

Diversity of fig glands is associated with nursery mutualism in fig trees¹

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PREMISE OF THE STUDY: Fig trees (Moraceae) have remarkable enclosed inflorescences called figs or syconia. The flowers are pollinated by host-specific fig wasps that enter the fig to lay their eggs. This nursery pollination system is one of the most studied of tropical mutualism interactions, but the source of the volatiles that attract fig wasps to their specific host figs has not been confirmed. The fragrance is the basis of host selection and, therefore, of reproductive isolation among sympatric *Ficus* species. This study locates and characterizes the glands likely to be responsible for pollinator attraction and also protection from herbivory in the figs of nine *Ficus* species representing all the major lineages within the genus.

METHODS: Figs with receptive pistillate flowers were examined using light and scanning electron microscopy. Tests for histolocalization of substances were employed to detect glandular activity throughout the figs.

KEY RESULTS: A great diversity of glands is found throughout the fig, and for the first time, the sites producing fragrances are identified. Scent glands are present on the ostiolar bracts and the outer layers of the fig receptacle. Laticifers and phenolic-producing idioblasts, epidermis, and trichomes associated with fig protection occur on the ostiolar bracts, the fig receptacle, and floral tissues.

CONCLUSIONS: The volatiles produced by glands on the ostiolar bracts are candidate sources for the long-distance attraction of pollinator fig wasps. Scent glands on the outer layers of the receptacle may also play a role in chemical perception of the figs or may be related to their protection. The high cost to the plants if the figs are eaten and the temperature conditions required for nursery pollination are likely the factors that led to the selection of phenolic glands and laticifers during the group's evolution.

KEY WORDS *Ficus*; flower anatomy; fragrance; glandular trichomes; laticifers; Moraceae; osmophores; phenolic-producing epidermis; phenolic-producing idioblasts; secretory structures

Fig trees (*Ficus* L., Moraceae) and their pollinating agaonid wasps constitute an extremely specialized and extensively studied nursery pollination mutualistic system (Weiblen, 2002; Cook and Rasplus, 2003). This mutualism seems to have arisen about 75 million years ago in Eurasia and subsequently dispersed to other continents during periods of expansion of tropical forests (Cruaud et al., 2012). *Ficus* encompass approximately 750 species (Cruaud et al., 2012)

divided into 19 infrageneric sections, distributed mainly in tropical regions of all continents (Rønsted et al., 2008). A large diversity of ecologies and habits is observed across sections (and in some cases within sections). The genus is characterized by the inflorescence named as fig or syconium, which consists of an urn-shaped receptacle internally lined with numerous small “unisexual” flowers and which opens to the exterior through a small ostiole, closed by apical bracts (Datwyler and Weiblen, 2004).

The pollinating fig wasps depend on the figs for reproduction as their offspring develop in the fig flowers, while the fig tree benefits from the pollinating female wasps, which transfer pollen from their natal fig tree to another, assuring the production of seeds (Anstett et al., 1997). During pistillate flower anthesis, the fig releases a scent that is responsible for attracting female agaonid wasps (Ware et al., 1993). When the pollinating wasps reach figs, they enter the fig through the ostiole and lay their eggs in the ovaries of some flowers. During this process, they transfer pollen (actively or passively,

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depending on the wasp species) to pistillate flowers. After the oviposition/pollination, wasp larvae and fig seeds develop for the next several weeks. The emergence of wasp offspring coincides with the maturation of staminate flowers. Female wasps loaded with pollen disperse from their natal fig to another fig tree bearing receptive figs, beginning the cycle anew (Galil and Eisikowitch, 1968).

The fig fragrances constitute a key factor in the fig–fig wasp mutualism, playing a role in pollinator orientation and reproductive isolation of sympatric species, as each wasp species seems to respond to specific chemical compounds of its associated fig tree species (Chen and Song, 2008; Chen et al., 2009). These chemical compounds released by figs comprise mainly mono- and sesquiterpenes (Grison et al., 1999; Grison-Pigé et al., 2002; Dudareva and Pichersky, 2006; Hossaert-McKey et al., 2010). Experimental studies have revealed that these volatiles are emitted through the ostiole in *Ficus burtt-davyi*, section *Galoglychia* (van Noort et al., 1989), and are, more precisely, produced inside the fig cavity in *F. carica*, section *Ficus* (Hossaert-McKey et al., 1994).

Knowledge of the volatiles responsible for the attraction of pollinators by figs and their role in maintaining pollinator specificity and gene flow rapidly expanded, but there was not a parallel increase in understanding of the location and structure of the glands responsible for producing the volatiles. Thus, the aim of this study was to identify the putative glands responsible for the production of fragrances attractive to pollinating wasps and also those associated with fig protection, topics that are central to the fig–fig wasp mutualism, in the receptive fig of nine species representing different lineages of *Ficus*. In addition, we tested the hypothesis that the type and distribution of glands in figs are associated with biological attributes of the fig trees. Specifically, we chose four biological attributes of fig trees potentially related to the fig–fig wasp mutualism: (1) life form, related to pollination functioning, because life forms use different environment strata, e.g., shrubs and creepers (understory species), hemiepiphytes (canopy species), and freestanding (emergent or understory species); (2) breeding system, related to dispersion distance of the pollinator, because monoecious fig tree species seem to have a wider pollen gene flow (Nazareno et al., 2013); (3) pollination mode (active/passive), expressing pollination efficiency; and (4) fig size, representing the number of pollinating wasps required to potentially pollinate all flowers in the inflorescence, because larger figs are colonized on average by more wasps (Herre, 1989).

To improve the understanding of the distribution and activity of glands in *Ficus*, we also analyzed the discoid inflorescences of *Castilla elastica*, an entomophilous species (Sakai, 2001), belonging to the Castilleae tribe (sister group of the tribe Ficeae, which includes a single genus, *Ficus*) (Clement and Weiblen, 2009).

MATERIALS AND METHODS

Nine species belonging to at least seven *Ficus* sections (Fig. 1, Table 1) according to Cruaud et al. (2012) were selected for this study, representing independent lineages for the group. Figs were collected and processed and voucher specimens deposited at SPFR and PUH herbaria (Table 1).

The figs were collected at the phase when pistillate flowers are ready to be pollinated and stigmas become receptive, corresponding to the stage of attraction of pollinating wasps (Galil and Eisikowitch,

1968). Stigma receptivity was confirmed with an indicator paper (Peroxtesmo Ko, Macherey-Nagel, Düren, Germany), which detects the activity of esterase enzymes present on receptive stigmas (Dafni and Maués, 1998). Small pieces of the paper were moistened in distilled water and placed in contact with pistillate flowers of fresh figs. The paper turns bluish when it detects enzymatic activity.

The collected figs were cut in half and fixed in buffered formalin (Lillie, 1965) for 72 h, washed in distilled water, dehydrated in an ethanol series, and stored in 70% ethanol.

The presence of glands in the outer and inner surface of the figs was checked with scanning electron microscopy. Previously fixed figs were dehydrated in an ethanol series (Tucker, 1993), critical point dried in a critical point dryer (Bal Tec CPD 030, Balzers, Oberland, Liechtenstein), placed on stubs over carbon adhesive tape, and sputter coated with gold (Bal Tec SCD 050). Images were obtained using a JEOL JSM-6610LV scanning electron microscope (Akishima, Tokyo, Japan).

