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Morphology, secretion composition, and ecological aspects of stipular colleters in Rubiaceae species from tropical forest and savanna

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Abstract Colleters are secretory structures that produce and release mucilage or a mucilage-resin mixture protecting meristems and young structures against desiccation, herbivores, and pathogens. The secretions may vary in colleters of same or different types, indicating that the functionality of colleters may be more specific than previously thought. In this study, we compared 17 Rubiaceae species from savanna and forest environment focusing on colleter secretions and its ecological role. First, we evaluated the morphology, distribution, and histochemistry of stipular colleters using light and scanning electron microscopy. Additionally, we investigated the phenology, microclimate, and the proportion of damaged apices in the savanna and forest species. We recorded standard-type colleters, variable in distribution and size, in 14 of the 17 studied species. The secretion varied from predominantly hydrophilic, mixed to predominantly lipophilic. During the budding period, secretion covered the vegetative apices. Savanna species had a prevalence of lipid secretion in habitats with higher luminosity, which had a lower proportion of damaged apices. In contrast, forest species occurred in habitats with lower luminosity and had a higher proportion of damaged apices, in general with the absence of lipids in the colleters. These results highlight that colleters with similar morphology clearly differed in secretions among species, especially between species from savanna and forest, in which the colleters appear potentially associated with protection against irradiation in savanna, but not in the forest environment.

Keywords Apical meristems · Colleters · Glands · Histochemistry · Microclimate · Phenology · Secretion · Shoot apex

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Introduction

Rubiaceae is the fourth largest family of angiosperms and shows a cosmopolitan distribution, with approximately half of its species occurring in the neotropics (Delprete and Jardim 2012). This family is especially diverse and well represented in the Brazilian savanna and tropical forests (Souza and Lorenzi 2005). The presence of interpetiolar stipules with colleters is one of the most important vegetative characteristics of the family Rubiaceae, which distinguishes it from most other families (Robbrecht 1988).

Colleters are secretory structures characterized by the production of mucilage or a mucilage-resin mixture (Fahn 1979) and are present on the adaxial surface of vegetative and reproductive organs, protecting meristems and young structures against desiccation (Kronestedt-Robards and Robards 1991), herbivores, and pathogens (Thomas 1991). Anatomically, colleters are described as emergences comprising a central, multiseriate, vascularized or not, parenchymatous axis covered by palisade-arranged epidermal cells that are specialized in secretion (Fahn 1979). This structural organization characterizes the most common colleter type, designated the standard type (Evert 2006). Based on morphology, the following other

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colleter types are recognized in Rubiaceae: reduced standard, dendroid, intermediate, brush-like, winged, filiform (Lersten 1974; Thomas 1991), and lachrymiform (Miguel et al. 2009). The presence of crystals is common in colleters and represents a taxonomically relevant characteristic (Evert 2006).

The shoot apex is essential for plant building and its damage is considered of high cost for plants (Coley and Kursar 1996). Therefore, the apices could be associated with specific morphological and chemical features that would increase their protection or resistance against harmful agents such as herbivores, desiccation, and solar radiation. In this case, the presence of colleters would be a potential character of protection to the apex of the plants. Colleters differentiate early in the shoot apices and are active in secretion during leaf development (Thomas 1991); however, there is no clear evidence of a relationship between their presence and accurate protection to apex in the budding stage of plants exposed to different environmental conditions. In addition, there are no data on the proportion of damage to the apex and the secretion characteristics or the environment in which the plants occur. These knowledge gaps reinforce the need for integrative studies that associate the morphological type of colleters and their variations, as well as the nature of the secretion with the environmental type where the species occur. At present, research applying this approach is scarce, and the studies conducted by Klein et al. (2004) and Vitarelli and Santos (2009), who investigated the variation in secretions among species of the same genus and between environments, respectively, are noteworthy.

In this study, we characterized the morphotypes and histochemistry of stipular colleters found in 17 Rubiaceae species distributed in two vegetation types with contrasting characteristics, savanna (continuous herbaceous and sparse woody typically named cerrado sensu stricto) and seasonal semi-deciduous forest. In savanna, Rubiaceae species occur in opened physiognomies (associated with dystrophic soils) and are exposed to the intense incidence of light that generates a drier and hotter microclimate with higher radiation (Coutinho 1978, 2002) when compared with the understory of the adjacent seasonal semi-deciduous forest (commonly associated with mesotrophic and eutrophic soils) (Scariot and Sevilha 2005), which is the stratum that harbors the Rubiaceae species. Thus, we assessed whether colleters of the same morphotype differ regarding the chemical nature of the secretions when comparing species from forest and savanna. Finally, we tested if there is an association among the proportion of damaged apices, chemical compounds of the colleters' secretion, and type of vegetation and microclimate around each individual of Rubiaceae sampled in the field.

