. 2017), both with perfect flowers and with pollen in permanent tetrads. Conversely, in FPF, the pollen development is outside of this general pattern. In the analysed Gardenieae species the pollen development stops at different stages: in the mmc stage (before meiotic division) in *R. heteromera*, in meiosis of the mmc in *G. americana*, and in tetrads with callose in *C. concolor* and *R. calycina*. In Rubiaceae, the only known case of a species with imperfect flowers in which pollen development has stopped was in *Mussaenda pubescens* (Li et al. 2010). In this species the pollen development in the pistillate flowers reaches the stage of free microspores and later, the nucleus of the microspores is fragmented.

In other Angiosperms it has been observed that the male sterility in pistillate flowers involves different cases: 1) the sporogenous tissue may not be formed, as is the case of *Schinopsis balansae* (Anacardiaceae, Gonzalez 2016); 2) pollen development is not completed, this is the case in *Consolea* spp. (Strittmatter et al. 2006) and *Opuntia stenotepala* (Flores-Rentería et al. 2013), both Cactaceae, in which the mmc abort; in *Actinidia deliciosa* (Actinidiaceae, Coimbra et al. 2004) the development reaches the microspore stage but with abnormalities in its cytoplasm and nucleus; 3) pollen development is complete but the anther is indehiscent, this occurs in *Pseuduvaria trimera* (Annonaceae, Yang & Xu 2018) and some Sapindaceae such as *Cardiospermum grandiflorum* and *Urvillea chacoensis* (Solís et al. 2010), *Allophylus edulis* (González et al. 2014), and *Magonia pubescens* (González et al. 2017).

Arrested pollen development in FPF of Gardenieae at different stages (depending on the species) could be interpreted as different degrees of transition to a morphological dioecy.

Anther wall

The persistence of the middle layers has shown variation between the anthers of the different types of flowers analysed. In PF and FSF these layers collapse early, as described for perfect flowers of other Rubiaceae (Dedecca 1957, Galati 1991, Hansson & El-Ghazaly 2000, Vinckier & Smets 2005, Magalhães Souza et al. 2008, Romero et al. 2017, Yue et al. 2017). However, in the FPF of *C. concolor, G. americana* and *R. calycina*, the anther middle layers may persist even when the endothecium of the anther has already thickenen. It has been observed that in flowers with fertile pollen and flowers with sterile pollen of *Actinidia deliciosa* (Actinidiaceae, Falasca et al. 2013) both the tapetum and the middle layers have secretory activity. Falasca et al. (2013) suggested that in *A. deliciosa* flowers with pollen sterility, the degeneration of the tapetum and the middle layers was retarded in comparison with the male-fertile pollen, even the middle layers persist after the collapse of the tapetum. According to these authors, when the middle layer does not degenerate earlier than the tapetum it can assume its activity. This delay of the two tissues might have prolonged secretion of exine components, resulting in microspore abnormalities. For the analyzed species of Gardenieae, a suggestion similar to that was proposed in *A. deliciosa* cannot be made since studies should be carried out with other types of techniques.

Anther dehiscence

Wilson et al. (2011) synthesize the sequence of key factors for the process of anther dehiscence to take place: a) the endothecium develops fibrous thickenings, b) the septum between the pollen sacs of a theca undergoes enzymatic lysis by a process of programmed cell death, and c) the stomium is broken by the tension caused by both the increase in size and volume of the pollen grains inside the pollen sacs and by the dehydration of the anther. This sequence of events is observed in the anthers of *T. formosa* (PF) and *Randia* (FSF), culminating in the dehiscence of the anthers and release of the pollen grains (monads or tetrads, respectively). However, in the anthers of the FSF of *C. concolor* and *G. americana* the septum develops thickening similar to that of the endothecium in the wall of the anther, it does not retract and consequently there is no fusion of the pollen sacs in a single locule per anther. In these species the lysis of the septum is not an essential factor for dehiscence to occur; the anthers open up since the rest of the factors associated with dehiscence are fulfilled.