For anatomical characterization of glands, some fixed figs were freehand cross-sectioned, and others were dehydrated in an ethanol series, embedded in methacrylate-based resin (Gerrits, 1991), and sectioned at 5 μm thick on a rotary microtome. The sections were cut throughout the entire fig, including the region of ostiolar bracts, receptacle, staminate and pistillate flowers (Fig. 2). To study the chemical nature of substances produced by the fig glands, the follow reagents were employed in situ: 0.05% toluidine blue in phosphate buffer as a general stain (O'Brien et al., 1964), ferric trichloride to detect phenolic compounds (Johansen, 1940), Sudan III (Sass, 1951) and Sudan black B (Pearse, 1980) to detect lipophilic substances, Nadi reagent (David and Carde, 1964) to detect terpenes, periodic acid–Schiff's (PAS) reagent (Feder and O'Brien, 1968) to detect polysaccharides and xyldine Ponceau (Vidal, 1977) to detect proteins. The anatomical sections were observed and photographed using bright field optics in a Leica DM 4500 B light microscope (Wetzlar, Germany) coupled to a Leica DFC 320 digital camera.

Inflorescences with pistillate flowers of *Castilla elastica* were collected (see Table 1) and subjected to the same procedures described for *Ficus* species.

To formally test the hypothesis whether the occurrence of glands in figs is associated with species biological attributes, we used Mantel's test based on 999 permutations (Manly, 2006) to correlate the pairwise similarity matrices among species based on anatomic structures and biological characters. The anatomy matrix included 11 presence–absence variables representing secretory structure types and their distribution in the fig (i.e., scent gland in the ostiole, scent gland in the receptacle, laticifer in tepals, laticifer in the style, phenolic-producing idioblast in the style, phenolic-producing epidermis in the receptacle, phenolic-producing epidermis in the ovary, the outermost layer of cells of the nucellus, glandular trichomes at the flower base, glandular trichomes at the ostiole and glandular trichomes on the receptacle). The biological matrix included eight presence–absence variables representing the species' life forms, their reproductive systems and fig size (i.e., freestanding life form, hemiepiphyte life form, shrub life form, monoecious vs. dioecious reproductive system, active or passive pollination, fig diameter < 1 cm, fig diameter = 1–1.5 cm, fig diameter > 3 cm). Both anatomic and biological pairwise similarity matrices were estimated using the Jaccard index. For these numerical analyses, we used the Vegan package (Oksanen et al., 2013) of the R software (R Core Team, 2014).



FIGURE 1 Inflorescences of the studied species of *Ficus* and *Castilla*. (A) *F. auriculata*. (B) *F. citrifolia*. (C) *F. lyrata*. (D) *F. microcarpa*. (E) *F. montana*. (F) *F. obtusiuscula*. (G) *F. pumila*. (H) *F. tikoua*. (I) *F. variegata* (image credits: J.-Y. Rasplus). (J) *C. elastica* (image credit: V. G. Leite). Bars: 1 cm.

RESULTS

We found five types of glands in the inflorescences of the studied species: scent glands, laticifers, phenolic-producing idioblasts, phenolic-producing epidermis, and phenolic-producing trichomes (Table 2). Overall, such structures secrete terpenes, other lipophilic substances, and phenolic compounds. Structures secreting polysaccharides and proteins were not found in the receptive phase.

The occurrence and morphology of fig glands were not related to the biological attributes of the study species. Pairwise similarities

among species based on gland characters (i.e., types and their distribution) and on biological features (i.e., life form, reproductive system, pollination mode and fig size) were not significantly correlated (Mantel statistic $r = 0.229$, $P = 0.092$).

Scent glands—Two sites of fragrance production were observed in *Ficus* species according to the test of terpenes histolocalization: ostiolar outer bracts (Fig. 3) and the outer layers of the fig receptacle (Fig. 4). Scent glands were not found in *Castilla elastica*.

TABLE 1. Species of *Ficus* and *Castilla* selected for the study, location, voucher, and biological traits. Sections are according to Cruaud et al. (2012) except for *F. variegata* that is placed within section *Sycomor* because the node separating it from the remainder of section is not so strongly supported (white square: BP > 70% or PPMrBayes or PPBEAST > 0.95), and its pollinating wasps belong to the group of pollinators of section *Sycomor*. Symbol: * = Brazilian native species.

Species	Section	Location	Voucher	Life form	Breeding system	Pollination mode	No. of sampled inflorescences	Studied inflorescence	Size of inflorescence (cm)
<i>Castilla elastica</i> Sessé		IAC, Campinas, Brazil	M.B. Carvalho 122 & J.F. Benedito (IAC)	Freestanding	Androdioecious		2	"Female"	2.5
* <i>Ficus citrifolia</i> Mill.	<i>Americana</i>	USP Campus, Ribeirão Preto, Brazil	S.P. Teixeira et al., 79 (SPFR)	Hemiepiphyte	Monoecious	Active	5–10		1
<i>F. microcarpa</i> L. f.	<i>Conosycea</i>	USP Campus, Ribeirão Preto, Brazil	S.P. Teixeira et al., 81 (SPFR)	Hemiepiphyte	Monoecious	Active	5–10		0.6
<i>F. pumila</i> L.	<i>Ficus</i>	Jardim Recreio neighborhood, Ribeirão Preto, Brazil	S.P. Teixeira et al., 83 (SPFR)	Creepers	Gynodioecious	Passive	3–6	"Male"	3
<i>F. lyrata</i> Warb.	<i>Galoglychia</i>	USP Campus, Ribeirão Preto, Brazil	S.P. Teixeira et al., 80 (SPFR)	Freestanding	Monoecious	Active	3–6		3.3
* <i>F. obtusiuscula</i> (Miq.) Miq.	<i>Pharmacosycea</i>	USP Campus, Ribeirão Preto, Brazil	S.P. Teixeira et al., 82 (SPFR)	Freestanding	Monoecious	Passive	5–10		1
<i>F. montana</i> Burm. f.	<i>Sycidium</i>	USP Campus, Ribeirão Preto, Brazil	S.P. Teixeira & C. D. Souza 85 (SPFR)	Shrub	Gynodioecious	Active	5–10	"Male"	0.5
<i>F. auriculata</i> Lour.	<i>Sycomor</i>	USP Campus, Ribeirão Preto, Brazil	S.P. Teixeira & C. D. Souza 78 (SPFR)	Freestanding	Gynodioecious	Active	3–6	"Female"	3.5
<i>F. tikoua</i> Bureau	<i>Sycomor</i>	Jardim Recreio neighborhood, Ribeirão Preto, Brazil	S.P. Teixeira et al., 84 (SPFR)	Shrub	Gynodioecious	Passive	5–10	"Female"	1
<i>F. variegata</i> Blume	<i>Sycomor</i>	University of the Philippines Diliman, Manila, Philippines	L.J.V. Rodrigues 228 (PUH)	Freestanding	Gynodioecious	Active	3–6	"Female"	1.5

In all *Ficus* species, except for *F. citrifolia*, the terpenes are produced on the ostiolar bracts (Fig. 3A–I). In *F. obtusiuscula* and *F. variegata*, only mesophilic cells of the bracts are involved in the production of volatiles, while both epidermal and mesophilic cells play such a role in the other species (Table 2). In *F. auriculata* and *F. pumila*, epidermal cells on both adaxial and abaxial surfaces of the ostiolar bracts produce terpenes; in *F. lyrata* and *F. microcarpa*, terpenes are produced only in the epidermal cells on the adaxial surface, and in *F. montana* and *F. tikoua*, they are produced only in the epidermal cells on the abaxial surface.

Cells located on the external side of the inflorescence receptacle also produce terpenes in most species (Fig. 4A–G), with the exception of *F. auriculata*, *F. lyrata*, and *F. variegata*. Terpene droplets were observed in epidermal and subepidermal cells in *F. montana* and *F. pumila*. In *F. citrifolia*, *F. microcarpa*, *F. obtusiuscula*, and *F. tikoua*, only subepidermal cells produced terpenes (Table 2).