Materials and methods

Species selection and study area

For the present study, based on field expeditions and herbarium collections, we selected 17 Rubiaceae species varying from herbs, shrubs, and trees (Table 1): nine species growing

Table 1 List of the studied subfamilies and species of Rubiaceae and their respective habits, vegetation types (forest and savanna), and record numbers in the BOTU herbarium in central-western São Paulo state, Brazil

Subfamily	Species	Habit	Vegetation type	BOTU record
Cinchonoideae	*Coccocypselum lanceolatum* (Ruiz & Pav.) Pers.	Herb	Savanna	28,502
	Guettarda viburnoides Cham. & Schltdl.	Tree	Savanna	28,482
	Warzewiczia sp.	Shr	Forest	28,513
	Cordiera concolor (Cham.) Kuntze	Shr	Forest	28,483
Ixoroideae	*Cordiera sessilis* (Vell.) Kuntze	Shr	Savanna	28,505
	Ixora gardneriana Benth.	Tree	Forest	28,488
	Ixora heterodoxa Müll. Arg.	Tree	Forest	28,492
	Tocoyena formosa (Cham. & Schltdl.) K. Schum.	Tree	Savanna	28,506
	Coussarea hydrangeifolia (Benth.) Benth. & Hook.f. ex Müll.Arg.	Tree	Savanna	28,499
Rubioideae	*Palicourea rigida* Kunth.	Shr	Savanna	28,484
	Palicourea sp.	Shr	Savanna	28,514
	Psychotria hoffmannseggiana (Willd. ex Schult.) Müll. Arg.	Shr	Forest	28,477
	Palicourea marcgravii (A.St.-Hil.) Spreng.	Shr	Forest	28,501
	Psychotria sp.	Shr	Savanna	28,512
	Randia sp.	Shr	Forest	28,511
	Richardia grandiflora (Cham. & Schltdl.) Steud.	Herb	Forest	28,476
	Simira corumbensis (Standl.) Steyerm.	Tree	Forest	28,490

herb herbs, *shr* shrubs, *tree* trees

in the understory of a seasonal semi-deciduous tropical forest and eight species growing in cerrado sensu stricto physiognomies, characterized by a continuous herbaceous and a sparse woody strata (savanna formations), all located in central-western São Paulo state, Brazil (48° 20′ to 48° 50′ W; 22° 40′ to 23° 05′ S). The south marginal areas of the cerrado domain and the seasonal semi-deciduous forest are both characterized by marked climatic seasonality. The selected species are unique to each environment as shown in Table 1.

The climate was characterized as Cfa hot climate with rains in the summer and drought in the winter, with average temperature in the hottest month above 22 °C, with small hydric deficiency in April, July, and August (Cunha and Martins 2009). Data of monthly average temperature and monthly accumulated rainfall were obtained from Estação de Radiometria Solar de Botucatu, Faculdade de Ciências Agrárias, UNESP (48° 27′ W; 22° 54′ S, 786 m altitude) to prepare a climate diagram (Fig. 1) according to Walter and Lieth (1967).

For each study species, five individuals located in the same area were identified with numbered labels and monitored monthly from May 2011 to April 2012 ($n=85$ plants, distributed in five study areas).

Voucher specimens were deposited in the Herbarium BOTU of the Department of Botany, Botucatu, Universidade Estadual Paulista (UNESP), SP, Brazil (Table 1).

Colleters' distribution, morphology, and histochemistry

We collected and dissected vegetative apex samples from the studied species during the budding period to evaluate the distribution, number, size, and morphology of the stipule colleters ($n=5$ per species). We analyzed three stipules per
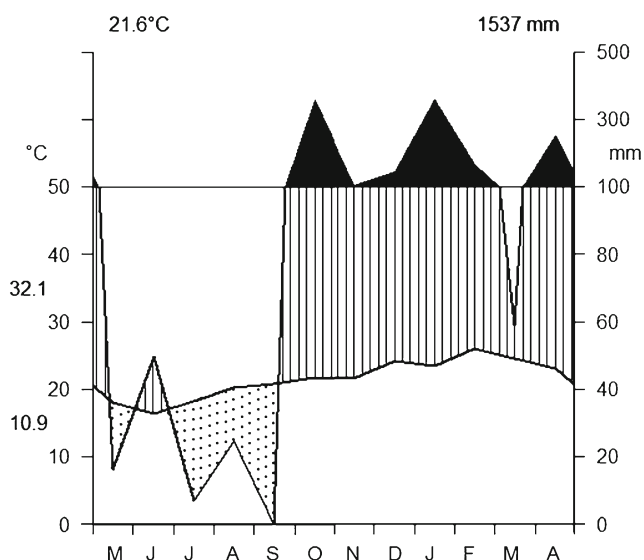
species under a stereoscopic microscope to count all the colleters on the stipule's adaxial surface and to measure their sizes using a system with image capture and software for image processing.

