RESEARCH ARTICLE
BRIEF COMMUNICATION

TIMING OF POLLEN RELEASE AND STIGMA RECEPTIVITY PERIOD OF *PIPER VICOSANUM*: NEW INSIGHTS INTO SEXUAL REPRODUCTION OF THE GENUS[1]

ADRIANO VALENTIN-SILVA[2], VICTOR PEÇANHA DE MIRANDA COELHO, MARÍLIA CONTIN VENTRELLA, AND MILENE FARIA VIEIRA

Departamento de Biologia Vegetal, Universidade Federal de Viçosa (UFV), Av. P.H. Rolfs, s/n, Campus Universitário, CEP 36570-900, Viçosa, Minas Gerais, Brasil

- *Premise of the study:* Dichogamy is a common characteristic among angiosperms, including *Piper* species. In this genus, the tiny flowers are morphologically similar and have an asynchronous stamen development. However, there is no information on the duration of stigma receptivity and whether it overlaps with pollen release. To better understand mechanisms of floral function in *Piper vicosanum*, we provide a detailed characterization of the timing of pollen release from the four stamens and the period of stigma receptivity and exposure mode of the receptive areas.
- *Methods:* We investigated plants of a natural population in a semideciduous seasonal forest (Viçosa, Minas Gerais State, southeastern Brazil), based on chemical tests, light microscopy, and scanning electron microscopy analyses.
- *Key results:* Incomplete protogyny—a mechanism that favors outcrossing—was recorded. The period of stigma receptivity was long (14 d), and the sequential exposure and senescence of stigmatic papillae occurred gradually and in a basipetal direction. Pollen release began 2–6 d after the beginning of the pistillate phase, with an average pollen viability of 87.7%, during the bisexual flower phase. Pollen was released for up to 6 d and occurred in one stamen at a time. The fruit set observed in tests of self-pollination indicated self-compatibility.
- *Conclusions:* The gradual and sequential exposure of stigmatic papillae in *P. vicosanum* flowers is described here as the mechanism for the long duration of receptivity. Anther development and pollen release were also sequential. These findings are yet unreported reproductive characteristics of the genus and offer new perspectives for future studies on the floral biology of other *Piper* species.

Key words: asynchronous pollen release; incomplete protogyny; self-compatibility; stigmatic exposure.

The genus *Piper* includes more than 1000 species (Jaramillo and Manos, 2001) and is easily identifiable by reproductive characteristics, such as its short spike inflorescences with hundreds of reduced flowers. These characteristics have made manipulative studies of reproduction in the genus difficult. The flower is perianthless and is protected by a bract; the androecium consists of 1–10 stamens and the gynoecium has 3–4 stigmas (Jaramillo and Manos, 2001).

Comparative analyses of floral ontogeny on this genus by Tucker (1982) detected asynchrony in stamen development in species with four and six stamens. According to this author, androecium development in species with four stamens begins with two, simultaneously, followed by a third, and finally the fourth stamen. In species with six stamens, the first four follow the same sequence as species with four stamens and the last two develop in the end, simultaneously. However, no studies have investigated whether the chronological order of stamen development also results in staggered pollen release.

Most studied species were described as dichogamous, with pollen release and stigma receptivity temporally isolated within the flower. Complete or incomplete protogyny (Menon, 1949; Martin and Gregory, 1962; Figueiredo and Sazima, 2000) and incomplete protandry (Figueiredo and Sazima, 2000; Kikuchi et al., 2007) are recorded; in addition, there are also reports of adichogamy (= homogamy; Figueiredo and Sazima, 2000). However, these studies provide no information on stigma longevity or the exposure mode of its receptive areas in relation to the timing of pollen release. Characterizing the relative timing of these floral functions is important for understanding aspects of sexual reproductive mechanisms in species with four or six stamens—particularly those with partial dichogamy, due in this case, to the possibility of self-pollination (Lloyd and Webb, 1986) in self-compatible species. Both self-compatibility (Marquis, 1988; Figueiredo and Sazima, 2000),

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[1] Manuscript received 23 September 2014; revision accepted 19 March 2015.

The authors thank the National Council for Scientific and Technological Development (CNPq) for scholarships for a Master's degree and research productivity, respectively, of the first and last authors. This study is part of the dissertation of the first author in the Graduate course of Botany, Federal University of Viçosa.

[2] Author for correspondence: adrianovalentin86@gmail.com

doi:10.3732/ajb.1400419

as well as self-incompatibility (Prakash et al., 1994; Figueiredo and Sazima, 2000; Kikuchi et al., 2007) have been reported in the genus.