Laticifers—Branched laticifers with thin walls (Fig. 5A, B) were found scattered throughout the parenchymatic tissues and near the vascular bundles in the inflorescence receptacle of all studied species and in the ostiolar bracts of *Ficus* species and inflorescence bracts of *Castilla elastica* (Table 2). In the pistillate flowers of *Ficus*, laticifers were also observed in the floral pedicel except in *F. pumila* (Fig. 5C); in the tepals of *F. obtusiuscula*, *F. microcarpa*, and *F. montana*; and in the style of *F. obtusiuscula*. Laticifers were also observed in the pedicels of all the staminate flowers. Seven of the nine *Ficus* species have lipophilic substances in the latex composition (*F. auriculata*, *F. citrifolia*, *F. microcarpa*, *F. montana*, *F. obtusiuscula*, *F. pumila*, and *F. variegata*) (Fig. 5D, E), and in three of these species (*F. auriculata*, *F. montana* and *F. variegata*; Fig. 5F), terpenes were also detected.

Phenolic-producing idioblasts—Phenolic-producing idioblasts occur in the mesophyll of ostiolar bracts in all *Ficus* species studied (Fig. 6A, B; Table 2) and in the inflorescence bracts of *C. elastica* (Table 2). With the exception of *F. montana*, they also occur scattered throughout the parenchymatic tissue of the inflorescence receptacle of *Ficus* species (Fig. 6C, D; Table 2) and *C. elastica* (Fig. 6E, Table 2). In *F. auriculata*, *F. citrifolia*, *F. obtusiuscula*, *F. pumila*, and *C. elastica* (Fig. 6F), they can be seen in stigmas and subepidermal tissues of the style (Table 2).

Phenolic-producing epidermis—Ostiolar bracts (Fig. 6A, B), fig receptacle (Fig. 6C), and floral tissues (Fig. 6G–I) of *Ficus* species have a uniseriate epidermis that produces phenolic substances.

The outer ostiolar bracts of the nine *Ficus* species studied show both adaxial and abaxial surfaces with phenolics. For the inner bracts, the location of phenolic-producing epidermis is variable, being present on both surfaces in *F. auriculata*, *F. lyrata*, *F. montana*, *F. tikoua*, and *F. variegata*; only on the abaxial surface in *F. citrifolia*, *F. microcarpa*, and *F. obtusiuscula*; and only on the adaxial surface in *F. pumila*.

The outer surface of the fig receptacle of *F. auriculata*, *F. lyrata*, *F. obtusiuscula*, and *F. tikoua* is covered by a phenolic-producing epidermis. In *F. pumila*, only the inner epidermis of the receptacle is secretory. In *F. variegata*, both epidermises that cover the receptacle externally and internally are phenolic (Table 2).

The tepals of the flowers also have phenolic-producing epidermis in all species, except in *F. montana*. Cells of the ovary wall in flowers of

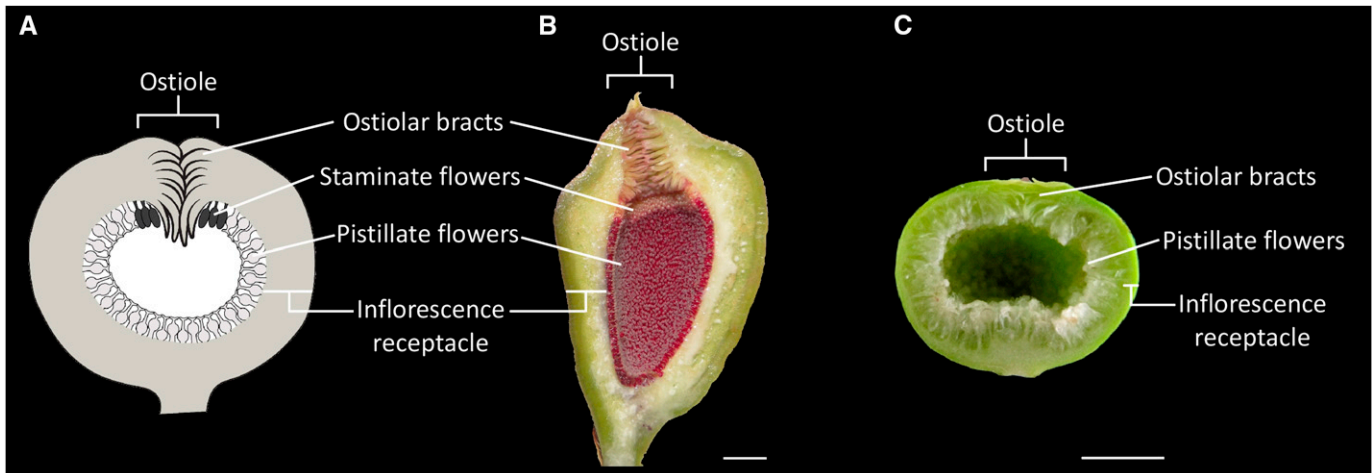


FIGURE 2 Internal view of the inflorescences of *Ficus* showing the flowers, bracts, and receptacle. (A) General scheme in a gynodioecious species. (B) *F. pumila*, a gynodioecious species. (C) *F. citrifolia*, a monoecious species. Bars = 1 cm.

F. tikoua and *F. variegata*, as well as the outermost layer of cells of the nucellus in flowers of *F. montana*, are also phenolic. In *F. microcarpa*, both cells of the ovary wall and the outermost layer of cells of the nucellus are phenolic (Table 2).

Phenolic-producing trichomes—Grouped glandular trichomes were observed between the innermost ostiolar bracts, on the inner surface of the fig receptacle, and between the tepals and ovary in *F. citrifolia* and *F. microcarpa* (Fig. 7A–D, Table 2). Such trichomes are composed of a short nonsecretory stalk (only one cell in *F. citrifolia* and one or two cells in *F. microcarpa*) and a biseriate, elongated head with four or more cells secreting phenolic compounds (Fig. 7D).

With the exception of *F. pumila* and *C. elastica*, the remaining studied species also exhibit glandular trichomes scattered throughout the outer side of the inflorescence receptacle (Fig. 7E–G, Table 2). In all of them, trichomes are composed of a unicellular stalk and a head secreting phenolic compounds with a variable number of cells: two to four in *F. auriculata*; three to four in *F. montana*; two in *F. citrifolia*, *F. tikoua*, and *F. variegata*; and one in *F. lyrata*. It was not possible to identify the number of head cells and the content of the glandular trichomes in *F. microcarpa* and *F. obtusiuscula*.

DISCUSSION

Figs of all studied *Ficus* species exhibit five types of glands traditionally reported for angiosperms (see Fahn, 1979, 1988, 1990, 2000, 2002): scent glands, laticifers, secretory idioblasts, secretory epidermis, and glandular trichomes. These structures are active in figs at the receptive phase, when the stigmas of pistillate flowers are receptive for pollination. Receptive fig fragrances generally include large amounts of terpenes (Grison et al., 1999; Grison-Pigé et al., 2002; Dudareva and Pichersky, 2006; Hossaert-McKey et al., 2010), and according to our results, scent glands producing terpenes were located in the ostiolar bracts and in the epidermis and subepidermal cells on the outside of the fig. As such, they are candidate production sites of the receptive fig fragrances responsible for pollinator attraction in the fig–fig wasp mutualism (Dudareva and Pichersky, 2006; Hossaert-McKey et al., 2010).

Laticifers, secretory idioblasts, secretory epidermis, and glandular trichomes are likely important in protecting the inflorescence and flowers against phytophagous animals, especially insects, as well as acting as a chemical barrier against sunlight radiation (Levin, 1973; Wagner, 1991; Beckman, 2000; Rodriguez-Saona and Trumble, 2000; Haslam, 2007; Ni et al., 2007; Agrawal and Konno, 2009; Konno, 2011; Silva et al., 2014).