We processed stipule samples ($n=5$ plants) for scanning electron microscopy (SEM) analyses. For this purpose, samples of the material were fixed in 2.5 % glutaraldehyde in 0.1 M phosphate buffer, pH 7.3, for 24 h then post-fixed in 1 % osmium tetroxide in 0.1 M phosphate buffer, pH 7.3, for 1 h, dehydrated in an ascending ethanol series, critical point dried using liquid CO_2, mounted on aluminum stubs, and gold sputtered (Robards 1978). The material was observed and documented using a FEI Quanta scanning electron microscope at 20 kV.

For general histology of the colleters, stipule samples ($n=5$ plants) were fixed in Karnovsky solution (4 % paraformaldehyde; 1 % glutaraldehyde in 0.1 M phosphate buffer, pH 7.2; 0.2 M phosphate buffer, pH 7.2) for 24 h (Karnovsky 1965). We kept the samples fixed in 70 % tertiary butyl alcohol for 7 days then dehydrated them in an ascending butyl series, transferred them to Histo-Clear®, and embedded them in Paraplast® (Johansen 1940). Next, we cut 8- to 10-μm-thick sections using a semi-automatic rotary microtome and stained the sections with Astra blue and safranin.

We prepared permanent slides using Entelan® synthetic resin and then viewed and photographed them under a microscope coupled to a digital camera. We measured the length and width of the colleters of each studied species in longitudinal sections of the vegetative apex ($n=5$ per species), using the LAS measuring system, version 3.8.

We used sectioned material for in situ identification of the main chemical classes of compounds present in the colleters. For this purpose, we performed tests using the following histochemical stains: Sudan IV for total lipids (Pearse 1980), the periodic acid/Schiff reagent (PAS) for neutral polysaccharides (Cortelazzo 1992), ruthenium red for acid polysaccharides (Jensen 1962), ferric chloride for phenolic compounds (Johansen 1940), mercuric bromophenol blue solution for proteins (Mazia et al. 1953), tannic acid/iron chloride III for mucilages (Pizzolato and Lillie 1973), the Dragendorff reagent for alkaloids (Svendsen and Verpoorte 1983), and cupric acetate for resin (Johansen 1940).

Phenology

We evaluated the phenology, according to Fournier (1974), focusing on the budding phase. We investigated the presence and aspect of exudates covering the shoot apices by the naked eye.

Microclimate

Temperature, relative humidity (RH), and luminosity were measured using portable thermometer, hygrometer, and

Fig. 1 Climate diagram for Botucatu region, central-western São Paulo state, Brazil. *Dotted area* corresponds to dry period (May–September), *stripes* and *black areas* correspond to wet period (October–April)

luximeter, respectively. Data were collected next to the branches of each specimen, monthly for a year.

Proportion of damaged apices

For herbs, shrubs, and small trees (Table 1) having less than 40 branches, all the vegetative apices were evaluated in loco. For bigger trees, *Coussarea hydrangeifolia*, *Guettarda viburnoides*, and *Simira corumbensis*, ten apices in each cardinal point of the plant were evaluated, totaling 40 apices sampled per tree. All the labeled plants were investigated for the presence of apex damage using a portable magnifying glass. The evaluations were performed in the dry (August/September) and wet (January/February) periods.

We considered 'damaged apices' as those having injury, necrosis, or total loss of tissue, regardless of their causes (e.g., herbivores, desiccation, or ablation). The mean proportion of damaged apices in relation to the total number of sampled apices per species ($n=5$ plants) was calculated in both periods, dry and wet.

