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Mixed pollination system and floral signals of *Paepalanthus* (Eriocaulaceae): insects and geitonogamy ensure high reproductive success

Edivaldo Rodrigues Martins Junior[1], Ana Carolina Galindo da Costa[1,2], Paulo Milet-Pinheiro[3], Daniela Navarro[4], William Wayt Thomas[5], Ana Maria Giulietti[6] and Isabel Cristina Machado[1,*]

[1]*Programa de Pós-Graduação em Biologia Vegetal, Departamento de Botânica, Universidade Federal de Pernambuco, Recife, Pernambuco, Brazil,* [2]*Instituto Tecnológico Vale, Belém, Pará, Brazil,* [3]*Universidade de Pernambuco, Campus Petrolina, Pernambuco, Brazil,* [4]*Departamento de Química Fundamental, Universidade Federal de Pernambuco, Recife, Pernambuco, Brazil,* [5]*The New York Botanical Garden, Bronx, NY, USA and* [6]*Universidade Estadual de Feira de Santana, Programa de Pós-Graduação em Botânica, Feria de Santana, Bahia, Brazil*

* For correspondence. E-mail imachado@ufpe.br

- **Background and Aims:** Eriocaulaceae exhibit a great variety of floral traits associated with insect (e.g. nectariferous structures) and wind pollination (unisexual flowers, exposed sexual organs and small pollen grains), as well as the 'selfing syndrome' (small flowers, short distance between stigma and anthers, and temporal overlap of male and female phases). *Paepalanthus bifidus*, *P. subtilis* and *P. tortilis* are related species that differ in form, size and colour of floral structures. We aimed to investigate the pollination and reproductive biology of these three species.
- **Methods:** We analysed the floral biology, floral visitors, pollinator behaviour, and the contribution of insects, wind and spontaneous geitonogamy to fruit set. We also evaluated the floral colour and scent of the species. Colour reflectance of capitula of each species was measured and plotted in models of insect vision. Floral scent samples were extracted and the compounds were compared to vegetative scent samples.
- **Key Results:** In all species, the staminate and pistillate flowers are arranged in alternating cycles with a temporal overlap between these phases. Ants were the most frequent floral visitors and were effective pollinators in *P. bifidus* and *P. tortilis*, while flies were occasional pollinators in *P. tortilis*. Floral visitors were not observed in *P. subtilis*. In all species, fruits were produced by spontaneous geitonogamy, with no evidence of wind pollination. According to the models of insect vision, the colours of the capitula of *P. bifidus* and *P. subtilis* are the most inconspicuous for ants and flies. We found no difference between the emission of volatiles of inflorescences and vegetative structures.
- **Conclusions:** This study suggests that ant pollination might be more widespread in Eriocaulaceae than currently assumed. Furthermore, for small monocarpic plants, mixed mating strategies are most favourable, by ensuring reproduction either by outcrossing when pollinators are abundant or by spontaneous geitonogamy when pollinations are scarce/absent.

Key words: *Camponotus crassus*, floral colour, floral volatiles, myrmecophily, *Paepalanthus bifidus*, *Paepalanthus subtilis*, *Paepalanthus tortilis*, selfing, *Solenopsis tridens*, spectral reflection.

INTRODUCTION

Flowering plants exhibits an exuberant and highly diverse array of floral traits that are related to different pollination systems. Floral shape, size, colour and scent, as well as their patterns across space and time, play an important role in mediating plant–pollinator interactions (Raguso, 2004; Friedman and Barrett, 2009; Harder and Johnson, 2009; Barrett, 2010; Goodwillie *et al.*, 2010; Schiestl and Johnson, 2013; Leonard and Masek, 2014; Van der Niet *et al.*, 2014; Torezan-Silingardi *et al.*, 2021). For plants that rely on animals for pollination, visual and olfactory floral signals usually transmit information that allows pollinators to locate a specific host flower, to discriminate between flowers of different species and to evaluate reward availability (Chittka and Raine, 2006; Wright and Schiestl, 2009; Dötterl and Vereecken, 2010; Junker and Parachnowitsch, 2015). On

the other hand, plants that are less dependent on biotic pollinators usually have inconspicuous flowers (Goodwillie *et al.*, 2005, 2010; Friedman and Barrett, 2009; Costa *et al.*, 2021).

Eriocaulaceae is the fourth largest family of the order Poales with ~1400 species, distributed in ten genera (Giulietti *et al.*, 2012). The family has a pantropical distribution, and its representatives are easily recognized by leaves arranged in a rosette, scapes with a capitulum at the apex, and capitula comprising small pistillate and staminate flowers and surrounded by involucral bracts, which can be the same size or larger than the flowers. Species of Eriocaulaceae display a variety of floral traits, and the inflorescence architectures are highly variable (Stützel and Trovó, 2013). The capitula can be considered conspicuous, usually more than 1 cm in diameter and/or with bracts larger than the whitish or cream flowers, as in some species of

Comanthera and *Paepalanthus* subg. *Xeractis*, or inconspicuous (usually about 2–5 mm in diameter including brownish, black or greenish bracts, which are smaller than the flowers). The emission of odour can be perceived by human olfaction in some species of *Comanthera* and *Actinocephalus* (Sano, 1996; Ramos *et al.*, 2005). Flowers offer pollen and nectar as a reward to pollinators (Stützel, 1981, 1998; Ramos *et al.*, 2005; Sawyer *et al.*, 2005; Oriani *et al.*, 2009; Del-Claro *et al.*, 2019). However, even with a wide diversity of floral traits, data on reproductive biology and pollination ecology are incipient in this family, representing only 2.2 % of all studies on Neotropical Poales (Wolowski and Freitas, 2015).

Given such diversity of floral traits, since Ruhland (1903) and Cronquist (1981) it has been questioned whether Eriocaulaceae are pollinated by wind or insects. Floral traits present in Eriocaulaceae species, such as unisexual flowers, anthers and stigmas above the perianth, small pollen grains and a relatively large number of staminate flowers in a capitulum, are frequently correlated with wind pollination (Friedman and Barrett, 2008, 2009). However, until now, there was no concrete evidence of wind pollination for the family (Ramos *et al.*, 2005).

Recent studies on the floral biology of Eriocaulaceae suggest that entomophily is the main pollination system of the family (Ramos *et al.*, 2005; Oriani *et al.*, 2009; Del-Claro *et al.*, 2019). Nectariferous structures, which are quite rare in Poales, evolved independently in Eriocaulaceae in its two subfamilies. In Eriocauloideae they are associated with the nectariferous carpellodes and petal glands in the staminate and pistillate flowers (Rosa and Scatena, 2003, 2006; Silva *et al.*, 2016, 2021). However, in Paepalanthoideae they are associated with nectariferous carpellodes in the staminate flowers, and nectariferous branches in the pistillate flowers (Rosa and Scatena, 2003, 2006; Silva *et al.*, 2016, 2021). Several nectar-seeking insects of different orders, such as flies, bees and beetles, are recorded as visitors and/or pollinators of *Comanthera curralensis* (Ramos *et al.*, 2005), *C. mucugensis* (Ramos *et al.*, 2005) and *C. elegans* (Oriani *et al.*, 2009). Ants were reported as the main effective pollinators in *Paepalanthus lundii*, representing a quite unusual interaction (Del-Claro *et al.*, 2019).