Glands involved in attraction of pollinating wasps—The scent glands identified in ostiolar bracts and/or fig receptacle of *Ficus* concur with the concept of Fahn (1979), Vogel (1990), and Dudareva and Pichersky (2006) that they consist of cells specialized in the production and simultaneous release of terpenes in the form of droplets. The set of ostiolar bracts producing fragrances constitutes a scent gland belonging to the restricted type (see Fahn, 1979; Vogel, 1983, 1990; Endress, 1994; Marinho et al., 2014) as production is restricted and localized. The glands of the fig receptacle constitute scent glands belonging to the diffuse type (see Fahn, 1979; Vogel, 1983, 1990; Endress, 1994; Marinho et al., 2014) because they are composed of cells distributed along the inflorescence axis that are responsible for secreting fragrances.

In addition, the scent glands of *Ficus* species are of the mesophilic type (Endress, 1994); not only the epidermis but also the subepidermal tissues act in scent production and/or release. The additional production of terpenes in deeper tissues explains the absence of terpene droplets on the fig epidermis in some species (*F. citrifolia*, *F. microcarpa*, *F. obtusiuscula*, and *F. tikoua*) since epidermal cells instantaneously release the volatiles to the environment (see Vogel, 1990).

These scent glands produce volatiles responsible for attracting pollinating fig wasps. Fig volatiles seem to play a double role in *Ficus* pollination (Gibernau et al., 1998), i.e., (1) chemotaxis to guide the newly emerged wasps toward a tree that bears receptive figs (Ware and Compton, 1994) and (2) chemical stimuli to trigger the wasp behavior of searching for the ostiole when wasps land on the fig and start to explore its surface (Frank, 1984; Hossaert-McKey et al., 1994; Gibernau et al., 1997, 1998; Wang et al., 2013). Thus, fragrances in *Ficus* seem to act both in long-distance attraction and in more precise guidance at short distances. This situation is different

TABLE 2. Distribution of the glands found in inflorescences of *Ficus* and *Castilla elastica*. Symbols: + = present, — = absent, na = not applicable (the *Castilla elastica* inflorescence does not have an ostiole nor flowers with tepals).

Distribution	Species										
	<i>C. elastica</i>	<i>F. citrifolia</i>	<i>F. microcarpa</i>	<i>F. tikoua</i>	<i>F. lyrata</i>	<i>F. obtusiuscula</i>	<i>F. pumila</i>	<i>F. montana</i>	<i>F. auriculata</i>	<i>F. variegata</i>	
Scent glands	na	—	+	+	+	—	+	+	+	+	
Ostiole bracts	na	—	+	+	+	—	+	+	+	+	
Epidermis	na	—	+	+	+	—	+	+	+	+	
Mesophyll	na	—	+	+	+	—	+	+	+	+	
Outer epidermis	—	+	—	—	—	—	—	—	—	—	
Subepidermal cells	na	+	+	+	+	—	+	+	+	+	
Laticifers	na	+	+	+	+	—	+	+	+	+	
Ostiole bracts	na	+	+	+	+	—	+	+	+	+	
Inflorescence receptacle	+	+	+	+	+	—	+	+	+	+	
Pistillate flower pedicel	na	+	+	+	+	—	+	+	+	+	
Tepals	na	—	+	+	+	—	+	+	+	+	
Style	+	—	—	—	—	—	—	—	—	—	
Ostiole bract mesophyll	na	+	+	+	+	—	+	+	+	+	
Inflorescence receptacle	+	+	+	+	+	—	+	+	+	+	
Stigma and subepidermal layer of style	+	+	+	+	+	—	+	+	+	+	
Phenolic-producing idioblasts	na	+	+	+	+	—	+	+	+	+	
Phenolic-producing epidermis	—	—	—	—	—	—	—	—	—	—	
Outer surface	na	—	—	—	—	—	—	—	—	—	
Inner surface	na	—	—	—	—	—	—	—	—	—	
Tepals	na	—	—	—	—	—	—	—	—	—	
Ovary	na	—	—	—	—	—	—	—	—	—	
Outermost layer of cells of nucellus	—	—	—	—	—	—	—	—	—	—	
Between ostiole bracts	na	—	—	—	—	—	—	—	—	—	
Phenolic-producing trichomes	na	+	+	+	+	—	+	+	+	+	
Inflorescence inner surface	na	—	—	—	—	—	—	—	—	—	
Between tepal and ovary	na	—	—	—	—	—	—	—	—	—	
Inflorescence outer surface	—	+	+	+	+	—	+	+	+	+	

from most phanerogam groups pollinated by animals, in which fragrances act in long-distance attraction and visual cues act in orienting pollinators to flowers (Giurfa and Lehrer, 2001; Raguso, 2001). Experimental data on *F. burtt-davyi* (section *Galoglychia*) show that when the ostiole is sealed by beeswax, pollinators are no longer attracted by receptive figs (van Noort et al., 1989). These data are compatible with a long-distance attractant emitted by glands in the ostiole bracts or in the fig cavity. Results for *F. carica* suggest that the site of attractant production is either the ostiole or the fig cavity but not the outside of the ostiole (Hossaert-McKey et al., 1994). Upon opening, receptive figs of some species, including some from section *Americana*, have clearly accumulated fragrance (to the human nose). This production is compatible with secretion by glands on the ostiole bracts because ostioles are most strongly sealed toward the exterior of the fig (*F. Kjellberg*, unpublished observation). Hence, a comparison between histological results and biological data suggests that the glands located on the ostiole are responsible for producing the olfactory message attractive to pollinators. If they are, then what is the role of the scent glands in the fig receptacle? Terpene emissions have other roles than pollinator attraction because they are often produced by leaves. In *F. septica* (section *Sycocarpus*), not only did the composition of receptive fig fragrances vary during the day, but also the composition of the blend of volatile compounds emitted by leaves varied in a similar way, suggesting a protective role (Conchou et al., 2014). Alternatively, the behavior of wasps on the fig suggests a role for chemical perception on the fig receptacle. Further studies are needed to ascertain the precise role of the different glands along the fig.

Five of the nine studied species (i.e., *F. microcarpa*, *F. montana*, *F. obtusiuscula*, *F. pumila*, and *F. tikoua*) had two sites of volatile production (i.e., ostiole bracts and fig receptacle) in the same fig. Multiple sites of a volatile source in a single inflorescence may create an odor gradient of different bouquets that guide pollinators toward receptive flowers, as reported for *Sauromatum guttatum* (Araceae, Hadacek and Weber, 2002). However, in *Ficus* the simultaneous occurrence of two osmophore sites is probably not related to an odor gradient, or if so, it is not universal for this plant genus since it does not occur in all species. Three other species studied here (i.e., *F. auriculata*, *F. lyrata*, and *F. variegata*) have scent glands only on the ostiole bracts, whereas *F. citrifolia* has them only on the fig receptacle.

Indeed, the number and position of sites producing fragrances seem not to be related to phylogenetic position of these *Ficus* lineages (Cruaud et al., 2012) nor with their biological features (e.g., life form, breeding system, pollination mode, and fig size). If scent gland distribution was phylogenetically constrained, we would have expected to find a more similar distribution in more closely related species, such as the species belonging to subgenus *Urostigma* (i.e., *F. citrifolia*, *F. lyrata*, and *F. microcarpa*) or to section *Sycomor* (i.e., *F. auriculata*, *F. variegata*, and maybe *F. tikoua*). Therefore, the distribution of scent glands in *Ficus* likely underwent rapid evolution despite their high functional value. The distribution of scent glands on the fig of a particular *Ficus* species can probably be selected by a complex combination of biological and ecological pressures. Indeed, *Ficus* has a great diversity of life forms, breeding systems, pollination modes, and ecological features (population density, spatial distribution, and characteristics of pollinating wasps). These differences certainly determine the reproductive attributes of individual *Ficus* species, leading to specific selective pressures.