On the other hand, anther dehiscence does not occur in species with FPF although in *C. concolor, G. americana* and *R. calycina* the endothecium develops thickening.The absence of completely developed pollen and the retraction of the sporogenic tissue may be related to the indehiscence of the anther.

Evolutionary trend of anthers in dioecious species of Rubiaceae

Based on the results of this study and in what was observed in *Mussaenda pubescens* by Li et al. (2010) we suggest a possible evolutionary sequence for the loss of staminate function in pistillate flowers of Rubiaceae (Figure 5p). The species show a trend for a reduction in the complexity of the anther, starting from a functional anther correspond to the PF of *T. formosa* or the FSF of any of the species here analysed (Figure 5p, left). Progressive changes in sterile anthers of the FPF comprise the loss of the anther dehiscence capacity, disappearance of endothecium thickening, and the detention of pollen development (Figure 5p, right). In the upper right part of figure 5p, it is represented the moment in which the development of the pollen stops (state X or arrested development) and in the lower part are the indehiscent anthers with the sacs of collapsed pollen and with the aborted tissues in the different studied species. In *Mussaenda pubescens* (Li et al. 2010), the anther is indehiscent, the endothecium presents thickenings, and the development of pollen reaches to the microspore stage; in *Cordiera concolor* and *Randia calycina*, the anther is indehiscent, the endothecium presents thickenings, and pollen development is up to tetrad stage; in *Genipa americana,* the anther is indehiscent, the endothecium presents thickenings, and the development of pollen reaches to the stage of meiosis of the mmc; and in *Randia heteromera*, the anther is indehiscent, the endothecium lacks thickenings, and the development of pollen reaches to the stage of mmc (Figure 5p, right).

These evidences supports the hypothesis that in the FPF of the dioecious species of Rubiaceae a total reduction of the anther is occurring, with a tendency to achieve a completely imperfect flowers in its structure.

Characters of anthers and pollen and their taxonomic relevance

Placentoid

This structure was defined by Chatin (1866) as a sterile anther wall tissue, either parenchymal or tapetal in nature, which projects from the septum and invades the anther locule.

It has been described in several families of angiosperms (Table III), but so far it has not been recognized in Rubiaceae. Of the species analyzed in this study, the placentoid is present in *Tocoyena formosa* and in imperfect flowers of the *Randia* species, being the first records for the family.

According to Chatin (1866) and Endress (2011), this tissue contributes to the formation of pollen by increasing the contact surface between the tapetum and the sporogenic tissue. The placentoid would disappear between the end of pollen ripening and the anther anthesis, when it loses its function, since, according to Chatin (1866) and Rezanejad (2013), if it persists it could obstruct the release of pollen. In contrast, Passarelli and Cocucci (2006) have observed that in the anthers of *Solanum stuckertii*, *S. confusum*, and *S. glaucophyllum* the placentoid expands and would push the pollen grains against the anther wall, favoring their dispersal.

In both *Randia* species, as well as in other Angiosperms species (Table III), the placentoid has its maximum development in the young anthers and then it retracts, whereas in *T. formosa* persists even after dehiscence and develops thickenings similar to those of the endothecium. This suggests that in *T. formosa* the placentoid would fulfill three functions: in the young anther it might collaborate with the formation of pollen, while in the mature anther it would push the pollen grains against the wall and would participate in the dehiscence of the anther together with the endothecium. The studies conducted here show that the placentoid is a character to be considered in future analyses in other Rubiaceae species involving anther ontogeny. By analyzing other families comparatively where placentoid tissue has been described (Table III), it is proposed to classify it in four types according to its structure for possible use as a taxonomic character: Type I: parenchymatic placentoid, Type II: tapetal placentoid, Type III: mixed placentoid (parenchyma + tapetal) and Type IV: similar to type III, but the parenchyma develops fibrous thickening at maturity. So far this last type has only been described in *T. formosa*.