Moreover, some species of Eriocaulaceae can be classified as having the 'selfing syndrome' (*sensu* Sicard and Lenhard, 2011), which is associated with a particular set of traits, such as small flowers, short distance between stigma and anthers, temporal overlap of male and female flowers within a capitulum, as well as reduction in floral display, nectar secretion and emission of volatiles. In terms of reproductive strategies, self-compatibility and production of seed through spontaneous geitonogamy between the staminate and pistillate flowers of the same capitulum have been reported in the family (Stützel, 1981; Sawyer *et al.*, 2005; Oriani *et al.*, 2009; Horiuchi *et al.*, 2020).

Paepalanthus is the largest and most morphologically diverse genus of Eriocaulaceae, Paepalanthoideae, with five subgenera and many infrageneric taxa (Körnicke, 1863; Ruhland, 1903; Costa *et al.*, 2018; Andrino *et al.*, 2020, 2021). This genus comprises ~400 species, of which 348 occur in Brazil (Andrino *et al.*, 2020). *Paepalanthus bifidus*, *P. subtilis*, and *P. tortilis* are three morphologically closely related species in *Paepalanthus* ser. *Leptocephali* (Körnicke, 1863, Ruhland, 1903) and are widely distributed in South America, occurring from Venezuela to Brazil (Giulietti and Hensold, 1990; Hensold, 1999; Andrino

et al., 2020). In Brazil, they occur in all regions except the south. Given the possibility of different pollination systems in Eriocaulaceae, such as insect, wind and self-pollination, we investigated the pollination and reproductive biology of these three species. We evaluated the role of insects and how they discriminate the floral structures in the capitula in Eriocaulaceae, contributing to our understanding of the evolutionary processes related to plant–pollinator interactions in the family. For this, we addressed the following questions: (1) What is the pollination and reproductive system of each of the studied species? (2) What are the floral visitors and do they all play a role in the pollination of the species? (3) Are visual floral signals conspicuous to pollinators? (4) Do flowers emit scents?

MATERIALS AND METHODS

Study area

The work was carried out in the Guaribas Biological Reserve (06°40′40″–06°44′59″S, 41°06′46″–41°08′00″W), Paraíba State, north-east Brazil, from May to August 2019. The vegetation is characterized by fragments of semideciduous forest and open areas covered by an herbaceous layer dominated by representatives of Poaceae, Cyperaceae, Eriocaulaceae and Xyridaceae (Barbosa *et al.*, 2011). Average annual temperature varies from 24 to 26 °C, with hottest months from December to February. The rainy season begins in February and ends in July, with average annual precipitation from 1750 to 2000 mm (Barbosa *et al.*, 2011).

Studied species

At the study site flowering of the three species studied occurs from June to August, peaking in July. *Paepalanthus bifidus* and *P. tortilis* grow in dense patches with many individuals (~60 individuals for *P. bifidus* and 40 individuals for *P. tortilis*; Supplementary Data Fig. S1). *Paepalanthus bifidus* grows under shaded conditions, mostly along the edges of trails, whereas *P. tortilis* occurs in open areas. *Paepalanthus subtilis* was the most abundant species, occurring from the edges of the trails along with *P. bifidus* and extending into the open areas. Vouchers of studied species were deposited in the herbarium UFP – Geraldo Mariz, Federal University of Pernambuco, PE, Brazil (*P. bifidus* UFP 87.397; *P. subtilis* UFP 87.399; *P. tortilis* UFP 87.398).

Floral biology

The time, sequence and duration of flower anthesis were monitored in 20 capitula of each species (one capitulum per individual plant). The receptivity of the stigma was evaluated in ten functional pistillate flowers of each species, always from different individuals. For this, we dripped 3 % hydrogen peroxide on to the stigma hourly from 0800 to 1700 h and checked for the presence of bubbles using a hand lens (40×) – the presence of bubbles indicates that the stigma is receptive (Dafni *et al.*, 2005). We counted the number of pistillate and staminate

flowers in ten capitula from different individuals under a stereomicroscope.

Floral visitors

Focal observations were conducted in early July and in August. In July, one patch of plants of each species was selected and observed (each patch in a delimited area of about 1 m²) for five consecutive days, from 0800 to 1700 h, totalling ~45 h of observation per species. In late August, observations were conducted in two plant patches about 1 km away from the patches previously studied. In one patch, *P. subtilis* and *P. bifidus* occurred together whereas in the other *P. tortilis* occurred alone. The patches were observed for 3 d (two consecutive days in the patch with *P. subtilis*–*P. bifidus* and 1 d in the patch with *P. tortilis*), from 0800 to 1100 h and from 1300 to 1700 h. We recorded all insects that reached the inflorescences and later left the observation area (absolute abundance) at intervals of 1 h using a hand-held counter and stopwatch. Similarly, the number of visits for each visiting insect species was recorded. We considered a visit when insects contacted a receptive flower and collected the floral resource (Ramos *et al.*, 2005; Oriani *et al.*, 2009). During this time, we described the behaviour of floral visitors.

According to the number of individuals of each insect species, visits to the nectariferous structures and behaviour of the visitor, the insects were classified as effective or occasional pollinators (Alves-dos-Santos *et al.*, 2016). Effective pollinators were those that were abundant and frequently visited the nectariferous structures, transferring pollen grains between different individuals and capitula, and contacting stigmas of the flowers. Occasional pollinators had a similar behaviour as described before, but were less frequently found on flowers (i.e. those that were not recorded on all days of focal observations). Flower visitors were captured with entomological nets, mounted, identified and deposited in the collection of insects of the Floral and Reproductive Biology Lab at the Federal University of Pernambuco, Brazil.

Pollination systems

To determine the pollination systems of the studied species, we used 35 capitula of different individuals for each treatment in each studied species ($N = 105$ capitula per species) and treated them as follows: (1) spontaneous geitonogamy (i.e. pollination between the staminate and pistillate flowers of the same capitulum) – inflorescences were covered with polyester bags (Oriani *et al.*, 2009), preventing the entrance of exogenous pollen carried by the wind and/or by floral visitors; (2) wind pollination – inflorescences were enclosed with a tulle fabric (500 × 500-µm mesh) (Oriani *et al.*, 2009) preventing access by insects while allowing free passage of pollen grains carried by the wind; and (3) natural pollination (control) – capitula were marked and left exposed under natural conditions enabling the action of both possible pollen vectors (i.e. wind and insects). The capitula used in this experiment were all bagged in flower-bud stage. After about 7 weeks, all capitula were collected and the numbers of unpollinated pistillate flowers and fruits formed

were counted. Reproductive success was calculated as the ratio of the number of unfertilized pistillate flowers in relation to the number of fruits formed. By comparing (1) with (2) we can infer the participation of wind, and (3) with (2) and (1) the participation of insects in reproductive success. Since the data did not meet the criteria for parametric statistics, differences in fruits formed in each capitula among the three treatments were compared by means of the Kruskal–Wallis test ($\alpha = 0.05$) followed by Dunn's multiple comparison post-hoc test ($\alpha = 0.05$).