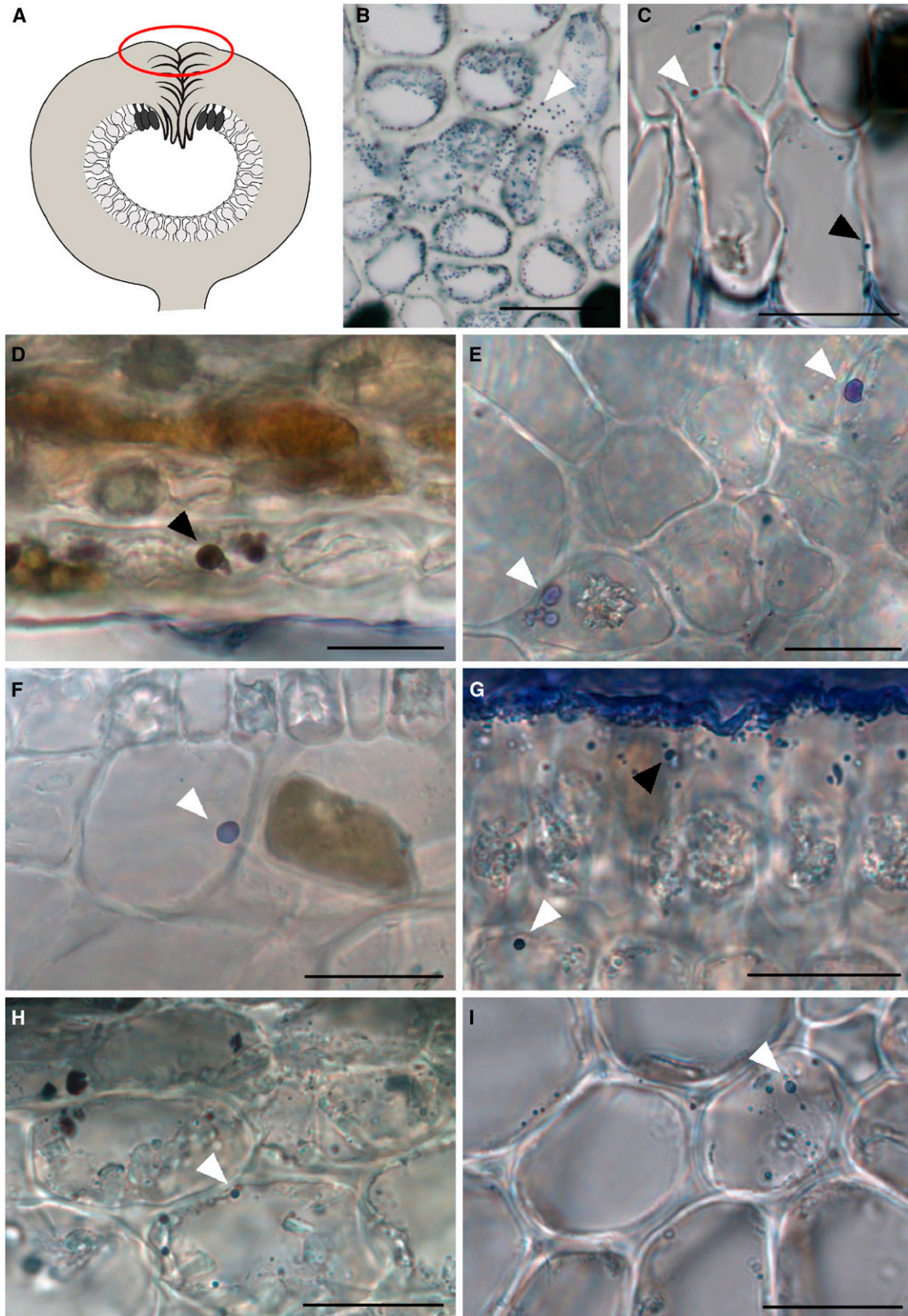


FIGURE 3 Schematic diagram of fig (A) and photomicrographs of terpene-secreting cells in ostiolar bracts of *Ficus* species (B–I). (A) Schema with red encircling the location of the scent glands in ostiolar bracts. (B) *F. auriculata*. (C) *F. lyrata*. (D) *F. microcarpa*. (E) *F. montana*. (F) *F. obtusiuscula*. (G) *F. pumila*. (H) *F. tikoua*. (I) *F. variegata*. White arrowheads: terpene droplets in bract mesophyll; black arrowheads: terpenes in epidermal cells. Nadi reagent. Bars: 50 μm (B), 20 μm (C, D, E, F, G, H, I).

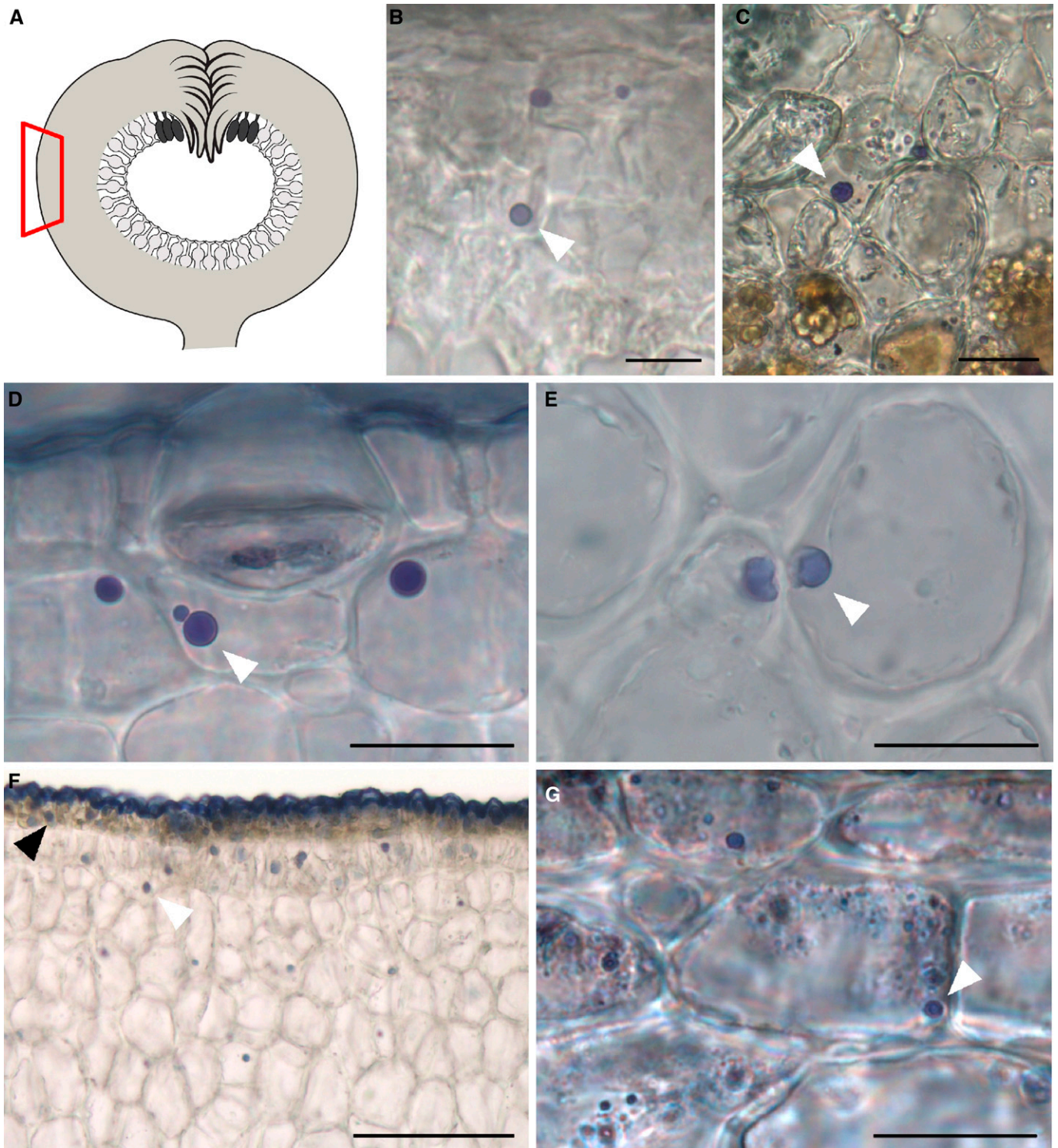


FIGURE 4 Schematic diagram of fig (A) and photomicrographs of terpene-secreting cells in inflorescence receptacle of *Ficus* species (B–G). (A) Location of the scent glands in the receptacle. (B) *F. citrifolia*. (C) *F. microcarpa*. (D) *F. montana*. (E) *F. obtusiuscula*. (F) *F. pumila*. (G) *F. tikoua*. White arrowheads: terpene droplets in subepidermal layers of the receptacle; black arrowhead: terpenes in epidermal cells. Nadi reagent. Bars: 10 μ m (B), 25 μ m (C), 20 μ m (D, E, G), 50 μ m (F).