Orbicules (Ubisch bodies)

Considering the number of genera in the Rubiaceae (620, Govaerts et al. 2011) there are few studies on orbicules in the family. The most comprehensive study that focused on Rubiaceae orbicules was conducted by Verstraete et al. (2011). They found orbicules in 40 of the 64 species analysed. Within the Rubiaceae family, orbicules were also reported in different tribes: Catesbaeeae, Cinchonae, Coptosapelteae, Isertieae, Rondeletieae, and Naucleeae (Huysmans et al. 1997), Catesbaeeae-Chiococceae-*Exostema* complex (Huysmans et al. 1999); Naucleeae and Hymenodictyeae (Verellen et al. 2007). The only study focused on the tribe Gardenieae found orbicules in nine of the 22 species studied (Vinckier et al. 2000), including *Cordiera pilosa*. In the present study, we confirm the presence of orbicules for the genus and describe it for the first time in *C. concolor*. In species of *Cordiera* orbicules have a spherical shape, which would place them in type 3 in the classification of Huysmans et al. (1997). Ultrastructural studies are necessary to give a more detailed description of the *C. concolor* orbicules.

Pollen

Pollen morphology: In Rubiaceae the pollen grain apertura type is an identifying characteristic and it is usually useful for making phylogenetic inferences (Dessein et al. 2005). The species of Gardenieae studied presented 3-porate pollen grains in *T. formosa* and in the *Randia* species, and 3-colporate pollen grains

Table III. Species with placentoid in the anther, type, duration and bibliographic citation. Abbreviations: pa: parenchymatic placentoid; ta: tapetal plancetoid; p+t: parenchymatic and tapetal placentoid.

Family	Species	Type	Retraction time	Thickenings	References
Bignoniaceae	*Jacaranda mimosifolia* (PF)	pa	no data	no	Galati & Strirrmatter 1999
	Tabebuia ochraceae (PF)	pa	tetrads stage	no	Bittencourt 1996
	Tabebuia pulcherrima (PP)	pa	tetrads stage or latter	no	Bittencourt & Mariath 1997
	Campis radicans (PF)	pa	no data	no	Tütüncü Konyar & Dane 2013
Caryophyllaceae	*Psammosilene tunicoides* (PF)	ta	pollen grains	no	Qu et al. 2010
Gentianaceae	*Gentiana lutea* (PF)	ta	after tetrads stage	no	Yankova-Tsvetkova & Yurukova-Grancharova 2009
	Gentiana cruciata (PF)	ta	no data	no	Yankova & Yurukova 2010
Paulowniaceae	*Paulownia tomentosa* (PF)	pa	no data	no	Erbar & Gülden 2011
Rubiaceae	*Tocoyena formosa* (PF)	p+t	post anthesis	yes	present paper
	Randia calycina (FPF + FSF)	ta	tetrads stage (FPF), pollen grains stage (FSF)	no	present paper
	Randia heteromera (FPF + FSF)	ta	mmc stage (FPF), pollen grains stage (FSF)	no	present paper
Solanaceae	*Petunia hybrida* (PF)	pa	no data	no	Rezanejad 2013
	Solanum confusum (PF)	p+t	just before dehiscence	no	Passarelli & Cocucci 2006
	Solanum glaucophyllum (PF)	p+t	just before dehiscence	no	Passarelli & Cocucci 2006
	Solanum stuckertii (PF)	p+t	houres after dehiscence	no	Passarelli & Cocucci 2006

in *C. concolor* and *G. americana*, this type being the one that prevails in Rubiaceae (Robbrecht 1988). In *Cordiera*, 3-colporate pollen grains are a characteristic of the genus that distinguishes it from its sister genus *Alibertia,* which has 3-porate pollen grains (Persson & Delprete 2010).

In the non-acetolized pollen grains, an outstanding structure of projected intine has been observed from each aperture, including a protoplast, called oncus (Hyde 1955, Tilney & Van Wyk 1997). This structure is considered to have evolved independently several times as it has also been observed in several Rubiaceae taxa of different tribes (including Gardenieae, Yue et al. 2017). It is mentioned for the first time here in *Cordiera*, *Genipa*, *Randia*, and *Tocoyena*.