Spectral floral reflectance and colour space

In order to evaluate the colour of the floral structures from the pollinator perspective, we analysed spectral reflection properties of the capitula (including flowers and involucral bracts), scapes and leaves of each species. We obtained wavelengths from 300 to 700 nm using a calibrated spectrophotometer (JAZEL200, Ocean Optics, USA). For each species and structure, five measurements were taken *in situ* to obtain the average of the spectrum. The reflectance of the scapes and leaves were similar, and the reflectance of both structures was considered the background colour. In *P. bifidus*, we observed that after dehiscence of the anthers, the filaments lose turgor and the anthers regressed toward the corolla tube, with a clear colour change from white to yellow-orange. Because of this colour change, as perceived from the human perspective, colour measurements were conducted at two distinct times: (1) before the colour change, in capitula starting to mature and borne on short scapes, when the first outer cycle of staminate flowers, with white anthers, are in anthesis, and the remaining, internal flowers of the capitulum are only in bud (Fig. 1A); and (2) after the colour change, in capitula in an intermediate phase of maturation and borne on long scapes, when the anthers of staminate flowers of the first outer cycle have become dehiscent and their colour has changed from white to yellow-orange (Fig. 1B).

Data obtained were plotted in colour space models, which are graphical representations that characterize how colour can be recognized by insects (Renoult *et al.*, 2017). In this approach, colours are represented in a locus in the insect model vision, and the position of each colour locus depends on the quantum catches captured by each photoreceptor that is determined by the stimulus reflectance spectrum, photoreceptor sensitivity curves, background reflectance and illuminant spectrum (Kelber *et al.*, 2003).

For the ant vision model, a dichromatic colour space was applied (Kelber *et al.*, 2003). We implemented this model estimating photoreceptor sensitivity using the wavelength of maximum sensitivity UV (350 nm) and green (510 nm) (Aksoy and Camlitepe, 2018). For flies, we used the colour space model developed by Troje (1993). This model was applied assuming the photoreceptor sensitivity of *Eristalis tenax* (Shrestha *et al.*, 2016) and we followed the adjustments recommended in Shrestha *et al.* (2016) and Hannah *et al.* (2019). As ambient light, we applied daylight illumination D65 (Wyszecki and Stiles, 1982) for *P. tortilis*, which occurs in the herbaceous layer of the study site, and the illumination *forestshade* (Maia *et al.*, 2013, 2019) for *P. bifidus*, which occurs in shaded areas along the trail edge. For *P. subtilis*, both ambient light illuminations (D65 and *forestshade*) were applied. Finally, we calculated the

Fɪɢ. 1. Capitulum details of the Eriocaulaceae species from the Reserva Biológica de Guaribas, Paraíba, Brazil. (A) Capitulum of *Paepalanthus bifidus* before the anthers change colour, indicated by an arrow. (B) Capitulum of *P. bifidus* after the colour change, indicated by an arrow. (C) Capitulum of *P. subtilis.* (D) Capitulum of *P. tortilis.* (E) Capitulum of *P. subtilis*; anthesis begins with the flowers almost completely surrounded by involucral bracts. Scale bars = 0.1 cm.

Euclidean distances between each colour locus and the achromatic centre of the colour space (background). Behavioural and electrophysiological experiments with *Camponotus blandus* (Formicidae) demonstrated that the greater the Euclidean distances from the centre of the colour space (0–1 unit), the better the colour discrimination (Yilmaz *et al.*, 2017). Similarly, Hannah *et al.* (2019) suggested a threshold of discrimination of 0.059 units to quadrant 'blue' and 0.021 units to quadrant 'green' in the model vision of Troje (1993). The analyses were performed in R (R Core Team, 2013) using the package 'PAVO' (Maia *et al.*, 2019).

Scent glands and floral volatiles

To check for osmophores (scent glands) in flowers of *P. bifidus*, *P. tortilis* and *P. subtilis*, we immersed flowers of different individuals ($N = 10$ per species) in a neutral red solution (1:1000) for 10 min, which should selectively stain odour glands (Vogel, 1990). We then verified the possible presence of odour glands stained with neutral red. Floral scent samples ($N = 3$ for each species) were collected *in situ* by using dynamic headspace methods (Dötterl *et al.*, 2005; Costa *et al.*, 2021). For

this, 20 inflorescences were put together and enclosed within PET film oven bags (WYDA® Assarápido Churrasco, Sorocaba, São Paulo, Brazil) for 60 min. After this time, the air inside the bags was drawn by a battery-operated rotary vane vacuum pump (model G 12/01 EB, ASF Thomas, Inc., Germany) at a constant flow rate of 150 mL min⁻¹ for 10 min and trapped in an adsorbent filter. The adsorbent filters consisted of miniaturized quartz glass cartridges containing 3 mg of a 1 : 1 mixture of the adsorbent polymers Tenax TA (80/100 mesh, Supelco, USA) and Carbopack™ X (20/40 mesh, Supelco). To control for ambient contaminants and for other non-floral volatiles in the inflorescence scent samples, vegetative material was enclosed within PET film oven bags ($N = 2$) and sampled following the same protocol as described above.

The samples were analysed on a gas chromatograph coupled to a mass spectrometer (GC–MS; Agilent 7890A gas chromatograph, Agilent 5975C Series MSD mass spectrometer; Agilent Technologies, Palo Alto, CA, USA) and equipped with a nonpolar HP-5ms column (Agilent J&W; 30 m × 0.25 mm i.d., 0.25-µm film thickness). A split/splitless inlet was fitted with an Agilent Thermal Separation Probe (TSP). For analysis, the quartz cartridges were directly inserted in the TSP vial holder with the inlet set to splitless mode and the injector temperature

at 250 °C. The GC oven temperature was set at 40 °C for 2 min and then increased at a rate of 4 °C min^{-1} to 230 °C. The final temperature was held steady for 5 min. Helium (He) carrier gas flow was maintained at a constant pressure of 7.0 psi. MS source and quadrupole temperatures were set at 230 and 150 °C, respectively. Mass spectra were taken at 70 eV (in EI mode) with a scanning speed of 1.0 scans s^{-1} from *m/z* 35 to 350. A homologous series of linear alkanes (C7–C40) was used to determine linear retention indices (RIs) of the ion chromatographic peaks (Van den Dool and Kratz, 1963). Compounds were identified by comparing their mass spectra and retention indices with those available in commercial mass spectral libraries (FFNSC 2, NIST14 and Wiley Registry™ 9th Edition), integrated in the software GCMSolution version 2.72 (Shimadzu Corporation).