An important question that comes up is whether the presence and distribution of scent glands are similar in both types of inflorescences found in gynodioecious species of *Ficus*, represented here by *F. auriculata*, *F. montana*, *F. pumila*, *F. tikoua*, and *F. variegata*. The

type of inflorescence in a gynodioecious species seems not to influence at least the occurrence of the scent glands since our anatomical data show the presence of scent glands in the “male” figs of *F. montana* and *F. pumila* and “female” figs of *F. tikoua*, *F. auriculata*,

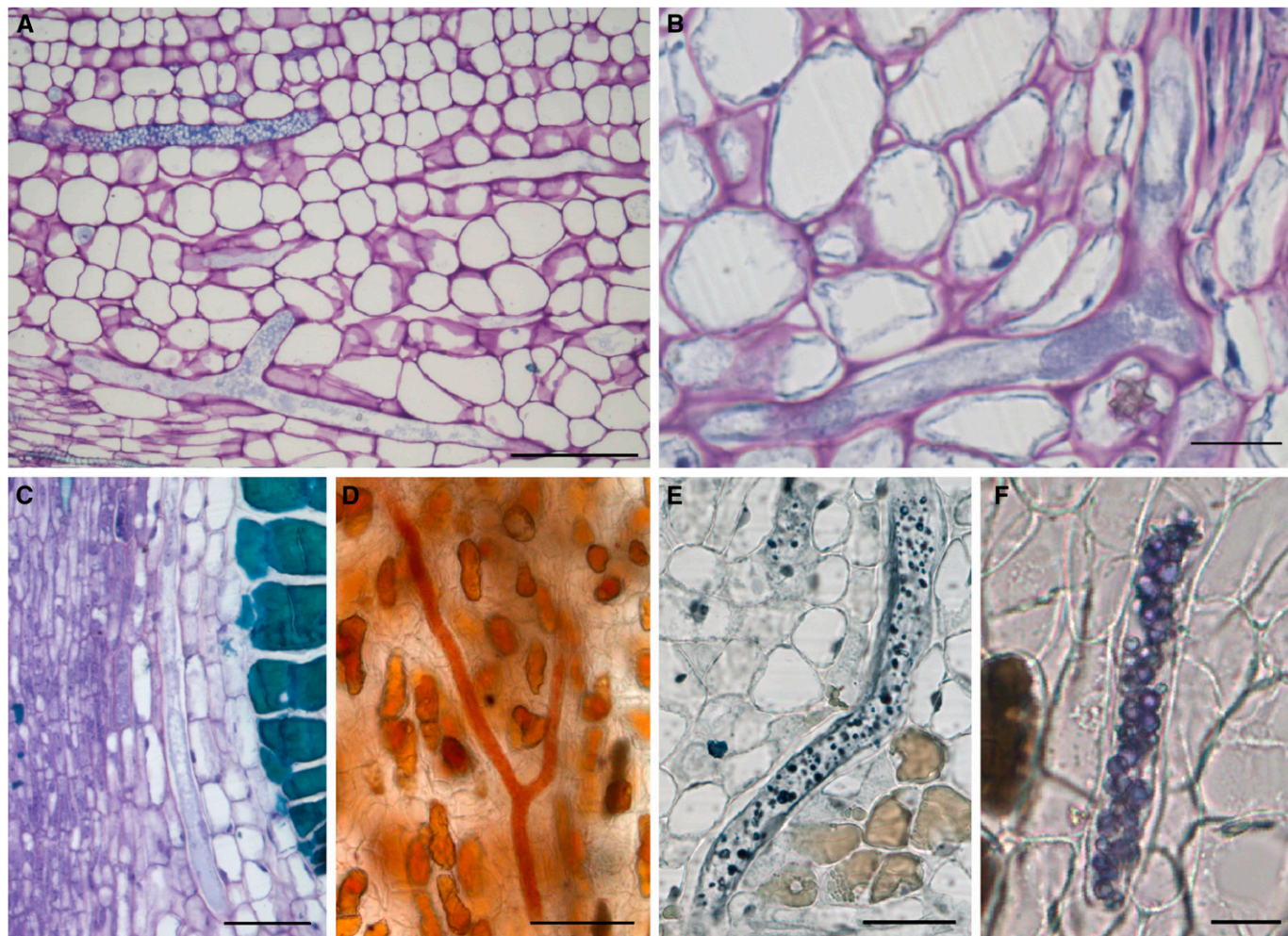


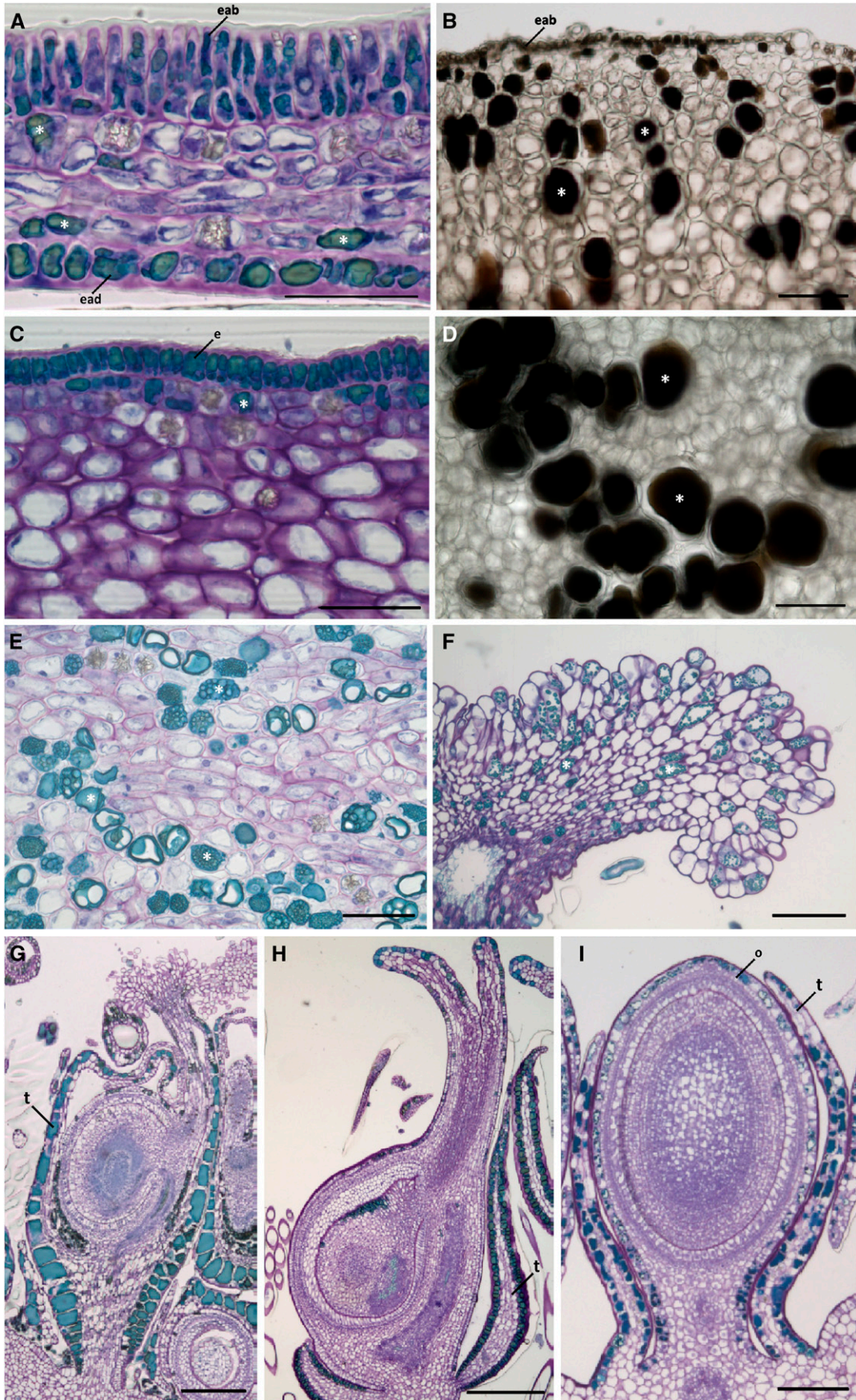
FIGURE 5 Photomicrographs of branched laticifers in inflorescences of *Ficus* species (A, C–F) and *Castilla elastica* (B). (A, B) Laticifers in the inflorescence receptacle of *F. montana* and *C. elastica*, respectively, showing the thin wall and ramifications (toluidine blue). (C) Laticifer in a floral pedicel of *F. citrifolia* (toluidine blue). (D, E) Lipophilic content evidenced in laticifers in the receptacle of *F. pumila* (Sudan III) and *F. auriculata* (Sudan black B), respectively. (F) Laticifer with terpenic content in *F. variegata* receptacle (Nadi reagent). Bars: 100 μm (A, C, D), 50 μm (B), 25 μm (E), 12.5 μm (F).