Data analysis

To analyze the temporal distribution of the vegetative apex budding, circular statistics was employed. For this approach, we consider the period of 1 year in a circular scale of measurements (Batschelet 1981; Zar 2010). To adapt the data to the circular scale, we converted the days to the specific angle of the sampling day. For this calculation, the total angles of a circle (360°) was divided by the total number of days of the year (365), obtaining the value of 0.98 for each day of the year. Since our data was not normal, the data variation was described based on the median and quartiles. To determine the intensity of seasonality, we use the length of the vector (r). This parameter is a measure of the data concentration around the mean angle and ranges from 0 (disaggregated data) to 1 (data concentrated in the same direction) (Zar 2010). The distribution of data was verified by Watson test based on the null hypothesis that the data follows the von Mises distribution (normal distribution analogous to the linear data). To investigate the occurrence of seasonality, we employed the Kuiper test based on the null hypothesis that the data presents uniformity, i.e., they are equally distributed around the circle (Jammalamadaka and SenGupta 2001; Zar 2010). The analyses were performed using the computing environment R packages "circular" (Agostinelli and Lund 2011) and "CircStats" (Jammalamadaka and SenGupta 2001).

To compare the differences in the proportions of species with each chemical compound (lipids, resin, phenols, and alkaloids) between the savanna and forest, a binomial proportion test was applied. In order to check for differences in microclimate factors (maximum and minimum RH, maximum and minimum temperature, and maximum and minimum

irradiation) around plants, we used analyses of variance (ANOVA, one-way), in which the response variables were each a quantitative descriptor of microclimate (maximum and minimum) and the independent variable was the vegetation type (savanna and forest). We also verified if the variation in the proportion of damaged apices could be explained by the presence of specific chemical compounds, the microclimate, or the vegetation type (forest and savanna). As there was no significant difference in the proportion of damaged apices between the dry and wet periods (paired t test; $t=1.3572$; d.f.$=16$; $p=0.19$), the mean value per species, considering both periods, was used as the overall proportion of damaged apices in the subsequent analyses. Therefore, to explain the variation in the overall proportion of damaged apices, we applied the ANOVA one-way and linear regression, depending on the nature of the independent variable considered, if discrete (e.g., vegetation type or chemical compounds) or continuous (e.g., maximum relative humidity). The assumptions of all analyses (normality and homogeneity of variances) were verified through graphical inspection of the distribution of the response variable values (histogram) and residues of each test individually. No transformation was applied to the original data before analysis. All analyses were performed in the computing environment R.

Results

Colleters' distribution, morphology, and histochemistry

We found that the examined colleters were associated with four morphological types of stipules: rounded, oval with bifurcate apices, fimbriate, and lanceolate. This information is summarized in Table 2.

Colleters varied regarding their distribution (Figs. 2 and 3a–d) and number (Table 2) on the adaxial surface of the stipules. We observed four patterns of distribution of colleters: colleters distributed across the entire surface of the stipules—*Cordiera concolor* (Fig. 2a), *Cordiera sessilis* (Fig. 2b), *Coccocypselum lanceolatum*, and *Richardia grandiflora*, with a lower density, occurring in the two latter species; colleters located within the concave region of the stipules occupying an area from the base to the apical region, in a triangular arrangement—*G. viburnoides* (Figs. 2f and 3b), *Ixora gardneriana*, *Ixora heterodoxa* (Fig. 2d), *Palicourea rigida* (Fig. 2e), *S. corumbensis*, *Tocoyena formosa*, and *Warszewiczia* sp.; colleters arranged in one or two longitudinal rows—*C. hydrangeifolia*, *Randia* sp. (Fig. 2g), *Psychotria hoffmannseggiana*, *Palicourea marcgravii*, *Psychotria* sp., and *Palicourea* sp.; and isolated colleters found in the distal region of the stipules in *Palicourea* sp. (Fig. 3d). The number of colleters per stipule and size (height and width) of colleters

Table 2 Stipule morphology, pattern of colleters' distribution in stipules, quantitative data for the colleters, colleter types, crystal types in colleters in Rubiaceae species from forest and savanna in central-western São Paulo state, Brazil