RESULTS

Floral biology

Paepalanthus bifidus, *P. subtilis*, and *P. tortilis* are small monocarpic, caulescent plants, with trimerous flowers, showing staminate flowers with three stamens with dithecous anthers and three carpellodes (Fig. 2C, H, M); the pistillate flowers have a three-carpelar, three-locular ovary, and each style is divided into two parts: nectariferous branches and stigmatic branches (Fig. 2E, J, P). However, the studied species have differences in form, size and colour of floral structures: *P. subtilis* is erect, unbranched, 4–12 cm tall, with one to tne floral scapes per individual, 2.5–6 cm tall scapes, bearing whitish capitula (~4 mm in diameter), whitish hyaline floral bracts and dark brown involucral bracts in two series. The glabrous bracts of the outer series are lanceolate and acuminate-cuspidate at the apex, while the bracts of the inner series are obovate and acuminate-cuspidate at the apex (Figs 1C and 2F–J). *Paepalanthus tortilis* is erect, 10–20 cm tall, with 5–10 cm tall scapes bearing whitish capitula (~5 mm in diameter), black floral bracts and small, obovate, ciliate, black involucral bracts, usually not seen in the mature capitulum (Figs 1D and 2K–P). *Paepalanthus bifidus* is 5–10 cm tall, with about two 20 floral scapes per individual, with 2–5 cm tall scapes bearing greenish capitula (~3.5 mm in diameter), whitish hyaline floral bracts and lanceolate, pilose, green involucral bracts that surpass the height of the flowers (Fig. 2A–E).

The floral organization in the capitulum and the process of anthesis of the three species studied are similar. The separate flowering periods of staminate and pistillate flowers are arranged in alternating phases, with the first outer flowers always being staminate flowers. In *P. tortilis* each capitulum shows three phases of staminate flowers alternating with three phases of pistillate flowers. In *P. bifidus* and *P. subtilis* each capitulum shows three phases of staminate flowers alternating with two phases of pistillate flowers. The staminate flowering phase lasts 3–4 d while the pistillate phase lasts 2 to 3 d. The capitulum produces more staminate flowers (*P. bifidus* 28.3 ± 1.8; *P. subtilis* 29.8 ± 2.8; *P. tortilis* 33.6 ± 2.9; mean ± s.d.) than pistillate flowers (*P. bifidus* 9.1 ± 0.9; *P. subtilis* 10.4 ± 0.84; *P. tortilis* 10.9 ± 0.87) in a ratio of about 3 : 1. The flowers undergo centripetal maturation and anthesis begins between 0800 and 0830 h. Floral anthesis lasts only 1 d, with an overlap between staminate and pistillate phases in the same capitulum. In *P. subtilis*,

anthesis begins with the flowers almost completely surrounded by involucral bracts that reflex, exposing the flowers during anthesis and maturation of the capitulum (Fig. 1E). Each day, two to three staminate and pistillate flowers opened. The anthers dehisced at around 0930h, and from the beginning of anthesis the stigmas are receptive after their exposure (between 0800 and 0830 h), remaining receptive until late afternoon.

Floral visitors

In observations conducted in July, we recorded two species of ants, *Solenopsis tridens* in *P. bifidus* and *Camponotus crassus* in *P. tortilis* (Fig. 3A), and an unidentified fly in *P. tortilis* (Fig. 3B). We did not observe floral visitors to the capitula of *P. subtilis*. In late August, at the end of the flowering period, no floral visitors were recorded in any of the species.

The beginning of activity of floral visitors, the number of observed individuals and the frequency of visits varied throughout the day depending on the species of floral visitors and the species of *Paepalanthus* (Fig. 4). Visits by individuals of *Solenopsis tridens* started between 0800 and 0900 h, at the beginning of anthesis of *P. bifidus* (Fig. 4A). Flies started to be active later, between 1001 and 1100 h (Fig. 4C). The peak of visitation by individuals and frequency of visits of *S. tridens* and the flies was from 1000 to 1100 h, whereas the peak of visitation by *Camponotus crassus* was from 1100 to 1200 h (Fig. 4).

During the visits, ants and flies displayed similar behaviour. In each capitulum, they leaned toward the nectariferous apex of the carpellodes (staminate flowers) and the nectariferous branch of the styles (pistillate flowers) to drink nectar. The nectariferous structures in both type of flowers are located below the anthers and the receptive portions of the stigmatic branches (Fig. 2C, E, J, P, M). Therefore, when they accessed the nectar, the visitors contacted the anthers and stigma, thereby removing or depositing pollen grains from/on male and female flowers, respectively. Pollen transfer occurred when the visitors moved over a capitulum, and the pollen load adhered to their legs, making transfer possible to the stigmas.

During the observations of floral visits, we verified that all floral visitors had a clear preference for mature capitula borne on the tallest and older scapes. Ants reached a capitulum by climbing the scapes and moved between different individuals either by descending and ascending a new scape or by directly moving from one capitulum to another if they were contacting each other (see Supplementary Data Video). Some individuals of *Solenopsis tridens* were observed trying to climb the scapes of *P. subtilis* without being able to reach the capitulum. The duration of each visit of *Solenopsis tridens* ranged from 2 s to 4 whereas for *Camponotus crassus*, it was 5 s to 3 min. The visits of flies lasted from 5 s to 2 min.

