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Morphological diversity and function of the stigma in *Ficus* species (Moraceae)

Simone Pádua Teixeira[a,*], Marina F.B. Costa[a,b], João Paulo Basso-Alves[b,c], Finn Kjellberg[d], Rodrigo A.S. Pereira[e]

[a] *Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo, Av. do Café, s/n, 14040-903, Ribeirão Preto, SP, Brazil*
[b] *PPG em Biologia Vegetal, Instituto de Biologia, Universidade Estadual de Campinas, Av. Bandeirantes, 3900, 14040-901, Campinas, SP, Brazil*
[c] *Instituto de Pesquisa do Jardim Botânico do Rio de Janeiro, DIPEQ, Rua Pacheco Leão, 915, 22460-030, Rio de Janeiro, RJ, Brazil*
[d] *CEFE UMR 5175, CNRS, Université de Montpellier, Université Paul-Valéry Montpellier, EPHE, 1919 route de Mende, F-34293, Montpellier Cédex 5, France*
[e] *Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, Universidade de São Paulo, Av. Bandeirantes, 3900, 14040-903, Ribeirão Preto, SP, Brazil*

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ABSTRACT

The stigma plays several roles such as pollen hydration and selection, and pollen tube nutrition. In the *Ficus*-fig wasp mutualism, stigmata have an additional, almost unknown, function by representing a physical interface for both plant and wasp reproduction. We used light and electron microscopy to compare the detailed morphology of the stigmata of nine *Ficus* species of different sections and with different pollination modes and sexual expressions. Figs were collected at the stage when the stigmata were receptive for pollination. Stigmata in actively pollinated monoecious species have well developed papillae concentrated on the adaxial surface exposed towards the fig cavity. Conversely, the passively pollinated monoecious species have the whole surface of the stigmata covered by somewhat smaller papillae. In both actively and passively pollinated monoecious species these features are consistent, irrespective of style length. In all actively pollinated gynodioecious species, the stigmata of pistillate flowers were tubular or infundibuliform whereas in almost all actively pollinated monoecious species (except *F. racemosa*) the stigmata were filiform, with one branch or two asymmetric branches. In gynodioecious species the short-styled flowers in "male" figs show a limited receptive surface with small papillae, while the stigmata of long-styled flowers in "female" figs are covered by papillae that extend down the sides of the style, increasing the stigmatic surface. In actively pollinated species, stigmata are cohesive, forming a common surface for pollen tube germination (= synstigma). The synstigma arrangement was quite variable: lax, cohesive or very cohesive, with entanglement by stigmatic papillae and stylar trichomes. Entanglement by stylar trichomes is common in gynodioecious species. The synstigma arrangement did not correlate with phylogeny or breeding system. This study is the first to report a very loose synstigma in actively pollinated monoecious *Ficus* species. Our analyses revealed that, in *Ficus*, the synstigma is functionally analogous to an extra-gynoecial compitum. Comparative studies will be required to test further hypotheses about the evolutionary determinants of such variation.

1. Introduction

The stigma is generally the apical extension of the carpel that receives the pollen grain and intercedes in the germination of the pollen tube (Heslop-Harrison and Heslop-Harrison, 1985; Heslop-Harrison, 1992). Thus, the stigma is the site where pollination ends and the journey of the pollen tube toward the ovule begins (van Went and Willemse, 1984). Although the function of the stigma is the same for all angiosperms, stigma structure (shape, cellular composition and secretion occurrence) and physiology are very diverse (Edlund et al., 2004; Endress, 1994; Heslop-Harrison, 1992). The shape of the stigma, for example, may be of taxonomic value (Bigazzi and Selvi, 2000; Brown and Gilmartin, 1989; Heslop-Harrison, 1981), in addition to being related to adaptations to pollen grain deposition, such as the plumose stigma in the anemophilous species of Poaceae (Heslop-Harrison, 1992), or requiring the visit/manipulation of an animal to become receptive to pollen germination (Basso-Alves et al., 2011; Sigrist and Sazima, 2004).

As a reflection of its morphological variation, the stigma can play several roles (Edlund et al., 2004), such as hydration of pollen grains

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[*] Corresponding author.
E-mail address: spadua@fcfrp.usp.br (S.P. Teixeira).