| Vegetation	Species	Stipule morphology	Colleters' distribution on the stipule	Colleters per stipule (mean±SE)	Colleter size (μm)		Colleter type	Crystals
					Height	Width		
Forest	*Cordiera concolor*	Rounded	Entire surface	95.8±2.43	60	30	Dendroid	n.o.
	Ixora gardneriana	Rounded	Triangular arrangement	8.8±1.08	1805	230	Standard	ra
	Ixora heterodoxa	Rounded	Triangular arrangement	33.4±1.73	440	110	Standard	ra
	Palicourea marcgravii	Oval with bifurcated apices	One or two longitudinal rows	33.4±2.01	590	115	Standard	ra
	Psychotria hoffmannseggiana	Oval with bifurcated apices	One or two longitudinal rows	37.2±1.99	480	160	Standard	ra
	Randia sp.	Rounded	One or two longitudinal rows	9.2±0.87	160	60	Standard	n.o.
	Richardia grandiflora	Fimbriate	Entire surface	4.6±0.7	190	50	Standard	n.o.
	Simira corumbensis	Rounded	Triangular arrangement	214.4±5.03	415	85	Standard	n.o.
	Warzewiczia sp.	Lanceolate	Triangular arrangement	35.8±2.30	550	30	Standard	n.o.
Savanna	*Cordiera sessilis*	Rounded	Entire surface	74.6±2.09	450	150	Dendroid	n.o.
	Coccocypselum lanceolatum	Rounded	Entire surface	4.8±0.63	340	60	Standard	n.o.
	Coussarea hydrangeifolia	Lanceolate	One or two longitudinal rows	27±1.29	1360	215	Lachrymiform	ra
	Guettarda viburnoides	Rounded	Triangular arrangement	24.6±1.55	588	295	Standard	n.o.
	Palicourea sp.	Lanceolate	One or two longitudinal rows and single in distal region	12.6±1.47	543	80	Standard	ra
	Palicourea rigida	Lanceolate	Triangular arrangement	337±2.90	1600	170	Standard	ra/pr
	Psychotria sp.	Oval with bifurcated apices	One or two longitudinal rows	35.6±2.38	535	45	Standard	ra
	Tocoyena formosa	Rounded	Triangular arrangement	46.6±1.77	450	165	Standard	n.o.

n.o. not observed, *ra* raphides, *pr* prismatic
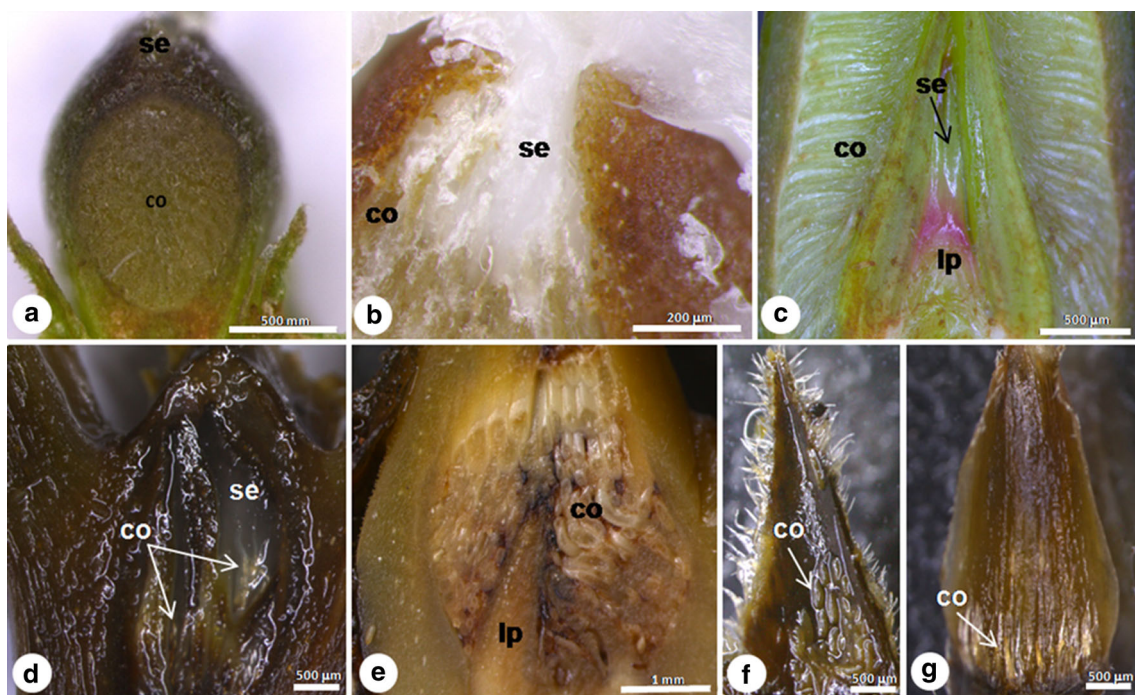
Fig. 2 Exudates and distribution of colleters on the adaxial surface of the stipules in vegetative apices of Rubiaceae species. **a–e** Longitudinal sections of apices. **f–g** Adaxial surface of stipules. **a–b** Resinous secretions (*se*) covering the vegetative apices in *Cordiera concolor* (**a**) and *Cordiera sessilis* (**b**). **c** Hydrophilic secretion (*se*) covering the apex and leaf primordium (*lp*) in *Simira corumbensis*. **d** Resinous secretions (*se*) covering the vegetative apex in *Ixora heterodoxa*. **e** Numerous colleters (*co*) projecting themselves onto other colleters and leaf primordium (*lp*) in *Palicourea rigida*. **f** Colleters (*co*) triangularly arranged in the concave portion of the stipules in *Guettarda viburnoides*. **g** Colleters (*co*) arranged in longitudinal rows in *Randia* sp. *co* colleters, *se* secretion, *lp* leaf primordium

ranged widely among the studied species, independently of the vegetation type (Table 2).