Pollination systems

We verified high fruit formation by spontaneous geitonogamy in all studied species (Table 1). The highest fruit set for this treatment occurred in *P. subtilis* (~67 %). In all species, fruit formed by spontaneous geitonogamy and wind pollination treatments
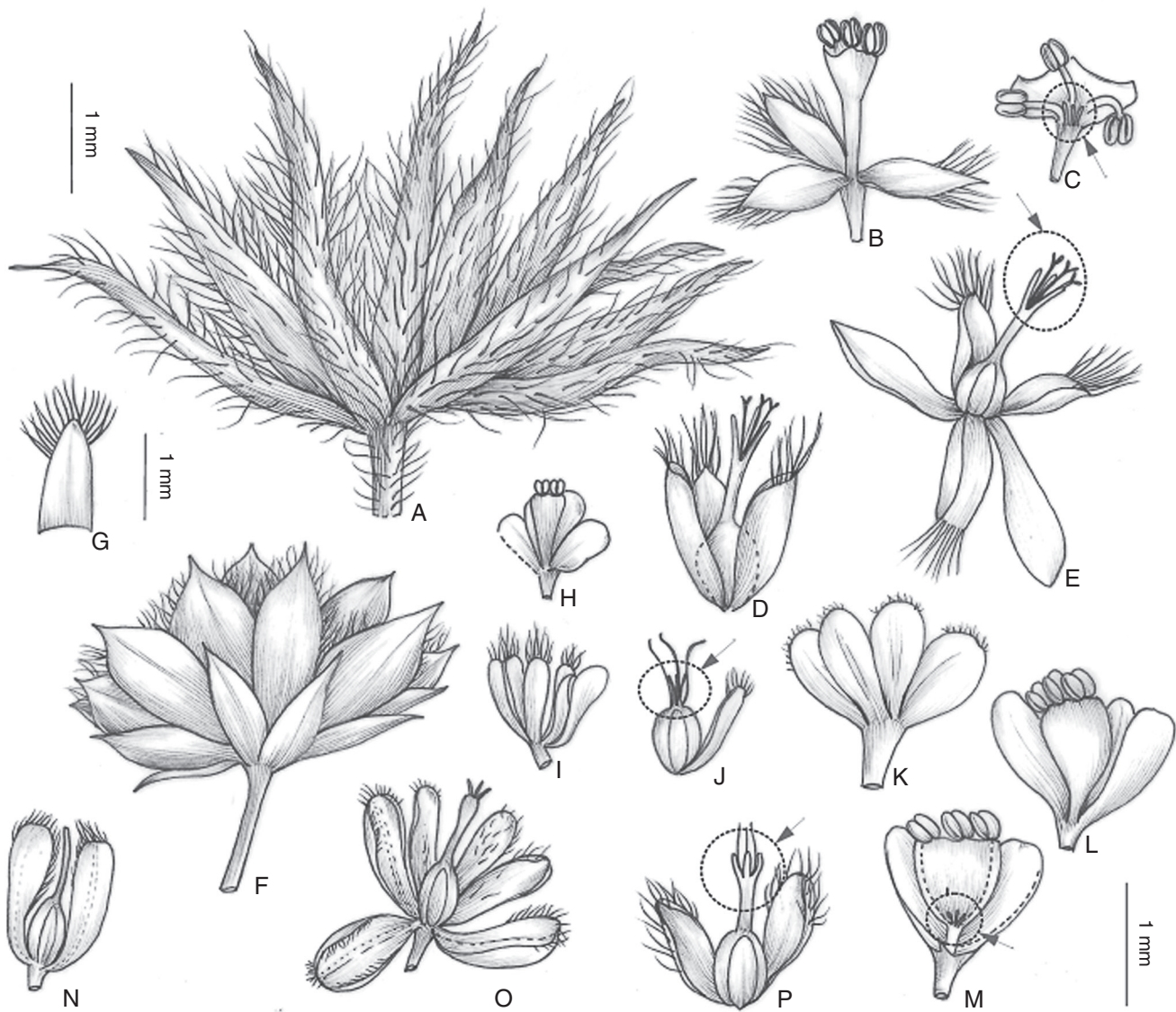
FIG. 2. (A–E) *Paepalanthus bifidus*. (A) Capitulum with lanceolate, acuminate, pilose involucral bracts. (B) Staminate flower with dialypetalous pilose calyx, corolla and tall anthophore. (C) Open staminate corolla showing stamens and carpellodes indicated by an arrow. (D) Pistillate flower showing ciliate petals and gynoecium. (E) Open pistillate flower with two glabrous sepals (one removed), three pilose petals and gynoecium with nectariferous and stigmatic branches of the style indicated by an arrow, with bifid stigmas. (F–J) *Paepalanthus subtilis*. (F) Capitulum with elliptic, acuminate, glabrous involucral bracts. (G) Pilose bract from staminate flower. (H). Staminate flower with calyx and corolla on short anthophore. (I) Pistillate flower showing two glabrous sepals (one removed) and three pilose petals. (J) A single petal and gynoecium with three style branches and simple stigma gynoecium, with nectariferous and stigmatic branches of the style indicated by an arrow. (K–P) *Paepalanthus tortilis*. (K) Young capitulum showing the involucral bracts each with a white stripe. (L) Staminate flower with glabrous sepals and corolla on short anthophore. (M) Open staminate flower showing anthophore and carpellodes (indicated by an arrow). (N) Pistillate flower showing two ciliate sepals with white stripe on dorsal surface. (O) Open pistillate flower with ciliate sepals and pilose petals. (P) Open pistillate flower showing two pilose petals and the gynoecium with style branches and simple stigmas indicated by an arrow [A–E, *Martius 555* (K); F–J, *Salzmann* s.n. (K); K–P, *M.J.G. Andrade 598* (HUEFS), drawings by A.M.G. inked in by C. Lima].

did not differ significantly ($P > 0.05$ after Kruskal–Wallis test). Therefore, wind did not contribute to reproduction (Table 1). Capitula under natural conditions showed the highest fruit set (>80 % of fruit set for all species) differing significantly from the other treatments ($P < 0.05$ after Kruskal–Wallis test) (Table 1).

Floral colour: spectral reflectance and colour space

The reflectance spectrum of *P. bifidus* (before the colour of the anthers changed), *P. subtilis* and *P. tortilis*

demonstrated that the capitula reflect wavelengths with a peak at 450 nm (Fig. 5A). *Paepalanthus bifidus* (after the colour change of the anthers) demonstrated an increase in the spectral reflectance curve, with one peak at 650 nm (Fig. 5A).

In the ant dichromatic colour space model, the colour loci of the capitula of *P. bifidus* (before colour change) and *P. subtilis* were located next to the central region (background) with a Euclidean distance of 0.17 and 0.19 units, respectively. Colour loci of the capitula of *P. tortilis* and *P. bifidus* (after colour change) were the most distant from
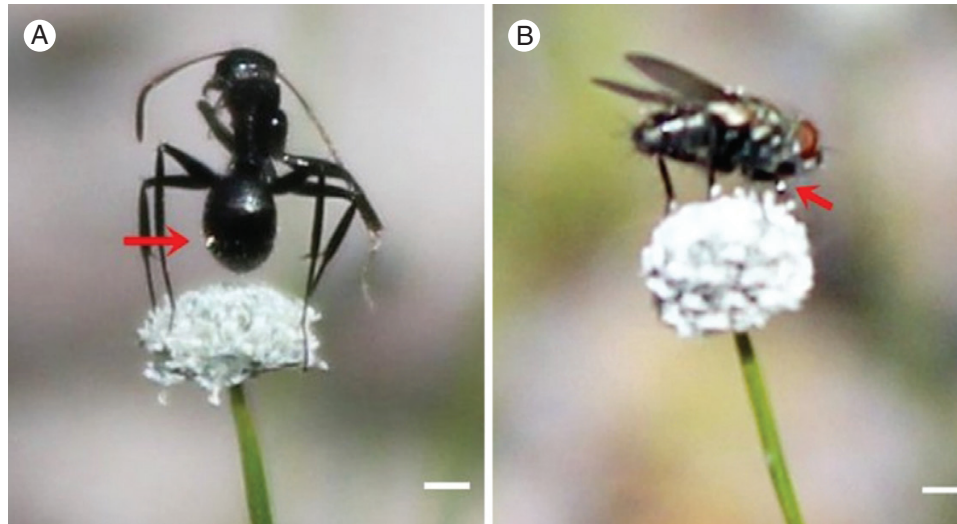
Fɪɢ. 3. Floral visitors of *Paepalanthus tortilis* in Reserva Biológica de Guaribas, Paraíba, Brazil. (A) *Camponotus crassus* and pollen load adhering to the abdomen (indicated by an arrow). (B) Fly and pollen load adhering to the legs (indicated by an arrow). Scale bars = 0.1 cm.
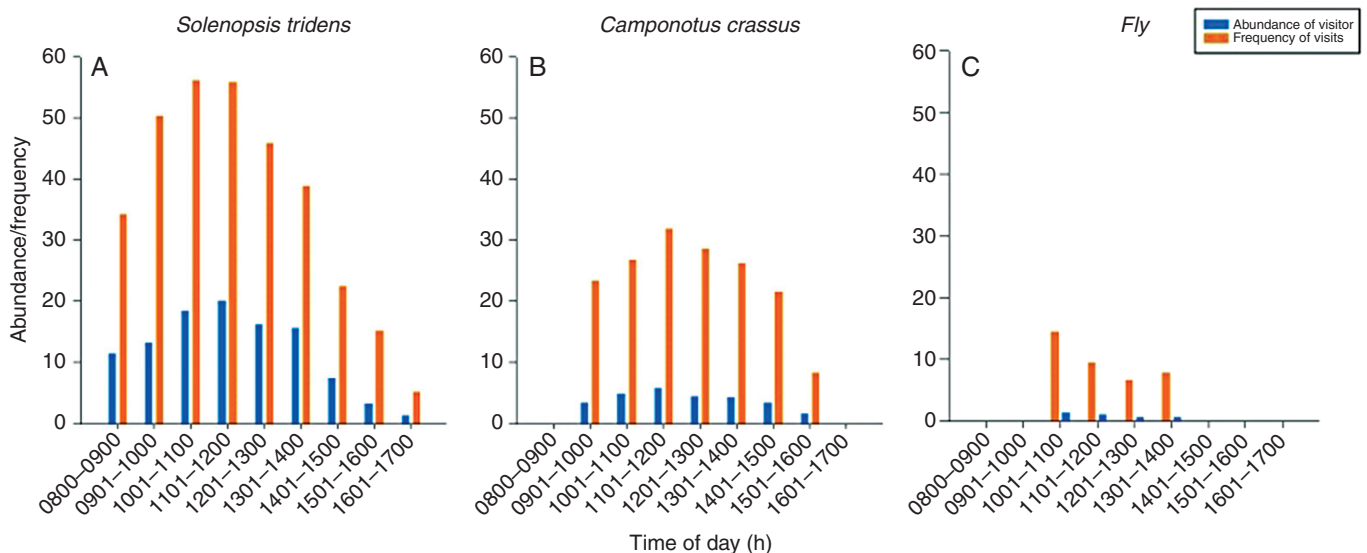