In *Vangueria infausta* (tribe Vanguerieae, Rubiaceae) an association has been found

between the outstanding oncus and the cellular structure of the epidermal stigmata that would facilitate the secondary presentation of pollen thanks to the connections provided by the protruding onci between the pollen grain and the epidermis of the pollen presenter (Tilney et al. 2014). Considering that the species analysed have a secondary presentation of pollen, future ultrastructural and histochemical studies of stigma and pollen may reveal whether a similar relationship exists.

Pollen dispersal units: two types of dispersal units have been found: 1) monads in *C. concolor, G. americana* and *T. formosa*, and 2) permanent tetrads in *Randia* species. Pollen dispersal in tetrad form is known in at least 52 families of Angiosperms (Copenhaver 2005). Rubiaceae, tetrads are found in *Gleasonia* (tribe Henriquezieae, Bremekamp 1957, De Block & Robbrecht 1998, Robbrecht & Manen 2006), and in 11 genera of Gardenieae (Hallé 1967, Robbrecht & Puff 1986, Robbrecht 1988, Persson 1993, Hansson & El-Ghazaly 2000), of which only *Casasia* is dioecious (Gustafsson & Persson 2002). Of the 17 families other than Rubiaceae in which pollen release in the form of permanent tetrads has been recorded species like *Randia* have been found whose flowers are also imperfect (Table IV). It has been suggested by some authors that the evolution of pollen dispersal from monads to different forms of aggregates (permanent tetrads, polyads, viscin threads, and pollinia) has occurred independently at least 39 times in angiosperm (Davies et al. 2004, Harder & Johnson 2008).

At least in *Randia*, the tetrad pollen dispersal has been mentioned by several authors (Fagerlind 1948, Brenekamp 1957, Hallé 1967, Persson 1993, Gustafsson & Persson 2002, Judkevich et al. 2015). Considering the observation with SEM, the acetolized tetrads of *Randia* are slightly acalymmate, as they have a moderate exine discontinuity between the joints of each monad. Recently, in *Gardenia jasminoides*, the ultrastructure of the tetrads revealed that the pollen grains that make it up are linked by the intertwining of the tectum and the columella in the opening areas, areas without openings, and center of the tetrad (Yue et al. 2017). In *Randia,* similar studies would be necessary to find out which stratum of the exine participates in the union between the grains.

Pollenkitt: it was found on the surface of pollen obtained from dehiscent anthers from PF and FSF, it is lipidic and pigmented (Knoll 1930) because reacts positively to Sudan III. Pollenkitt is found in all families of the angiosperms and is synthesized by the cells of the tapetum (Hesse 1981, Pacini & Hesse 2005). It is the first time that the presence of pollenkitt, has been reported for the four genera treated in this paper. In these species we have observed that the pollen is agglutinated by pollenkitt, which suggest the adhesion capacity of this substance (Dobson & Bergtröm 2000, Pacini & Hesse 2005).

Secondary pollen presentation

In this mechanism the pollen is presented to the pollinators in a structure different from the anther such as style or stigma (Imbert & Richards 1993). It was described by Sprengel (1793) in *Campanula* (Campanulaceae) which was used as a model of study. In this genus the style and stigmatic branches have retractable trichomes. When the anthers open at the button stage the pollen is retained by these trichomes. As the corolla opens the style lengthens leaving the pollen exposed to the pollinators, after which the stigma branches spread out (Leins & Erbar 1990, Howell et al. 1993, D'Antraccoli et al. 2019). Secondary pollen presentation has been recorded in more than 16 families of Angiosperms which include Araceae, Asteraceae, Campanulaceae, Cucurbitaceae, Fabaceae, Myrtaceae, Proteaceae, Santalaceae,

Table IV. Species with imperfect flowers and pollen release in the form of permanent tetrads. Abbreviations: PF: perfect flower; D: dioecious; PF: pistillate flower; FPF: Functional pistillate flower; FSF: functional staminate flower; M: monoecious; SF: Staminate flower.