The purpose of this study was to describe the timing of pollen release from the four stamens of *Piper vicosanum* Yunck. flowers, associating it with the period of stigma receptivity and exposure mode of the receptive stigmatic areas. We sought answers to the following questions: (1) Does the sequence of pollen release from the four stamens follow the same sequence as that of androecium development? (2) What is the duration and timing of stigma receptivity, and to what degree are flowers dichogamous? (3) If dichogamy is partial, is self-pollination possible?

MATERIALS AND METHODS

Study species and area—*Piper vicosanum* is a 1–3 m tall shrub; its spikes are upright and 2.5–4.5 cm long (Carvalho-Okano and Alves, 1998). The flowers are bisexual and have four stamens and a tricarpellate gynoecium with three sessile stigmas, and a superior ovary with one ovule (Yuncker, 1972). The spikes are produced throughout the year, but anthesis and fruiting occur in the rainy season in October and November–December, respectively (Valentin-Silva, 2013). Voucher specimen is deposited in the Herbarium of the Federal University of Viçosa (Herbarium VIC), in Viçosa, Minas Gerais, Brazil, with the codes 35605 (Silva 01), 35606 (Silva 02), and 35608 (Silva 04).

Plants of a natural *P. vicosanum* population in a forest fragment at the Station of Research, Environmental Training and Education Mata do Paraíso, were analyzed. This area is located in Viçosa (20°48′07″S, 42°51′31″W), state of Minas Gerais, in southeastern Brazil, in an area of 194 ha, at 690–870 m asl. It is the largest forest fragment of Viçosa (Silva et al., 2014) and the vegetation was classified as “mountainous semideciduous seasonal forest” (Veloso et al., 1991).

Floral biology, pollination tests, and observations of flower visitors—We analyzed spikes in different stages (with flowers in bud, anthesis, or senescent), fresh or stored in 70% ethanol, under a stereomicroscope (model EZ4D; Leica Microsystems, Deerfield, Illinois, USA), to deepen the understanding of the morphology and flower biology.

The stigma receptivity of 35 fresh spikes with flowers between preanthesis and senescence was tested using Peroxtesmo KO (Machery-Nagel, Düren, Germany), according to a method proposed by Dafni and Maués (1998). In each spike, flowers in three areas (lower, middle, and upper third) were tested. In addition, the stigma receptivity period of plants of a natural population was monitored over 19 consecutive days (total 46 h). With a hand lens (30× increase), we observed the exposure of stigmatic surface in flowers of 20 spikes, from preanthesis to senescence.

We tested the pollen grain viability according to the sequence of stamen development suggested by Tucker (1982). For this purpose, 15 spikes were fixed in FAA (formaldehyde, acetic acid, and 70% ethanol, 1:1:18 v/v/v) for 48 h and then stored in 70% ethanol (Johansen, 1940). We used completely exposed stamens without bract protection. On each slide, five anthers of flowers of the same spike were squashed in a drop of acetic carmine (Radford et al., 1974). Per slide, 200 grains were counted and separated into viable and nonviable grains (Kearns and Inouye, 1993). The period of pollen grain availability on the flowers was observed on the same days and flowers used to verify the stigma receptivity period of plants of a natural population.

To verify the dependence on pollinators for fruiting, spontaneous self-pollination was tested on 20 spikes isolated with tissue (fully closed mesh) bags before the beginning of stigma receptivity, preventing pollen arrival by biotic or abiotic vectors; this isolation was maintained until abortion of spikes or fruiting. Another 20 spikes were labeled and left exposed to visitation (open-pollination or control). The number of flowers per spike was counted in 15 spikes to estimate the fruit sets. During the fieldwork, flower visitors were observed from 8 AM until 5 PM.

Analysis of flower by light and scanning electron microscopy—We also analyzed aspects of flower biology with the aid of light microscopy (LM) performed at the Laboratory of Plant Anatomy, Federal University of Viçosa, and scanning electron microscopy (SEM) at the Center for Microscopy and Microanalysis at the same institution. To this end, spikes collected from flower buds to senescence were fixed in 2.5% glutaraldehyde (sodium phosphate buffer 0.1 M, pH 7.2) for 24 h, washed in buffer and subsequently dehydrated in ascending ethanol series and stocked in 70% ethanol (Gahan, 1984).