and *F. variegata* (see Table 1). “Male” and “female” figs of *F. asperifolia* are also anatomically similar at the receptive phase (Verkerke, 1987). In addition, receptive “male” and “female” figs of *F. carica* emit the same blend of volatile compounds, differing only in the quantities and proportions (Grison-Pigé et al., 2001), thus supporting our findings.

Our results demonstrate that the production and emission of fragrances attractive to pollinators in *Ficus* were transferred over time from the floral perianth (Fahn, 1979; Vogel, 1990; Dudareva and

Pichersky, 2006) to external parts of the fig, probably due to the enclosure of tiny flowers inside an urn-shaped structure with a small opening to the outside. Fragrance production in ostiolar bracts and in the receptacle would be more effective in distributing the scent through the external environment. This functional transfer to extrafloral structures has independently evolved in other plant groups. In *Sauromatum guttatum* (Araceae), the function of fragrance production was transferred from its tiny flowers to the appendices at the spadix apex and to the club-shaped organs near

FIGURE 6 Photomicrographs of phenolic-producing idioblasts (A–F) in inflorescences of *Ficus* species and *Castilla elastica* and phenolic-producing epidermis (A–C, G–I) in figs of *Ficus* species. (A) *F. citrifolia* ostiolar bract. (B) *F. auriculata* ostiolar bract. (C) *F. obtusifolia* receptacle. (D) *F. lyrata* receptacle. (E, F) Inflorescence receptacle and stigma of *C. elastica*, respectively. (G–I) Pistillate flowers of *F. citrifolia*, *F. pumila* and *F. tikoua*, respectively. Note the phenolic-producing epidermis on tepals (G–I) and ovary (I). Reagents: toluidine blue in A, C, and E–I; ferric trichloride in B and D. Symbols: * = phenolic-producing idioblasts; e = epidermis; eab = epidermis on the abaxial surface; ead = epidermis on the adaxial surface; o = ovary; t = tepal. Bars: 50 μm (A, C, E), 1 mm (B), 100 μm (D, F), 250 μm (G), 200 μm (H, I).



the pistillate flowers (Skubatz et al., 1996; Hadacek and Weber, 2002). The dwarf palm *Chamaerops humilis* (Arecaceae), which has small flowers without conspicuous visual cues, represents an extreme case of changing the site of scent emission, with the scent glands located in leaves to attract the pollinating weevil (Dufaÿ et al., 2003). It seems, therefore, that the transfer of osmophore location and the appearance of special, more complex systems of pollinator attraction may have developed in some species that have inconspicuous, less attractive flowers. But this hypothesis is based on a small sample size.

The precise location and characterization of glands responsible for fragrance production are novel data for the fig–fig wasp system, as the current knowledge of chemistry ecology of fig wasps has focused on composition analysis of the volatiles released by figs or on attraction experiments (van Noort et al., 1989; Hossaert-McKey et al., 1994; Grison-Pigé et al., 2002; Dudareva and Pichersky, 2006; Proffit et al., 2008; Hossaert-McKey et al., 2010). In this context, our study is the first to use more precise research tools (anatomy and histolocalization of substances) to locate the glands responsible for fragrance production in the group.

Glands involved in fig protection—The control of the phytophagous microbiota (Nadel et al., 1992; Pereira et al., 2000) that may inhabit the figs and, consequently, the protection of the entire pollination unit represented by this inflorescence is probably performed by laticifers, idioblasts, epidermis, and glandular trichomes that secrete phenolic compounds; such structures are often associated with protection against herbivores and pathogens (laticifers: Pickard, 2008; Agrawal and Konno, 2009; Konno, 2011; idioblasts: Rodriguez-Saona and Trumble, 2000; Ni et al., 2007; glandular trichomes: Levin, 1973; Wagner, 1991; phenolic glands: Castro and Demarco, 2008). These glands, unlike the scent glands, may persist through several phases of the fig development protecting not only the flowers but also the fruits and seeds (Bajjnath and Naicker, 1989; Jansen-González et al., 2012; C. R. Marinho, unpublished data). Laticifers even may be present from the beginning of the plant life itself, in the embryo (Fahn, 1979). It is worth noting that latex and the majority of phenolic compounds are released only after injury, acting as deterrents (Agrawal and Konno, 2009; Castro and Demarco, 2008).

The wide occurrence of laticifers in Moraceae (Judd et al., 2009) is corroborated here by expanding the study to 10 other species. The composition of latex is known in some *Ficus* species and contains bioactive substances that act in reducing herbivory, such as digestive proteases, oxidases, and alkaloids (Fahn, 1979; Pickard, 2008; Agrawal and Konno, 2009; Konno, 2011; Castro and Machado, 2012). Other substances commonly found in the latex of angiosperms are terpenes, here recorded in *F. auriculata*, *F. montana*, and *F. variegata*. A type of terpene well known in laticiferous plants is rubber, which occurs in the form of particles of varying size in a colloidal suspension, reported in large quantities in *F. elastica*, a species known as the India rubber plant that has been cultivated for its rubber production (Esau, 1974; Fahn, 1979; Castro and Machado, 2012).

Secretory structures of phenolic compounds are widely distributed and predominate in the fig tissues of the studied *Ficus* species. These compounds have great chemical diversity and may perform other functions besides defense against herbivory (Harborne, 1999;

Carvalho et al., 2007; Taiz and Zeiger, 2009; Silva et al., 2014). One of these functions is providing a barrier against ultraviolet and infrared rays (Harborne, 1999; Silva et al., 2014). Thus, they could additionally be involved in fig protection against ultraviolet rays and maintenance of the ideal temperature inside the syconium, providing the necessary conditions for the creation of the larvae of pollinating wasps. Experimental data show that temperatures slightly above room temperature within the fig are sufficient to kill larvae of pollinating fig wasps (Herre, 1996).