In most Rubiaceae species, colleters were accompanied by non-glandular trichomes (Fig. 3a–c, E). Non-glandular trichomes were scarce in *C. concolor* (Fig. 3a), *C. sessilis*, *I. gardneriana*, *I. heterodoxa*, *P. hoffmannseggiana*, *Psychotria* sp., *Randia* sp. and abundant in *C. lanceolatum*, *G. viburnoides* (Fig. 3b), *R. grandiflora*, *T. formosa* (Fig. 3e), and *Warszewiczia* sp. (Fig. 3c). Non-glandular trichomes were lacking in *Palicourea* species, *C. hydrangeifolia*, and *S. corumbensis*.

The colleter type and the presence and type of crystals were indicated for each examined species (Table 2). Standard colleters occurred in 14 of the 17 studied species (Table 2). Standard colleters exhibited a main body (Fig. 3e) and a constriction at the base (Fig. 3e, f), forming a peduncle. The main body has a smooth surface, which can have grooves on its distal portion (Fig. 3g). Dendroid colleters were recorded in *C. concolor* and *C. sessilis*, and lachrymiform colleters were exclusively found in *C. hydrangeifolia* (Table 2). Calcium oxalate crystals in colleters were observed in 47 % of the studied species (Table 2). Crystals were predominantly of raphide type, occurring in clusters (Fig. 3h, i), except in *P. rigida* that contains both prismatic crystals and raphides (Table 2).

Standard colleters were quite similar in histological organization. The main body has a sharp distinction between the secretory epithelium comprising palisade-arranged epidermal cells, and a central axis formed by axially elongated parenchyma cells (Fig. 4a–d). Standard colleters had considerable variation in height, width, and number of both epithelial cells and central parenchyma cells (Fig. 4a, b, d). The peduncle can have a variable size (Fig. 4a, b, d). Vascularization of the central axis was observed in colleters of *P. rigida* (Fig. 4e).

Lachrymiform colleters had a similar histological organization to standard colleters, being characterized by a pointed apex and a peduncle with a wide base (Fig. 4f).

Dendroid colleters exhibited a central axis comprising axially arranged parenchyma cells surrounded by a secretory epithelium formed by conical, elongated epidermal cells with irregular sizes, only attached at the base (Fig. 4g).

Colleters active in secretion were observed in the vegetative apex during the budding phase in species from both the forest and savanna. These colleters were turgid with a green-yellowish color and exhibited abundant secretions that varied from hyaline to milky, covering the entire vegetative apex (Fig. 2). In the first case, the colleters were initially green and smaller. Colleters inactive in secretion become brown and loose