For the LM analysis, samples of some of the spikes stored in 70% ethanol were dehydrated in increasing ethanol series and embedded in hydroxyethyl methacrylate resin (Historesin, Leica Microsystems, Heidelberg, Germany). The material was cut in cross- and longitudinal sections by an automatic advance rotary microtome (model RM2155, Leica Microsystems, Deerfield, Illinois, USA), thickness 5μm, stained with toluidine blue, pH 4.4 (O’Brien et al., 1964), and mounted under thin coverslips in synthetic resin (Permount, Fisher Scientific, New Jersey, USA). The slides were examined under a light microscope (AX-70 TRF, Olympus Optical, Tokyo, Japan) equipped with a U-Photo photographic system and digital camera (AxioCamHRc; Zeiss, Göttingen, Germany).

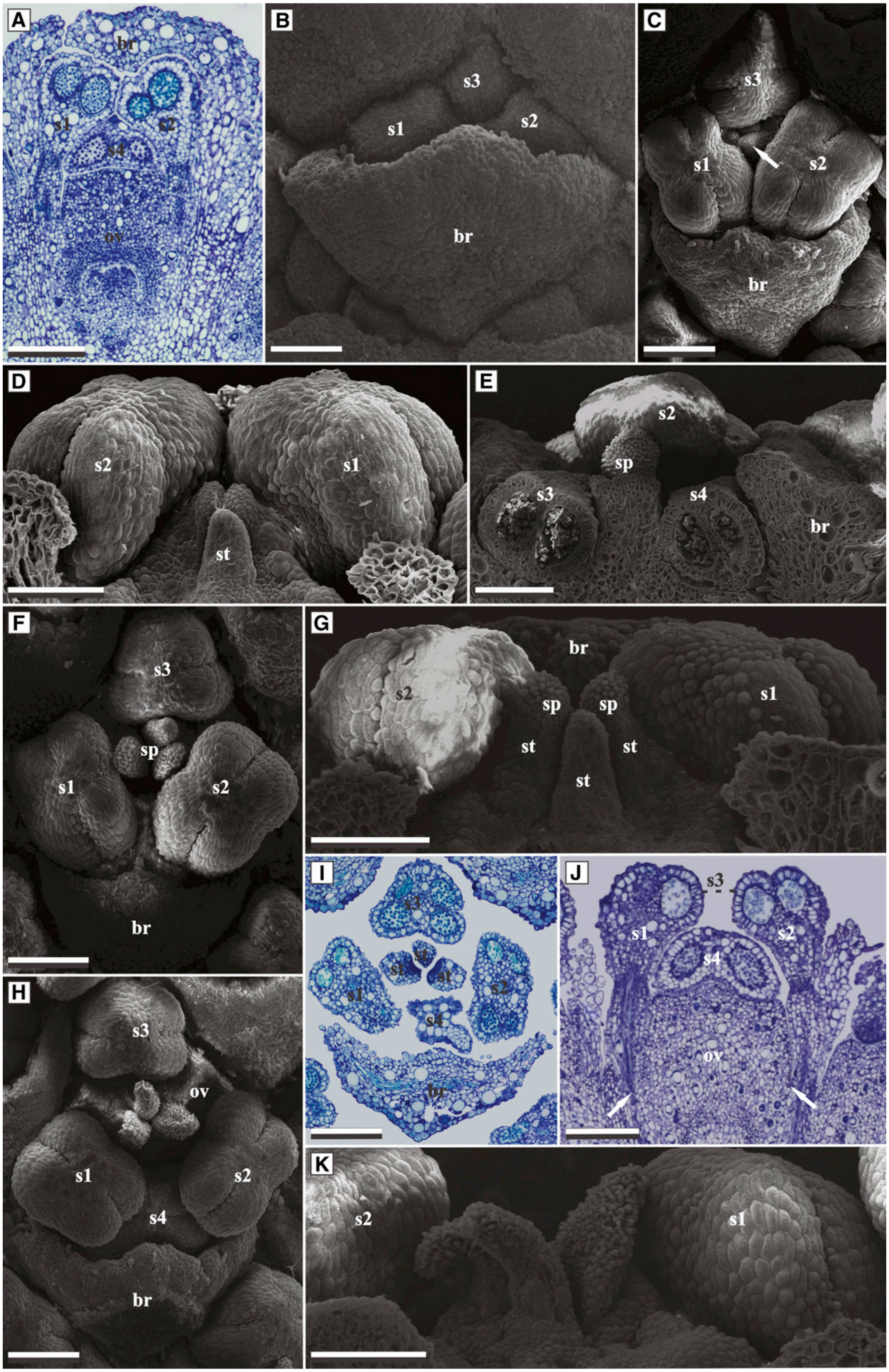
Samples of other spikes were also prepared for SEM analysis. For this purpose, samples were dehydrated in an ethanol series and subjected to critical-point drying using CO_2 (CPD 020, Bal-Tec, Balzers, Liechtenstein). After sample fixation on supports with a mixture of enamel and graphite powder, metal deposition with gold was performed (FDU 010, Bal-Tec, Balzers, Liechtenstein). The images were examined and captured in a scanning electron microscope (model LEO 1430 VP, Zeiss, Cambridge, England).