Family	Species	Sexuality	Reference
Annonaceae	*Pseuduvaria trimera*	M: FSF/FPF	Yang & Xu 2018
Araceae	*Xanthosoma sp.*	M: SF/PF	Ali 1988, Mayo & Bogner 1988
	Chlorospatha sp.	M: SF/PF	Croat & Hanno 2015
Cucurbitaceae	*Borneosicyos sp.*	D: SF/PF	Schaefer & Renner 2010
	Gurania sp.	M: SF/PF	Ali 1988, Schaefer & Renner 2010
	Psiguria sp.	M: SF/PF	Schaefer & Renner 2010
Cytinaceae	*Cytinus sp.*	M/D: SF/PF	Johnson et al. 2011, Burgoyne 2006
Datiscaceae	*Datisca glomerata* and *D. cannabina*	D: SF/PF	Ali 1988, Davidson 1973
Ebenaceae	*Dyospyros manni* and *D. longiflora*	D: SF/PF	Geeraerts et al. 2009
Empetraceae	*Ceratiola sp., Corema sp., Empetrum nigrum,* and *E. rubrum*	D: SF/PF	Kim et al. 1988
Ericaceae	*Pernettya rigida*	D: FSF/FPF	Anderson et al. 2000
Hydrocharitaceae	*Elodea Canadensis*	D: SF/PF	Wylie 1904
Juncaceae	*Distichia sp., Patosia sp.*, some species of *Oxychloe*	D: SF/PF	Balslev 1998, Gonzáles et al. 2016
Melicaceae	*Trichilia catigu., T. elegans,* and *T. pallida*	D: SF/PF	Souza et al. 2001
Monimiaceae	*Hedycarya angustifolia* and *H. arborea*	D: SF/PF	Sampson 1977, Ali 1988
Myrothamnaceae	*Myrothamnus sp.*	D: SF/PF	Kubitzki 1993
Nepenthaceae	*Nepenthes sp.*	D: SF/PF	Kato 1993, Adam 1998
Sapindaceae	*Magonia pubescens*	M: FSF/FPF	González et al. 2017
Typhaceae	*Typha caspica, T. latifolia, T. minima, T. shuttleworthii, T. lugdunensis*	M: SF/PF	Hamdi et al. 2010
Winteraceae	*Tasmannia sp.*	D: SF/PF	Vink 1988

and Zingiberaceae, among others (Howell et al. 1993, Jadeja 2015, Fan et al. 2015).

Secondary pollen presentation is common in Rubiaceae (Hallé 1967, Howell et al. 1993, Yeo 1993, Robbrecht 1988). Puff et al. (1996) recognizes four types for the family: 1) placement of pollen on the style, 2) placement of pollen on the style and on the outer surface of the stigma, 3) placement of pollen on the outer surface of the stigma, and 4) placement of pollen partially or largely on the inner surface of the stigma. Unlike the basic model of *Campanula* (Sprengel 1793, D'Antraccoli et al. 2019) in the studied species of Gardenieae the style does not lengthen, and there are trichomes only in *C. concolor* which are not retractable. The pollen is retained in the grooves on the surface of the pollen presenter organ. Taking into account the proposed classification

for the family, type 1 of secondary presentation of pollen occurs in *C. concolor*, whereas in the other species type 3 occurs. Also, the secondary presentation of *T. formosa* pollen is part of a more complex reproduction mechanism, because it occurs simultaneously with the staminate period of the flower during the protandry, when the stigma is not yet receptive. De Block & Igersheim (2001) have described in detail the anatomical basis of this combination of mechanisms in the species of *Rutidea* and *Nichaella* (tribe Pavettea). According to Bremekamp (1966), this combination of mechanisms occurs in Gardenieae and related tribes and has more taxonomic importance than other single reproductive strategies.

CONCLUSIONS

Most studies on dioecious species of Rubiaceae lack detailed anatomical analysis of the floral organs. With the present work, it was possible to find the structural cause that generates differences in the functioning of an anther of a perfect flower or of a staminated one in comparison with that of a pistillate in species of the tribe Gardenieae. It was found that the anthers lose the function of producing pollen because an arrested development of pollen occurs. The results obtained may be useful as a basis for future studies of programmed cell death in other dioecious species of Rubiaceae.