Fɪɢ. 4. Average abundance of visitors and average frequency of visits in Reserva Biológica de Guaribas, Paraíba, Brazil, during five consecutive days (~45 h of observation per species). (A) *Solenopsis tridens* in individuals of *Paepalanthus bifidus*. (B) *Camponotus crassus* in individuals of *Paepalanthus tortilis*. (C) Fly in individuals of *Paepalanthus tortilis*.

the background, with a Euclidean distance of 0.33 and 0.44 units, respectively (Fig. 5B).

In the fly colour space, the colour locus of the capitula of *P. tortilis* was located at the green quadrant with a Euclidean distance of 0.03 units from the background (Fig. 5C).

Floral volatiles

The neutral red test did not indicate the presence of osmophores in *P. bifidus*, *P. subtilis* or *P. tortilis*. In the head-space samples, we did not detect any compound that was exclusive to the inflorescences of any of the three species.

DISCUSSION

Our study demonstrated that *P. bifidus* and *P. tortilis* have a mixed system of reproduction, involving spontaneous geiton-ogamy and pollination by insects. *Paepalanthus subtilis*, in turn, reproduced only by spontaneous geitonogamy without attracting any floral visitor. In addition, we noted floral traits that might be attractive to visiting insects and those that could favour spontaneous geitonogamy.

Floral visitors

Based on the number of observed individuals, the frequency of visits and behaviour of the insects, the ants *Solenopsis tridens*

and *Camponotus crassus* were considered effective pollinators and flies an occasional pollinator.

Ants were the most abundant floral visitors and pollinators in *P. bifidus* and *P. tortilis*, although ant pollination can be considered rare and restricted to few species of flowering plants (Domingos-Melo *et al.*, 2017; Kuriakose *et al.*, 2018). One of the hypotheses explaining why ants are rare pollinating agents is based on their secretion of antibiotic substances (antibacterial and fungicidal) produced by the metapleural glands on the body surface that might interfere with pollen viability (Beattie, 1985; Peakall *et al.*, 1991). However, this is not corroborated by some experimental studies that demonstrate the absence of inhibition of pollen viability (Garcia *et al.*, 1995; de Vega *et al.*, 2009; Ibarra-Isassi and Sendoya, 2016; Delnevo *et al.*, 2020). Furthermore, the time of exposure of the pollen grains to the secretions is very low, because the ants move quickly from one flower to another (Mayer and Gottsberger, 2002). Therefore, other aspects, such as a low ability to transport pollen in

comparison to other pollinating hairy insects (e.g. bees), as well as a low ability to promote long-distance cross-pollination (given their poor dispersal ability in comparison to winged insects), might be a better explanation for the poor representativeness of ant pollination among angiosperms.

Other cases of ant pollination have been reported in Eriocaulaceae, such as *Comanthera elegans* that has *Solenopsis* sp. as one of the most abundant pollinators (Oriani *et al.*, 2009). As observed here in *P. tortilis*, *Camponotus crassus*, an ant species that does not have metapleural glands, was considered the most effective pollinator of *P. lundii* (Del-Claro *et al.*, 2019). Like many other species of Eriocaulaceae, *P. bifidus* and *P. tortilis* have floral traits correlated with ant pollination, such as small plants, open flowers, inflorescences near ground level, grouped floral scapes and individuals, nectar as the main reward, few ovules per ovary, and occurrence in dry environments (Hickman, 1974).

Although *P. subtilis* also has floral traits related to ant pollination and some individuals occur together, ants did not visit the capitula of *P. subtilis*, and, indeed, ants were unable to climb its floral scapes. This may be due to its scapes being thinner, glabrous and less robust than those of *P. bifidus*, which might prevent or hinder movement of the ants from one inflorescence to another and access to the floral resource.

TABLE 1. *Number of pistillate flowers (*n = 35 *individuals) of three* Paepalanthus *species and percentage of fruit formed and standard error in each treatment*

Species	Treatment	Pistillate flowers	Fruits (%)
P. bifidus[1]	NP	320 ± 1.16	86.25 ± 5.16[a]
	WP	337 ± 1.49	42.72 ± 4.67[b]
	SG	324 ± 1.19	41.66 ± 4.92[b]
P. tortilis[2]	NP	392 ± 0.75	82.39 ± 2.70[a]
	WP	388 ± 0.85	34.02 ± 3.31[b]
	SG	384 ± 0.82	35.67 ± 3.85[b]
P. subtilis[3]	NP	372 ± 0.94	89.36 ± 6.13[a]
	WP	375 ± 0.98	65.03 ± 6.57[b]
	SG	371 ± 0.97	67.54 ± 5.12[b]

NP = natural pollination; WP = wind pollination; SG = spontaneous geitonogamy. Kruskal–Wallis (1. $H^2 = 70.66$; 2. $H^2 = 72.57$; 3. $H^2 = 72.57$; $P < 0.0001$. Pairwise comparisons using Dunn's test $P < 0.05$). Different letters represent differences by Dunn's test ($P < 0.05$).