RESULTS

In the bud stage, the androecium and gynoecium are fully covered by the bract (Fig. 1A). As the bract begins to open, the androecium is partially exposed, i.e., the apical portion of the anthers of three of the four stamens. Two stamens flank the bract laterally (stamens 1 and 2) and the third stamen (stamen 3) is located between the first two (Fig. 1B). On average, six days after the beginning of bract opening, the three stigmas, juxtaposed and upright, become visible between the three stamens, when the anthers are almost completely exposed (Fig. 1C, D). At this point, the fourth stamen of the flower, still in bud, is hidden by the bract.

Anthesis begins with the receptivity of the stigmas, which have papillae on their ventral surfaces (Fig. 1E), in flowers with three exposed stamens (stamens 1–3) that have indehiscent anthers (Fig. 1F). In this early stage, the stigmas are partially separated from each other in the distal region, and the receptive papillae (positive test with Peroxtesmo KO) are turgid and positioned in the upper third of the stigmas; the remaining papillae are hidden by the portion of the still-juxtaposed stigmas (Fig. 1F, G). The stigmas are opposite from stamens 1, 2, and 3, and one of them (the one opposite from stamen 3) is shorter than the other two (Fig. 1F, G). The onset of stigma receptivity prior to pollen release indicates protogyny.

One or two days after the beginning of anthesis, the anther of the fourth (and last) stamen (stamen 4), located between the bract and the ovary, becomes partially exposed because of complete bract opening (Fig. 1H, I). On this occasion, we observed that the stamens have three different heights: stamens 1 and 2 are the highest, followed by 3, and lastly, stamen 4 (Fig. 1J). These differences in height are related to the dynamics of bract opening and the sequence of stamen development in the flower, observed by the timing of their exposure, partial (only anther) and total (filament and anther). Although all stamens are partially exposed, anthers are still indehiscent. At this time, the distance between the stigmas has increased and the turgid and receptive papillae are almost completely exposed (Fig. 1H, K); the upper portion of the ovary is now visible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(Fig. 1H). The pistillate phase lasted 2–6 d. When this phase lasted longer than two days, the flower remained in the state shown in Fig. 1H.

The anther of stamen 4 becomes visible when the spacing between stamens 1 and 2 increases (Fig. 2A). The stigmatic papillae are almost completely exposed and still receptive; however, the papillae of the distal portion of the stigmas have begun the process of cellular senescence, becoming plasmolyzed and unresponsive (Fig. 2B). At this stage, pollen release begins, and the flower is functionally bisexual. Pollen release usually occurs during the hottest hours of the day, between 10 AM and 1 PM. The anthers are whitish and turgid before pollen release, and become yellowed and wilted thereafter. The white, released pollen form clumps that often remained on the anther from one day to the next.

Stamens 1 and 2 are the first to release pollen, but not simultaneously (Fig. 2C). Usually, one of the stamens becomes higher than the other, due to its total exposure (filament and anther); the higher one is the first to release pollen (Fig. 2D). Two patterns were distinguished (Fig. 3). The stamens release pollen on the same day but at different times: the first in the morning and the other in the early afternoon, with a difference of 1–3 h. In the second pattern, pollen release occurred in the morning, but on separate days, about 24 h apart (Fig. 2E; see the filament scar of the stamen that already released pollen). In this stage, pollen from stamens 1 and 2 was 92.3% and 91.4% viable, respectively.

The anthers of stamens 1 and 2 are aborted one day after pollen release (Fig. 2F; see abscission tissue in Fig. 2D). Stamen 3, with 77.4% of viable pollen, can release pollen on the day immediately after release by stamens 1 and 2 or one day later (Fig. 3). On this occasion, the stigmatic papillae are fully exposed and still receptive. The papillae of the distal and median portions of the stigmas are already plasmolyzed (Fig. 2G). It was also observed that the plasmolyzed papillae begin to accumulate phenolic compounds, initially located in the distal portion of the stigmas (Fig. 2H).