Fig. 3 Distribution and morphology of colleters in Rubiaceae species under scanning electron microscope. **a** Dendroid colleters (*co*) between massive non-glandular trichomes (*nt*) in *Cordiera concolor*. **b–c** Standard colleters (*co*) between non-glandular trichomes (*nt*) in *Guettarda viburnoides* (**b**) and *Warszewiczia sp.* (**c**). **d** Single standard colleter (*co*) in the distal region of the stipule's adaxial surface in *Palicourea* sp. **e–g** Standard colleters in *Tocoyena formosa*. **e** General aspect. **f** Proximal region of the colleter showing peduncle (*arrow*). **G** Grooves in surface (*arrowhead*) on the distal region of the colleter. **h–i** Standard colleters in *Palicourea marcgravii*. **h** Idioblasts containing raphides in the parenchyma axis. **i** Detail of the cluster of raphides (*ra*). *co* colleter, *nt* non-glandular trichome, *ra* raphides
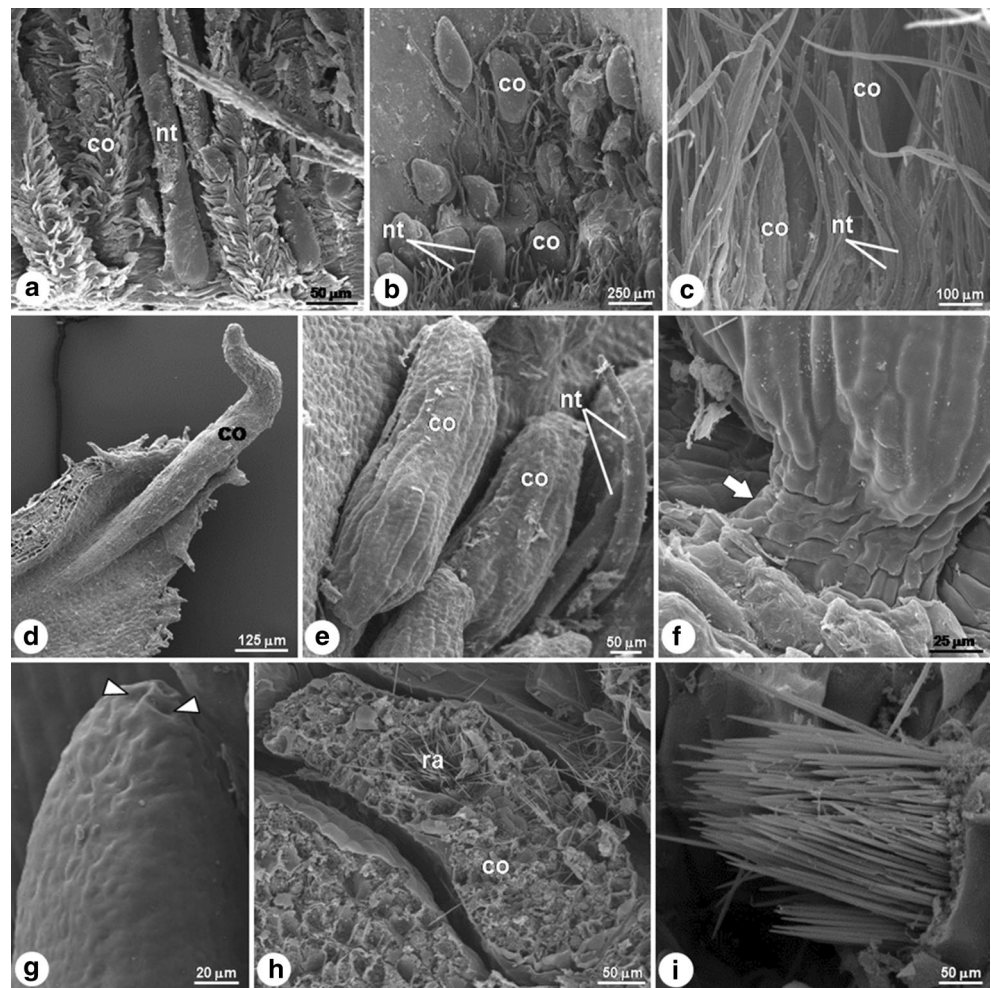
in aspect. In both cases, the changes in the colleters follow the senescence process of the stipules, which may be deciduous or may persist until the development of new apices.

Neutral polysaccharides, proteins, and pectic substances were histochemically detected in the epithelial cells of colleters in all of the studied species (Table 3). Whereas, lipids, alkaloids, phenolic compounds, and resins were detected in the epithelial cells of some species, with a prevalence of lipids in the species from the savanna (Table 3).

Temporal variation in budding and in the presence of exudates in the vegetative apices

The studied species showed continuous budding. For the forest species, the distribution of this event was more homogeneous with a peak around 20 % of budding in August (dry season) (Fig. 5a) and two peaks of greater intensity in the savanna, with approximately 40 % of budding concentrated in the months of October and February (rainy season) (Fig. 5b).

Analyzing all species, regardless of the type of vegetation, 64 % had a budding peak at the end of the rainy season (February, March, and April) and at the beginning of the dry season (May) (Table 4). During the dry period, budding peaks were recorded in four species of forest *C. concolor*, *I. heterodoxa*, *R. grandiflora*, and *Warzewiczia* spp. and two species of savanna *C. lanceolatum* and *T. formosa* (Table 4).

In all Rubiaceae species, secretion covering the vegetative apices was recorded during the budding period. The exudate ranged from translucent to off-white if composed by mucilage or resin, respectively. At the initial stages of leaf development, an exudate accumulation was detected inside the chamber formed by the stipules involving the vegetative apex. With the separation of the stipules, the leaf primordia and young leaves were exposed and the exudate covering all the new leaf created a translucent film on both surfaces. After the complete leaf expansion, the exudate remained as isolated patches throughout the blade.