On the other hand, many cases have been studied in Rubiaceae in which the dioecy evolves from heterostyly. The present study reveals, from an anatomical point of view, cases in which dioecy could be related to an origin from a perfect flower in this family. Also, the results found can be used to make comparisons with other Rubiaceae to evaluate whether there is a tendency to reduce anthers in species of the family with staminate flowers.

Our data provide information on anther and pollen morphology, pollen dispersal units, and pollen presentation mechanisms, which may be taxonomically relevant in future evolutionary studies in the family Rubiaceae.

Additionally, with this anatomical analysis it was possible to describe a new type of placentoid, until now proper to the species *Tocoyena formosa*.

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Appendix

List of the analyzed species of Gardenieae and their voucher information.

Cordiera concolor (Cham.) Kuntze. Argentina. Misiones, San Ignacio, Teyú Cuaré, 01 Mar 2013, Judkevich MD & Salas RM 11 (FPF). Idem, 23 Apr 2016, Judkevich MD & Salas 73 (FSF). Idem, 23 Apr 2016, Judkevich MD & Salas 74 (FPF).

Genipa americana L. Argentina. Formosa, Guaycolec, Estancia "Bella Mar", 11 Sep 2014, Judkevich MD & Salas RM 53 (FPF). Idem, Estancia Miriquiná, 28 Jan 2015, Judkevich MD & Salas RM 58 (FPF). Idem, Judkevich MD & Salas RM 59 (FPF). Idem, 22 Dic 2015, Judkevich MD et al.. 73 (FPF). Idem, Reserva Biológica Guaycolec, 16 Nov 2016, Judkevich MD et al.., 84 (FPF). Paraguay. Asunción, Campus UMA, 24 Nov 2016, Judkevich MD et al.. 85 (FSF).

Randia calycina Cham. Argentina. Formosa, Guaycolec, Estancia "Bella Mar", 10 Sep 2014, Judkevich MD & Salas RM 49 (FPF). Idem, Judkevich MD & Salas RM 52 (FSF). Idem, "Monte Lindo Chico", 11 Sep 2014, Judkevich MD & Salas RM 54 (FSF). Chaco, Primero de Mayo, Colonia Benitez, 28 Nov 2014, Judkevich MD & Salas RM 57 (FPF). Idem, 01 Oct 2015, Judkevich MD & Salas RM 70 (FPF). Idem, 01 Oct 2015, Judkevich MD & Salas RM 71 (FPF). Idem, 01 Oct 2015, Judkevich MD & Salas RM 72 (FPF).

Randia heteromera Argentina. Corrientes, Riachuelo, 17 Sep 2014, Judkevich MD & Salas RM 55 (FSF). Idem, Judkevich MD & Salas RM 56 (FPF). Idem, Puente Pexoa, 14 Sep 2016, Judkevich MD et al.. 75 (FSF). Idem, 14 Sep 2016, Judkevich MD et al.. 77 (FPF). San Cosme, Las Lomas, Ensenada Grande, 29 Aug 2015, Judkevich MD et al.. 61(FSF). Idem, Judkevich MD et al.. 62 (FPF). Dpto: Dan Miguel, Estancia "Tranquita", 11 Sep 2015, Judkevich MD et al.. 63 (FPF). Idem, 11 Sep 2015, Judkevich MD et al.. 64 (FSF). Idem, 11 Sep 2015, Judkevich MD et al.. 65 (FPF). Idem, Estancia "Santa Julia", 11 Sep 2015, Judkevich MD et al.. 66

(FSF). Idem, 11 Sep 2015, Judkevich MD et al.. 67 (FPF). Idem, 11 Sep 2015, Judkevich MD et al.. 68 (FSF).

Tocoyena formosa (Cham. & Schltdl.) K. Schum. Paraguay. Asunción, Cerro Tobatí, 25 Nov 2016, MM et al.. 187 (PF). Idem, 25 Nov 2016, MM et al.. 188 (PF).

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Author contributions

RMS provided the plant material and supplied taxonomic data of the species. MDJ processed the plant material to make the histological preparations, took the microscopy photos, and prepared the figures. MDJ and AMG did the anatomical interpretations. MDJ and AMG contributed to the discussions and approved the final manuscript.