On the day after pollen release by stamen 3, only stamen 4 is left on the flower (Fig. 2I). The variation in pollen release from stamen 4 is similar to that of stamen 3 (Fig. 3). On stamen 4, 89.8% of the pollen grains were viable. In this stage, the stigmas are separated from each other at the base (Fig. 2I) and some of the papillae may still be receptive, although the stigmas of some flowers are unreceptive at this stage. On receptive stigmas, the largest part of the stigmatic surface had papillae containing phenolic compounds.

Because of sequential anther opening, pollen release time varied from 3–6 d (Fig. 3). After anther abscission of stamen 4, part of the stigmatic surface of some flowers may remain receptive for up to two days. On this occasion, many papillae are collapsed and the papillae that remained receptive were those located in the proximal third of the stigmas (cells still turgid; Fig. 2J). Then, as ovary size increased during fruit formation, the stigmatic papillae became completely collapsed (Fig. 2K).

On the spike, the exposure of the reproductive organs begins in proximal flowers, but within a few days the others initiate opening of the bract. When the proximal flowers are in the pistillate phase, most flowers on the spike are in this phase as well, with few exceptions that persist as buds on the apex. During pollen release, there is no specific release sequence among the flowers (e.g., from the spike base to the apex), which indicates a minor time difference in anthesis along the spike. On the same plant, the anthesis of flowers of different spikes may be synchronous, such that geitonogamous pollination is possible.

The spikes had on average 463.7 flowers. Fruits were observed in all spikes of both pollination tests. Fruit set by spontaneous self-pollination was 77% and 81.5% by open-pollination. Apart from some weevils, which mated on the flowers, there were no other insects visiting the inflorescences. During weevil mating, couples often remained on a single spike without moving, which is an unfavorable behavior for a role as pollinator.

DISCUSSION

Protogynous dichogamy, recorded in *Piper vicosanum,* was also described for other species of the genus (Menon, 1949; Martin and Gregory, 1962; Figueiredo and Sazima, 2000). In representatives of the "non-monocot paleoherbs" and the "Magnoliid complex" plant groups that include Piperaceae (Judd et al., 2007), protogyny is a common trait, aside from being considered an ancestral condition in angiosperms (Endress, 2010). Protogyny favors cross-pollination (Lloyd and Webb, 1986; Bertin and Newman, 1993), although incomplete protogyny, as found in this study, does not prevent self-pollination due to the bisexual phase of the flower. Incomplete protogyny is the most commonly observed type of dichogamy in *Piper* species. Figueiredo and Sazima (2000) observed this trait in 11 species in southeastern Brazil.

Incomplete protogyny in *P. vicosanum* flowers seems to be a consequence of the long period of stigma receptivity (up to 14 d). This longevity, in turn, seems to result from the gradual and sequential exposure of the receptive stigmatic papillae. Other authors mentioned long-lived receptive stigmas (up to 10 d) in *Piper* species (Menon, 1949; Martin and Gregory, 1962; Figueiredo and Sazima, 2000), but provided no details on exposure of receptive area or any other stigmatic characteristic. The gradual and sequential exposure of stigmatic papillae is described here as the mechanism for the long duration of receptivity. The pattern of sequential exposure indicates that the three stigmas, with apparently continuous receptive areas, are actually fragmented. Each fragment consists of a set of receptive papillae exposed in a basipetal direction.