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(Edlund et al., 2004; Heslop-Harrison and Heslop-Harrison, 1985) and nutrition of growing pollen tubes (Rejón et al., 2014). Because of its ability to stimulate or prevent the emission of pollen tubes, the stigma also plays a selective role in the germination of pollen grains (Heslop-Harrison, 2000; Rejón et al., 2014). This control over the access of pollen tubes to style and ovary and, consequently, to ovules, is evidenced by systems of pollen-stigma recognition and compatibility (Heslop-Harrison, 1982; Heslop-Harrison et al., 1975). It should be noted that the selective role in pollen tube growth is not restricted to the stigma (Erbar, 2003; Lora et al., 2016) but is also performed by the compitum (Endress, 1982; Wang et al., 2012). The compitum (from the Latin crossing or crossroads) is a term applied to places of convergence of several growing pollen tubes, forming a competition arena. It is found at the intersection of a pistil transmitting tissue in flowers with a syncarpic gynoecium (Carr and Carr, 1961; Endress, 2011; Erbar, 2003).

Therefore, it seems evident that stigma shape and organization are highly associated with the type of pollination (Edlund et al., 2004). However, much greater effort has been expended on elucidating the relationship between pollen and pollination (*e.g.*, Banks and Rudall, 2016; Hesse, 2000; Osborn et al., 1991) than between stigma and pollination (Heslop-Harrison, 1992). The study of the diversity of stigmata related to various types of pollination (Basso-Alves et al., 2011; Jousselin and Kjellberg, 2001) and breeding systems (Katinas et al., 2016; Lora et al., 2011) is thus of great interest in general and for obligate mutualisms such as between fig trees (*Ficus*) and their agaonid pollinating wasps in particular (Galil and Eisikowitch, 1968).

Fig trees (*Ficus* L.) belong to the largest genus of Moraceae, comprising 841 of 1217 described species in the family (The Plant List, 2013). Its representatives are widely distributed in tropical, subtropical and temperate regions (Berg, 2005). The genus has as synapormorphy a distinct type of inflorescence called a syconium or fig, that consists of an urn-shaped receptacle. Internally the fig is covered by diclinous flowers (= unisexual) (Berg, 1990; Verkerke, 1989) and presents a single opening to the outside, called an ostiole (Berg, 1990; Galil and Eisikowitch, 1968). These plants are involved in a notable specialized pollination mutualism with agaonid wasps (Galil and Eisikowitch, 1968).

About half the *Ficus* species are monoecious. Their figs contain staminate and pistillate flowers and therefore they produce pollen and seeds and provide a breeding place for the pollinating wasps. The other *Ficus* species are structurally gynodioecious but functionally dioecious, *i.e.*, some individuals carry figs with long-styled pistillate flowers which produce only seeds ("female individuals"), while others ("male individuals") have figs with staminate flowers and short-styled flowers that are used by the pollinating wasps to deposit their eggs and which produce practically no seeds. Therefore figs in "male" fig trees produce pollen and wasp offspring (Kjellberg et al., 2005). Monoecy is probably the ancestral character-state in the genus, with one (or more) change to gynodioecy and two reversals to monoecy (Datwyler and Weiblen, 2004; Jousselin et al., 2003b; Weiblen, 2000).

The *Ficus*-fig wasp mutualism probably exerts a strong selective pressure over stigma morphology, as the stigma constitutes a physical interface for both plant and wasp reproduction. Indeed, stigma morphology is correlated with the pollination mode of different fig tree species, *i.e.* active or passive pollination (Jousselin et al., 2003a; Jousselin and Kjellberg, 2001). About 3/4 of all *Ficus* species are actively-pollinated. In these species females of pollinating wasps actively collect pollen from anthers before leaving their natal figs and place it in special thoracic structures called pollen pockets, in order to transport it to another fig whose pistillate flowers are receptive to pollination. Once inside a receptive fig, the female wasp removes some pollen from its pollen pockets each time it lays an egg and deposits it on the stigma surface (Galil and Eisikowitch, 1969; Ramírez -Benavides, 1969). In actively pollinated *Ficus* species, stigmata can be cohesive, forming a common surface for pollen tube germination (= synstigma). Since actively pollinating wasps are selected to pollinate the flower in which they have just laid an egg, the synstigma seems to play a functional role ensuring seed production by allowing lateral growth of the pollen tube and hence limiting the capacity of wasp to control which flower is fertilized (Jousselin and Kjellberg, 2001). However, in passively pollinated species stigmata are separated and elongated, brushing the surface of the wasp and thus the synstigma is absent.