Pollination systems

Ant and fly pollination were the main pollination systems of *P. bifidus* and *P. tortilis*, increasing the possibility of outcrossing in both species, even though they are self-compatible and geitonogamous. According to Goodwillie *et al.* (2005), about 45 % of animal-pollinated species exhibit a mixed mating system (selfing and outcrossing). In other species of Eriocaulaceae, such as *Eriocaulon heleocharioides*, a mixed mating system also seems to occur, as seeds were produced by outcrossing (flies were the main pollinators) and self-pollination (Tanaka *et al.*, 2015; Horiuchi *et al.*, 2020).
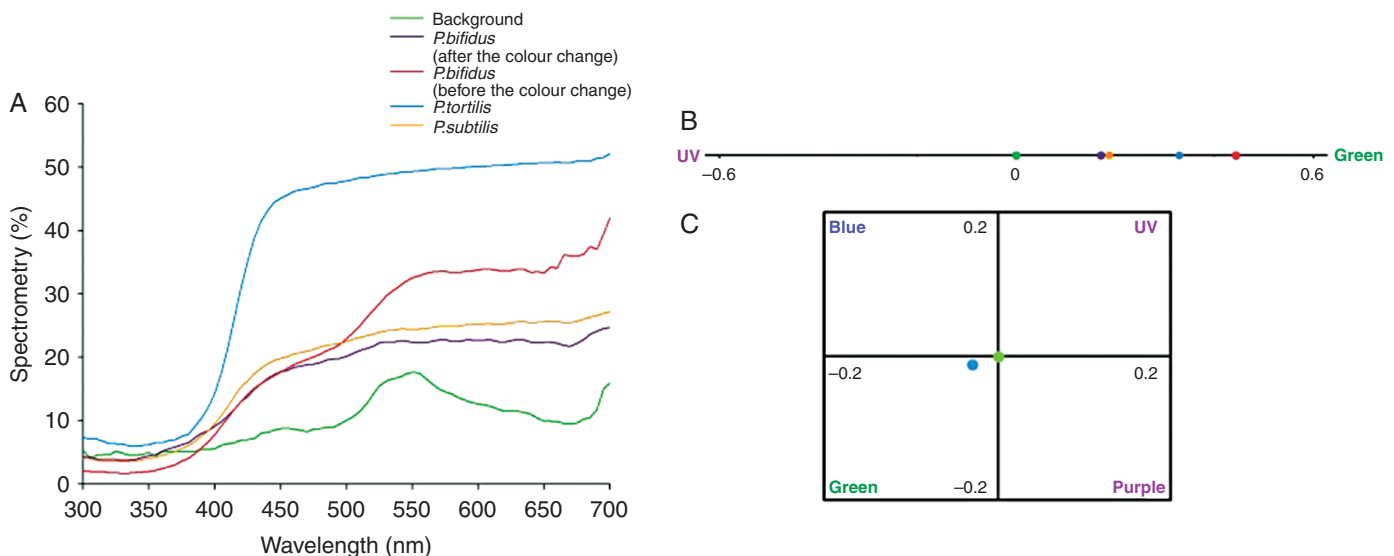

FIG. 5. (A) Colour spectrum reflected by capitula, leaves and scapes (background). (B) Dichromatic colour space model for vision in ants. (C) Fly colour space of Troje (1993).

Although wind has been suggested as a possible pollen vector in Eriocaulaceae (Cronquist, 1981), this pollination system did not play a role in the reproduction of the three species investigated here. Similar results were found by Ramos *et al.* (2005) in *Comanthera curralensis* and *C. mucugensis*, and by Oriani *et al.* (2009) in *C. elegans.*

Pollination ecology is usually assumed to be a major factor in the evolution of selfing and the maintenance of mixed mating systems (Lloyd, 1992; Barrett and Harder, 1996; Kalisz and Vogler, 2003; Goodwillie *et al.*, 2005). The hypothesis of reproductive assurance suggests that selfing evolves because it guarantees a reasonable seed set when pollinators are scarce or absent (Lloyd, 1992; Kalisz *et al.*, 2004; Busch and Delph, 2012). Environments in which pollination is unpredictable and varies over space and time are common in plant populations (Burd, 1994; Utelli and Roy, 2000; Kalisz and Vogler, 2003; Price *et al.*, 2005) and can favour self-pollination and contribute to mixed mating as a stable state (Kalisz and Vogler, 2003; Kalisz *et al.*, 2004; Goodwillie *et al.*, 2005). As observed in the present study, over about 1 month, pollination varies from periods of high pollinator abundance to periods of total absence of pollinators. High variation in ant pollination during the flowering period was also noted by Oriani *et al.* (2009) in *C. elegans*. It may be associated with variations in pollinator activity, as pollinators are abundant at the peak of the flowering season but scarce at the end of the flowering season (Price *et al.*, 2005).

The foraging strategy of the floral visitors is also related to the evolution of selfing rates (Devaux *et al.*, 2014). Populations of *P. bifidus* and *P. tortilis* have individuals with short scapes, close to the ground, and individuals with long scapes, farther from the soil surface. All floral visitors observed preferred to forage on capitula on long floral scapes and avoided capitula on shorter scapes. This may be due to optimal foraging behaviour (Pyke, 1984) of flower visitors that, upon reaching the taller scapes, need to move only a short distance to forage on an adjacent capitulum. Some studies have shown that plants with long scapes have more pollinator visits, probably because they are more visible than plants with short scapes (Vanhoenacker *et al.*, 2006; Anderson, 2010). Therefore, because individuals with short floral scapes are rarely visited, the outer cycle of pistillate flowers in anthesis usually only receive pollen from the same capitulum.

The species investigated in this study also have a set of floral traits associated with spontaneous geitonogamy, such as reduced flower size and floral display (Goodwillie *et al.*, 2010), short distance between staminate and pistillate flowers (Webb and Lloyd, 1986; Herlihy and Eckert, 2007), and temporal overlap of staminate and pistillate flowers within the capitulum (Bertin and Newman, 1993; Totland and Schulte-Herbrüggen, 2003). Although self-pollination is often reported in hermaphroditic flowers (Goodwillie *et al.*, 2005), in the unisexual flowers of *P. bifidus*, *P. subtilis*, and *P. tortilis*, the transfer of pollen grains between staminate and pistillate flowers of adjacent cycles in the same capitulum is facilitated by the overlap of staminate and pistillate flowering times, and the proximity of anthers and stigmas (see figure 7 in Stützel, 1981). According to Stützel (1981), the long trichomes on the petals of the pistillate flowers also play a role in spontaneous geitonogamy. This author demonstrated that pollen grains released by anthers are retained in these trichomes (see figure 10 in Stützel, 1981) and eventually, due to the proximity of the reproductive structures of different flowers, these retained pollen grains can be transferred to a stigma. In *P. subtilis*, due to the flowers being almost completely surrounded by involucral bracts at the beginning of anthesis, anthers and stigmas were even closer, favouring the transfer of pollen grains from one flower to the stigma of another of the same capitulum. Furthermore, this species showed an increased fruit set under natural conditions. From field observations, we verified that after the occurrence of rain at dawn, the capitulum of *P. subtilis* was surrounded by raindrops and remained so during the morning until their evaporation (Supplementary Data Fig. S2). Even surrounded by raindrops the staminate and pistillate flowers have their anthesis during the same period as when they are not wet. *Eriocaulon parkeri*, an aquatic plant with high percentage of seed set (~89 %) by spontaneous geitonogamy, preserves its pollen grains as viable and able to germinate even when exposed to water (Sawyer *et al.*, 2005). Therefore, we believe that the pollen grains (retained in the long trichomes on the petals and of the dehiscence of the anthers) can be suspended in a raindrop and transported to stigmas of pistillate flowers, resulting in geitonogamy, and that the role of rain as a pollen vector of species of Eriocaulaceae should be investigated in future studies.