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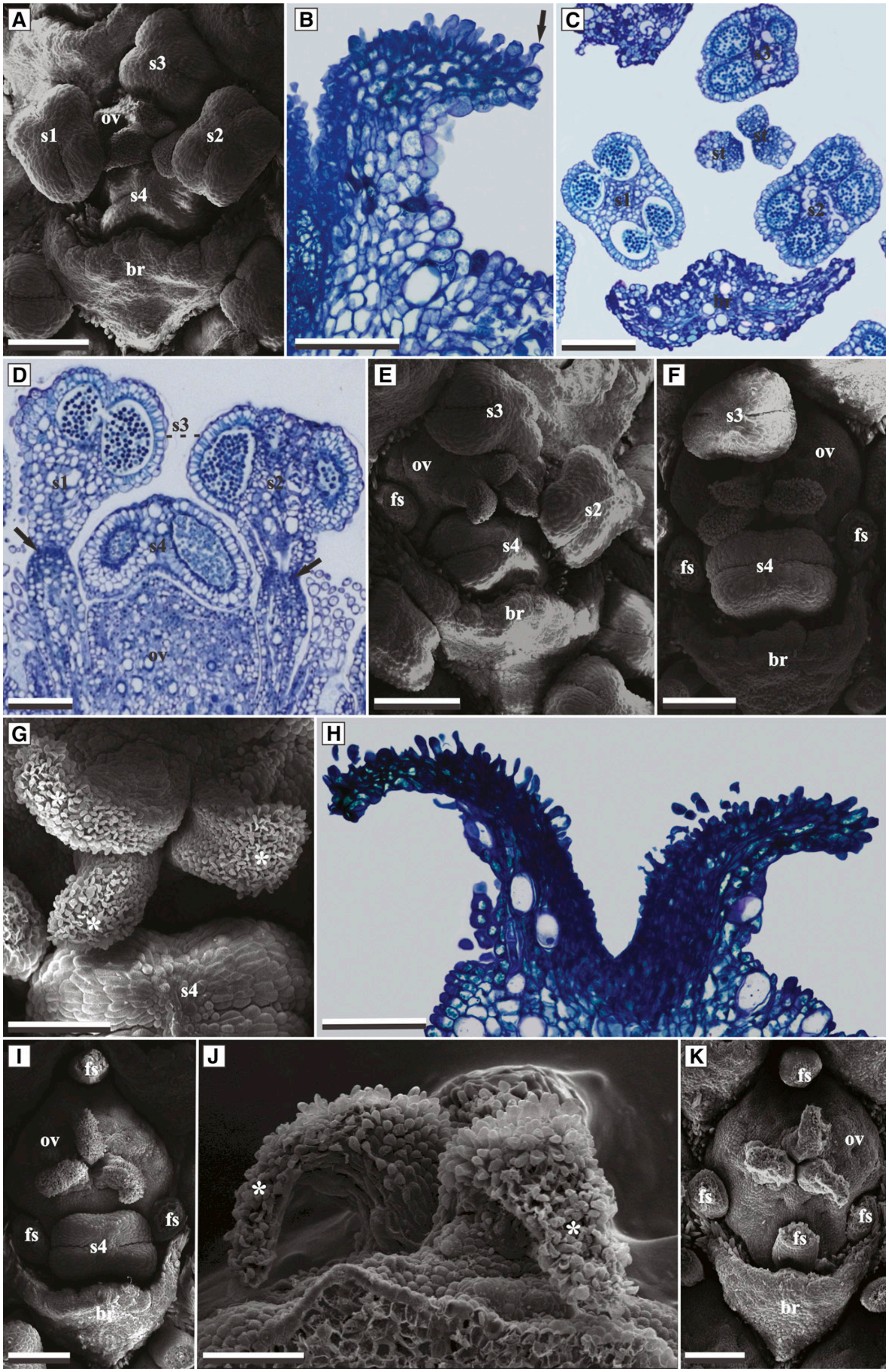
Fig. 1. Flowers of *Piper vicosanum* as buds and in the pistillate phase: (A) longitudinal cut of a flower bud, (B) beginning of stamen exposure, (C) flower bud with stigmas between the anthers (arrow), (D) upright and juxtaposed stigmas (stamen 3 removed), (E) flower with three stamens and one of the stigmas with papillae on the ventral surface (stamen 1 and two stigmas removed), (F) flower with three exposed anthers, (G) papillae exposure in the distal portion of the stigmas (stamen 3 removed), (H) partial exposure of stamen 4, (I) flower with four exposed anthers in a transversal cut, (J) height difference between stamens (stamen 3 in a series of cuts; filament insertion indicated by arrows), in a longitudinal cut, and (K) almost completely exposed stigmatic papillae (stamen 3 removed). The bract apex is directed toward the distal region of the spike. br, bract; ov, ovary; sp, stigmatic papillae; st, stigma; s1, stamen 1; s2, stamen 2; s3, stamen 3; s4, stamen 4. Scale bars: 150 μm (B, D, G, K), 200 μm (A, C, E, F, H, J), 250 μm (I).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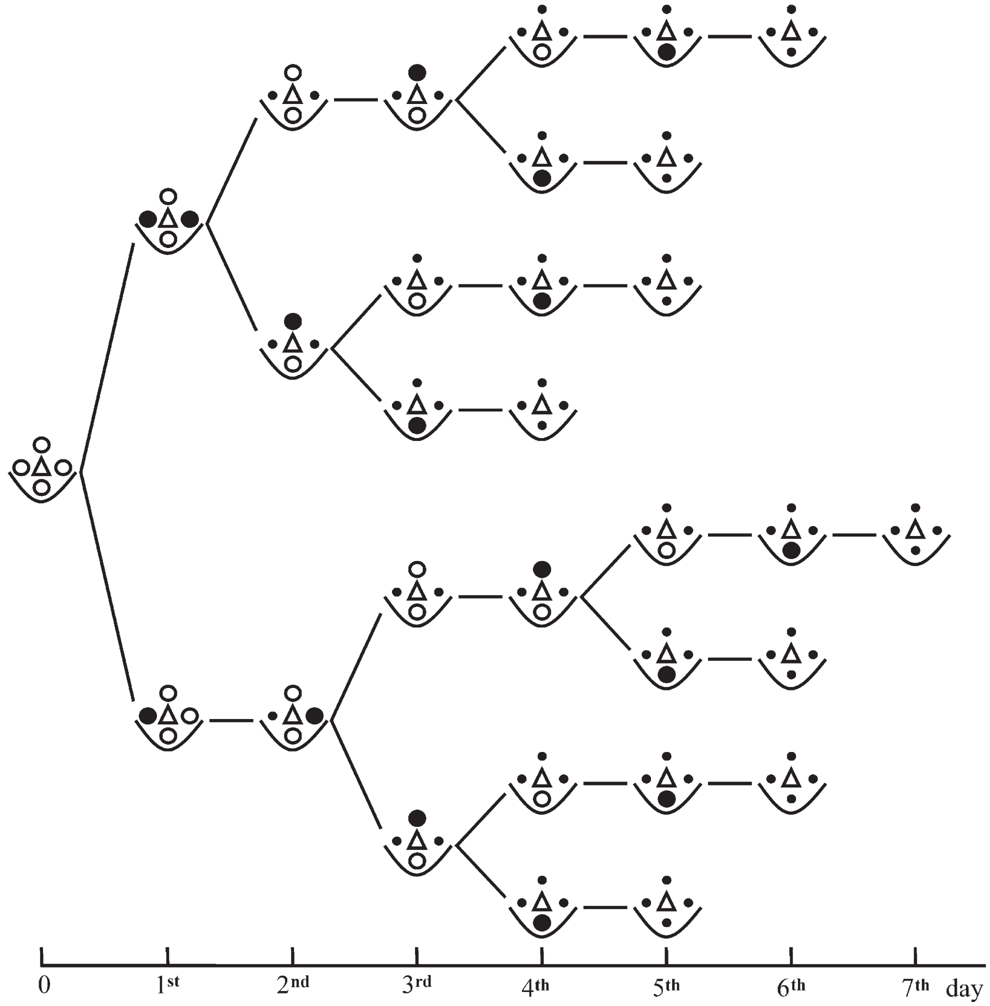