The objective of the present study was to check at a detailed morphological level the stigmatic features selected in response to the *Ficus*-fig wasp mutualism. To achieve this objective nine species of *Ficus* from different sections and with different modes of pollination and sexual expressions were compared in terms of stigma morphology. In addition, the morphology of stigmatic papillae in the highly cohesive synstigma of *F. religiosa* and *F. racemosa* was evaluated at the ultrastructural level.

2. Materials and methods

Nine species from eight *Ficus* lineages (sections) - *F. auriculata* (section *Sycomorus*), *F. crocata* (section *Americanae*), *F. lyrata* (section *Galoglychia*), *F. microcarpa* (section *Conosycea*), *F. obtusiuscula* (section *Pharmacosycea*), *F. racemosa* (section *Sycomorus*), *F. religiosa* (section *Urostigma*), *F. septica* (section *Sycocarpus*) and *F. ulmifolia* (section *Sycidium*) (Table 1) - were studied. *Ficus auriculata* and *F. racemosa* were included as they represent a gynodioecious and a monoecious species, respectively, in sect. *Sycomorus*. Species names were based on C.C. Berg (Berg and Villavicencio, 2004; Berg and Corner, 2005).

For monoecious *Ficus* species, stigmata of both shorter- and longer-styled pistillate flowers were analyzed. For gynodioecious species we studied long-styled flowers from figs of "female trees", except *F. septica* for which we studied short-styled flowers from "male trees" and long-styled flowers from "female trees".

Five figs (= inflorescences) of each species studied (from one individual/species) were collected at the stage when stigmata are receptive for pollination. Figs were cut in half and fixed in FAA 50 (formalin:acetic acid:alcohol) (Johansen, 1940) or in buffered formalin (Lillie, 1954) for 24–48 h, dehydrated in an ethanol series and processed for observation by light microscopy (LM), scanning electron microscopy (SEM) and transmission electron microscopy (TEM).

The external morphology of the stigmata was assessed by SEM. Previously fixed samples were dehydrated in an ethanol series, critical point dried in a Bal Tec CPD 030 critical point dryer, placed on stubs over carbon adhesive tape, and sputter coated with gold with a Bal Tec SCD 050 instrument. Images were obtained using a Jeol JSM-6610LV SEM.

Stigma anatomy was studied in samples embedded in methacrylate-based resin (Gerrits and Horobin, 1991) and cut into 5 μm thick sections with a Leica RM 2245 rotary microtome. Sections were stained with 0.05% toluidine blue in phosphate buffer as a general stain (O'Brien et al., 1964) and with Sudan black B (Pearse, 1980) to detect lipophilic substances. Anatomical sections were observed and photographed using a Leica DM 5000 B light microscope coupled to a Leica DFC 320 digital camera. Crystals were observed and shot with a Leica DM5000 B light microscope provided with a polarizing filter (PLM, Johansen, 1940).

The ultrastructure of stigmatic cells was analyzed under TEM in *Ficus religiosa* and *F. racemosa*. Samples were fixed in Karnovsky's fixative (McDowell and Trump, 1976) for 24 h, post-fixed in 1% osmium tetroxide in 0.1M phosphate buffer (pH 7.2) and embedded in araldite. Ultrathin sections (60 nm thick) were obtained using a Leica Reichert Ultracut S ultramicrotome, collected on copper grids covered with Formvar, contrasted with 2% uranyl acetate and lead citrate for 15 min each (Reynolds, 1963), and examined with a Jeol 100CXII TEM.

Synstigmata were classified into cohesive or lax according to the degree of difficulty in separating fixed samples using a needle.

For simplicity, in gynodioecious species, the fig tree containing figs with long-styled pistillate flowers which produce only seeds we called a "female plant". The one in which figs have staminate flowers producing