Spontaneous geitonogamy recorded here for *P. tortilis* (=*P. tatei*) was previously reported by Stützel (1981) as occurring in a glasshouse, thus demonstrating the persistence of the capacity for spontaneous geitonogamy regardless of the environment (natural/wild or controlled). In other species, such as *Eriocaulon parkeri* (Sawyer *et al.*, 2005) and *E. heleocharioides* (Tanaka *et al.*, 2015; Horiuchi *et al.*, 2020), spontaneous geitonogamy has also been reported. However, this pollination system may not occur or may contribute less to fruit set in species of Eriocaulaceae that have pistillate flowers arranged at the periphery and staminate flowers in the centre (or vice versa) (Stützel, 1981, 1984), or that have a strong temporal separation of the pistillate and staminate phases (Hensold, 1988; Ramos *et al.*, 2005). In protandrous species, such as *Comanthera elegans*, spontaneous geitonogamy can occur during the period of change from the staminate to pistillate cycle (Oriani *et al.*, 2009). Therefore, the arrangements of staminate and pistillate flowers in alternating cycles, and the concomitant anthesis of both types of flowers in the same capitulum might be a selective response to promote selfing.

Floral signalling

Colour is one of the most important floral signals in the communication between plants and pollinators (Shrestha *et al.*, 2013; Lunau *et al.*, 2017). In ants, although current knowledge about their colour vision system is still in its infancy, studies on behaviour and electrophysiology have demonstrated that these insects have cognitive ability to learn and discriminate colours (Camlitepe and Aksoy, 2010; Yilmaz *et al.*, 2017; Aksoy and Camlitepe, 2018). From analysis of dichromatic colour space, we found that ants have better capacity to distinguish the capitulum of *P. tortilis* and the capitulum of *P. bifidus* after the colour change. The capitulum of *P. bifidus* before the colour change and the capitulum of *P. subtilis* are more inconspicuous for these insects against the background. Since the capitula of *P. subtilis* appear to be inconspicuous to ants and these insects seem to be unable to climb the floral scapes of this plant species, individuals of *Solenopsis tridens* do not visit the capitula of *P. subtilis*.

Changes in flower colour, as observed in the anthers of *P. bifidus*, is a common phenomenon in angiosperms (Weiss and Lamont, 1997). The anthers of *P. bifidus* have a notable colour change from white at the beginning of anthesis to yellow-orange at the end. The filaments lose turgor and the anthers regress toward the corolla tube, making the nectariferous structures inaccessible. In other species of Eriocaulaceae, this phenomenon has also been observed (Ramos *et al.*, 2005; Oriani *et al.*, 2009), with colour change in the nectariferous structures from whitish to yellowish in both staminate and pistillate flowers, establishing a clear contrast with the other whitish parts of the flowers. Studies have shown that the presence of non-functional old flowers that change colour at the end of anthesis but no longer have any reward contribute to visual signalling over long distances for pollinators (Gori, 1989; Weiss, 1991; Oberrath and Böhning-Gaese, 1999; Niesenbaum, 1999; Kudo *et al.*, 2007). Once the pollinators reach the inflorescences, they might visit predominately new functional (non-pollinated) flowers, as insects are known to learn to associate colour with reward availability (Weiss and Lamont, 1997; Oberrath and Böhning-Gaese, 1999; Schaefer *et al.*, 2004; Brito *et al.*, 2015; Makino and Ohashi, 2017).

Flies are the most common floral visitors and pollinators reported in Eriocaulaceae (Ramos *et al.*, 2005; Sawyer *et al.*, 2005; Oriani *et al.*, 2009; Tanaka *et al.*, 2015; Del-Claro *et al.*, 2019). The fly colour space model (Troje, 1993) indicates that flies are able to discriminate the capitula of *P. tortilis* from the background. Many flies have been frequently observed visiting white flowers such as those of Eriocaulaceae (Lunau, 2014; Woodcock *et al.*, 2014; Domingos-Melo *et al.*, 2017). Thus, colour preferences for white flowers (Lunau, 2014; Woodcock *et al.*, 2014; Klecka *et al.*, 2018) might be related to the visual attraction and visits of flies in representatives of Eriocaulaceae.

Like floral colours, floral volatiles are normally relevant components of floral signalling (Wright and Schiestl, 2009). Ants generally use chemical signals as a source of information (Heil *et al.*, 2010; Nelson *et al.*, 2019). The studies by Schiestl and Glaser (2012) and De Vega *et al.* (2014) demonstrated that ants use floral volatiles as cues to the location of the floral resource. In our study, however, the absence of volatiles exclusive to inflorescences in the two studied species that are visited by ants, i.e. *P. bifidus* and *P. tortilis*, suggests either that visual signals are sufficient for the perception of flowers by these insects or that ants can rely on the synergy of visual and olfactory cues, detecting volatile chemical compounds which were not discovered in our experiments using dynamic headspace techniques. During food searching, ant antennae can detect trail pheromones to reach a food source (Knaden and Graham, 2016) and ants are also able to detect and associate less volatile chemical compounds, which are not properly sampled by headspace techniques (Milet-Pinheiro *et al.*, 2021), with the food rewards (Gronenberg and Hölldobler, 1999; Wanjiku *et al.*, 2014).

For monocarpic plants, which reproduce only once in their lifetime, to have strategies that ensure successful reproduction is crucial for population survival. The pollination services provided by ants play a key role in fruit production and reinforce previous findings showing that entomophily is an important pollination system in Eriocaulaceae. In addition, ant pollination may be more frequent in Eriocaulaceae than is currently assumed. On the other hand, in situations of pollinator absence, as observed in *P. subtilis*, *P. tortilis* and *P. bifidus* at the end of the flowering season, it is fundamental for monocarpic species to provide reproductive insurance. This, together with both flower arrangement and anthesis dynamics in the capitulum, allows spontaneous geitonogamy to occur and might ensure a constant high reproductive success. Moreover, in this study we have provided the first analysis of floral signals (colour and scent) for the family. The diversity of floral traits in Eriocaulaceae provides an excellent model for studies of the evolution of pollination biology, and future studies might provide novel advances in our understanding of floral traits that mediate plant–pollinator interactions.

SUPPLEMENTARY DATA

Supplementary data are available online at https://academic. oup.com/aob and consist of the following. Figure S1: Habit of the three studied species. Figure S2: Capitula of *P. subtilis* surrounded by raindrops. Video: *Solenopsis tridens* during nectar-collecting in individuals of *P. bifidus*.

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CONFLICT OF INTEREST

We declare that there is no conflict of interest.

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