Fig. 3. Diagrams of *Piper vicosanum* flowers with possible pollen-release sequences from the four stamens. ○, indehiscent anther; ●, pollen-releasing anther; •, filament scar; △, gynoecium; ⌣, bract.

In the stigmatic papillae, the changes in the cells from turgid to plasmolyzed and the subsequent accumulation of phenolic compounds also occurred gradually and in sequence. According to Beckman (2000), there is a relationship between cells that undergo programmed cell death (a process that includes senescence), and those that accumulate phenolic compounds. Furthermore, one of the functions of these compounds is resistance to pathogens (Beckman, 2000). It is possible that the accumulation of phenolic compounds in the plasmolyzed papillae prevents contamination of these cells by pathogens that may be transported, for example, by flower visitors (Elzinga et al., 2007); in this way, it would indirectly protect the still-functional papillae.

The asynchronous pollen release in *P. vicosanum* was first described in the genus and differed from the developmental sequence of stamens proposed by Tucker (1982); stamens 1 and 2 do not release pollen simultaneously. Tucker mentioned that anther dehiscence in *Piper* occurs at different times, but did not show the sequence of pollen release. Arias and Williams (2008) described the sequence of pollen release in *Manekia naranjoana* (C. DC.) Callejas ex N. Zamora, Hammel & Grayum—another representative Piperaceae with four stamens per flower. In this species, the two lateral stamens (1 and 2) released pollen simultaneously, followed by the basal stamen (4) and then the apical stamen (3). However, Arias and Williams were not able to distinguish sequential release of pollen from stamens 1 and 2 on the same day.