Some types of phenolic compounds have been identified in the fragrance released by scent glands, thus participating in pollinator attraction (Skubatz et al., 1996; Jürgens et al., 2006; Knudsen et al., 2006; Castro and Demarco, 2008). In *Ficus*, however, phenolic compounds have never been found among the volatiles (Grison et al., 1999; Grison-Pigé et al., 2002; Dudareva and Pichersky, 2006; Hossaert-McKey et al., 2010); therefore, they are probably not involved in receptive fig fragrances.

It is worth noting that the presence of laticifers and phenolic-producing idioblasts scattered through the receptacle and also of glandular trichomes on the outer receptacle of the fig (except in *F. pumila*) is frequent in *Ficus*, also reported in *F. sycomorus* (Galil et al., 1970), *F. ottoniifolia* (Verkerke, 1986), and *F. asperifolia* (Verkerke, 1987).

Ficus citrifolia and *F. microcarpa* (present study) and *F. enormis* (Machado et al., 2013) share the presence of glandular trichomes located internally in the figs. Despite the morphological and positional similarities, the produced exudate has a different chemical composition. In *F. enormis*, it contains mucilaginous substances that are apparently involved in lubrication of developing organs (Machado et al., 2013).

The distribution of glands putatively related to defense in *Ficus* is quite variable. The fig of these species at the receptive phase consists of a specialized inflorescence containing pistillate flowers and developing staminate flowers, thus representing a pollination unit, which certainly increases the cost exerted by phytophagous animals. Because the fig functions as a pollination unit, it may become inviable from even a relatively small injury caused by herbivory. This disproportionately higher cost probably does not occur in plant species with less specialized inflorescences, in which an injury caused by herbivory in one part of the inflorescence usually does not affect the whole unit, such as species of Araceae and Arecaceae in which beetles pollinate while feeding on floral parts (Endress, 1994; Paulino-Neto, 2015). Thus, the results of this study suggest that the several protective structures (idioblasts, epidermis, and trichomes that secrete phenolic compounds; and laticifers) present in the studied species may have been selected by the generalized pressure of phytophagous animals. The hypothesis that these protective structures are related to the high cost of herbivory for the fig is supported by the comparison with *Castilla*, sister group of *Ficus*. *Castilla elastica*, with a less specialized, discoid inflorescence and a less intricate relationship with the pollinating thrips (Sakai, 2001), in fact, had less diversity in its protective structures.

We conclude that *Ficus* species exhibit a great diversity of glands important in maintaining the interaction with their pollinators as well as their protection. The scent glands responsible for producing fragrances attractive to pollinating fig wasps were identified and located in the current study. Apparently, the diversity of types and distribution of the scent glands are strictly related to the particular pollination mutualism of *Ficus* because they were not observed in

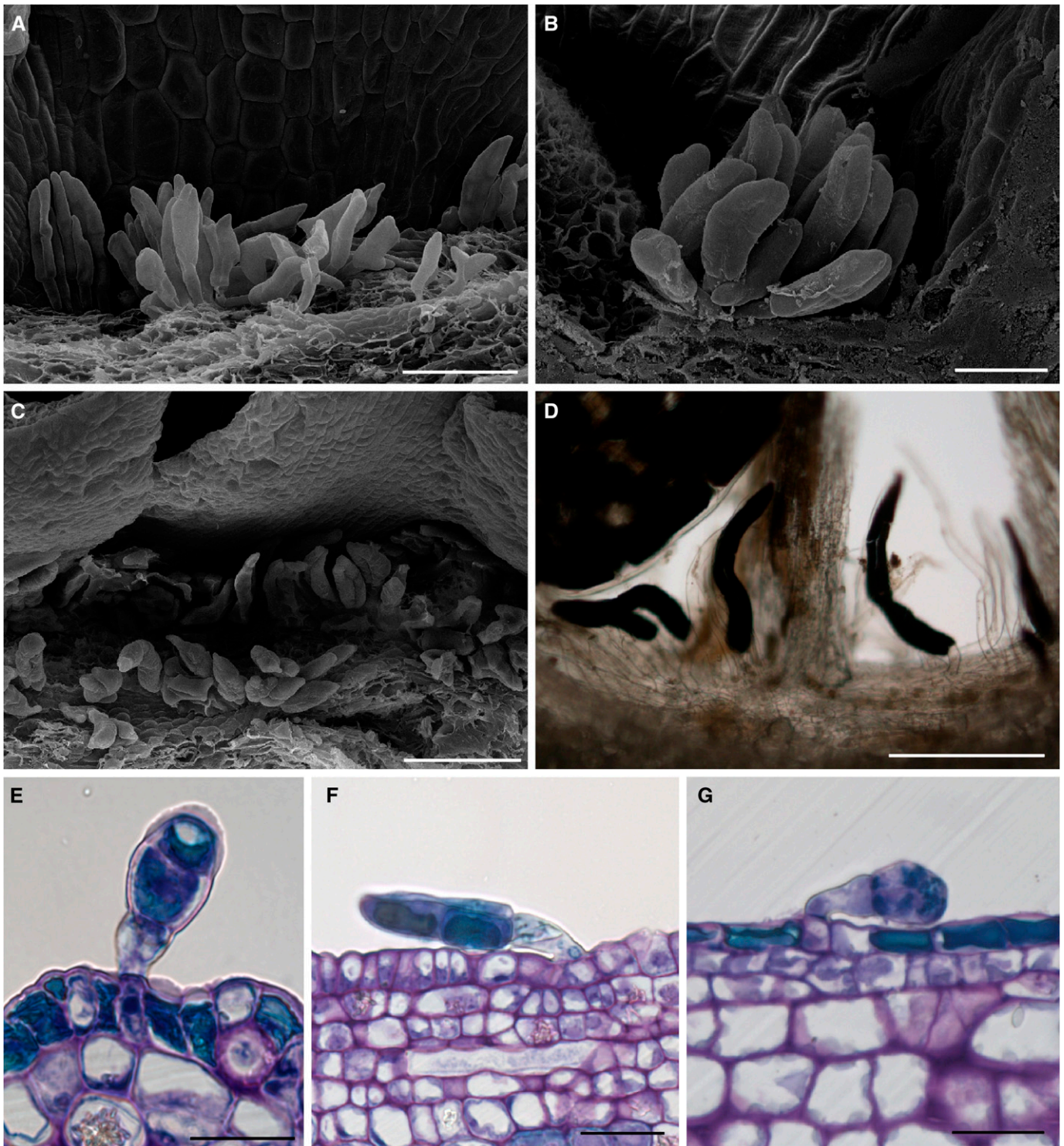


FIGURE 7 Electron micrographs (A–C) and photomicrographs (D–G) of the trichomes secreting phenolic compounds in *Ficus* species. (A, B) Glandular trichomes between the ostiolar bracts (A) and on the inner surface of the fig receptacle (B) of *F. citrifolia*. (C, D) Glandular trichomes between the ostiolar bracts (C) and between the tepal and the ovary (D) of *F. microcarpa*. (E–G) Glandular trichomes on the outer surface of the fig receptacle of *F. auriculata* (E), *F. citrifolia* (F), and *F. tikoua* (G). Reagents: ferric trichloride in D; toluidine blue in E–G. Bars: 100 μm (A, C, D), 50 μm (B, G), 25 μm (E, F).

C. elastica. In addition to the scent glands, the investigated species have other secretory structures, especially of phenolic compounds, which may play a role in protecting the fig, an extremely important unit for the reproduction of the fig tree as well as for the specialized

pollen-dispersing fig wasps. Taking into account that *Ficus* is a large and extremely diverse genus, we believe that further studies comparing the activity of the fig glands before and after pollinator entry will highlight the ecological role of the fig glands in the mutualism.

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