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Fig. 2. *Piper vicosanum* flowers in the bisexual phase and after pollen release: (A) flower with four stamens, (B) stigma with plasmolyzed papillae in the distal portion (arrow), in a longitudinal cut, (C) asynchrony at anther opening of the stamens 1 and 2, in a transversal cut, (D) height difference between stamens 1 and 2 during pollen release (stamen 3 in a series of cuts) and abscission tissue in the filament (arrows), in a longitudinal cut, (E) flower after pollen release of stamen 1, (F) flower with stamens 3 and 4, (G) detail of the stigmas, with most papillae plasmolyzed, (H) accumulated phenolic compounds (greenish), mainly in the distal portion of the stigmas, in a longitudinal cut, (I) flower with stamen 4, (J) receptive papillae in the proximal third of the stigmas, and (K) beginning of fruit formation. The bract apex is directed toward the distal region of the spike. br, bract; fs, filament scar; ov, ovary; st, stigma; s1, stamen 1; s2, stamen 2; s3, stamen 3; s4, stamen 4; *, plasmolyzed papillae. Scale bars: 100 µm (B, H, J), 150 µm (D, G), 250 µm (A, C, E, F, I, K).

The sexual attributes observed in *P. vicosanum*—dichogamy, gradual maturation of stigmas and sequential pollen release—are not commonly recorded together in flowers of other plant species. According to Ashman and Schoen (1996), each of these attributes is a mechanism that is related to increased floral longevity, as observed in the studied species.

Protogynous species, such as *P. vicosanum*, are usually pollinated by wind or beetles (Bertin and Newman, 1993; Sargent and Otto, 2004); this association between dichogamy and pollination syndrome seems to be the product of convergent evolution (Sargent and Otto, 2004). The conclusion was drawn that pollination in this species is biotic (entomophilous), based on the persistence of pollen grains on the anthers from one day to the next, as also observed by Semple (1974) in entomophilous *Piper* species. Additionally, in *P. vicosanum*, the position of the reproductive flower structures, i.e., stigmas closely surrounded by stamens, is unfavorable for anemophilous pollination (see the characteristics of anemophilous species in Culley et al., 2002; Friedman and Barrett, 2008). In fact, the most commonly observed pollination system in *Piper* is entomophily (Martin and Gregory, 1962; Semple, 1974; Fleming, 1985; Figueiredo and Sazima, 2000; Thomazini and Thomazini, 2002; Kikuchi et al., 2007). Wind has been cited as a pollen vector for some species, as well as ambophily (Martin and Gregory, 1962; Figueiredo and Sazima, 2000), but there have been no experimental tests for abiotic pollination in these plants. For example, anemophily was inferred in *Macropiper excelsum* Miq. (now *Piper excelsum* G. Forst.), a dioecious species, because of the absence of biotic pollinators and high pollen:ovule ratio (Merrett et al., 2007).

In *P. vicosanum*, as well as in many other species of distinct families of basal angiosperms, pollen is the resource offered to pollinators (Endress, 2010). During the pistillate phase of this species, no flower resource is available, which could result in the absence of visitations. However, it is possible that the stamens exposed in the pistillate phase provide pollinator attraction, because the mature, but unopened anthers (white) change the color of the spikes (initially greenish). Thus, spikes with flowers in the pistillate phase are visually similar to those with flowers in the bisexual phase, when pollen (also white) is available. This similarity may represent self-mimicry sensu Pasteur (1982). In this way, if visitation occurs in the pistillate phase, pollination occurs "by mistake", by the nutritive deception described by Dafni (1984). According to Dafni, the visual cues are learned by the visitors, and flowers have weak or no odor, decreasing the ability of pollinators to recognize the flower without access to pollen as a food source, which favors the maintenance of this deceptive mechanism. The flowers of *P. vicosanum* have no detectable odor (personal observation), reinforcing this hypothesis of pollination by mistake, but additional studies are needed for confirmation.

Despite the foregoing, in the study area, the probable absence of pollinators and high fruit set obtained in isolated spikes indicate that *P. vicosanum* must be self-compatible and pollinator-independent. The gradual and sequential exposure of the receptive stigmatic papillae and long-lived stigma associated with asynchronous pollen release seem to increase the chances of self-pollination, and consequently, autogamy ensures reproductive success. Autogamy as a substitute reproductive strategy for allogamy, due to the absence of pollinators, has been reported in other plants (Herlihy and Eckert, 2002; Kalisz and Vogler, 2003; Kalisz et al., 2004). Autogamy is possibly the prevailing reproductive mechanism in *P. vicosanum*, and therefore, this species may have reached the "end of the line in evolution", as explained by Barrett (2002). Studies in other natural populations where pollinators are active will shed new light on this subject.

In *P. vicosanum* flowers, we found gradual and sequential exposure of stigmatic papillae, a mechanism for stigma longevity, and asynchronous pollen release, which are yet unreported reproductive characteristics of the genus. These characteristics, when in association, explain the incomplete protogyny observed. These sexual attributes seem to ensure the reproductive success of the study species and provide new insights that call for future studies on the floral biology of other *Piper* species.

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