Fig. 4 Colleter types in Rubiaceae species. **a–e** Standard colleters. **a** *Tocoyena formosa*, observe the central axis (*ca*) surrounded by epidermal cells (*ep*), peduncle (*arrow*), and colleters' exudates (*asterisk*). **b–c** *Ixora heterodoxa*. **d** *Guettarda viburnoides*. **e** *Palicourea rigida*, note vascular elements (*arrowhead*) in central axis (*ca*). **f** Lachrymiform colleter in *Coussarea hydrangeifolia*, note the pointed apex and a peduncle with a wide base. **g** Dendroid colleters in *Cordiera concolor*, observe that the epidermal cells (*ep*) showed irregular sizes and they were attached only at the base. **a**, **b**, **d–g** Longitudinal sections. **c** Transverse section. *ca* central axis, *ep* epidermal cells
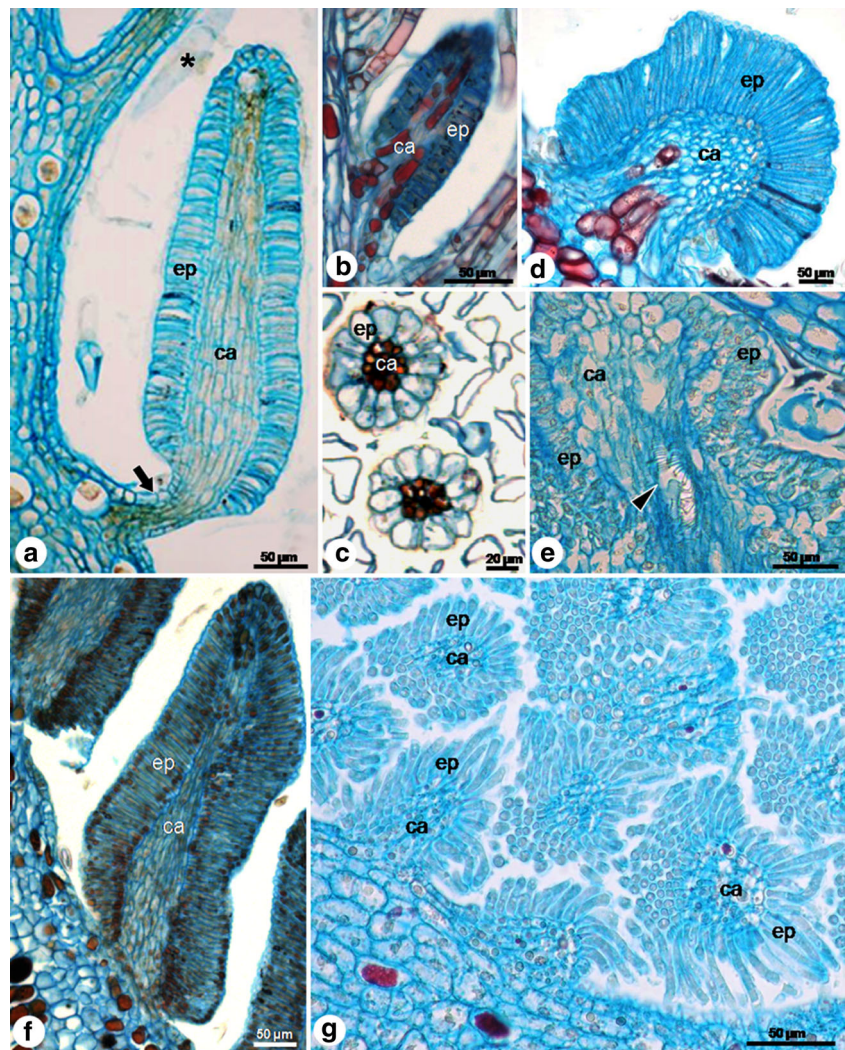
Microclimate around the studied plants in forest and savanna

In general, the relative humidity maximum RH ($F_{(1,15)}=0.03$; $p=0.86$) and minimum RH ($F_{(1,15)}=0.2$; $p=0.68$) around the studied plants did not differ between the two vegetation types (Fig. 6a). The maximum temperature did not differ between vegetation types ($F_{(1,15)}=0.3$; $p=0.59$). The minimum temperature ($F_{(1,15)}=7.5$; $p=0.01$) and the temperature amplitude ($F_{(1,15)}=6.5$; $p=0.02$) differed significantly between the two vegetation types (Fig. 6b). The temperature amplitude was 6–7 °C higher in the savanna than in the forest environment (Fig. 6b). The maximum luminosity was the variable that most differed between the two vegetation types ($F_{(1,15)}=41.4$; $p<0.001$), and the savanna showed about 600 % more light than the forest (Fig. 6c). The minimum luminosity (values close to zero in both vegetation types) was on average higher in the savanna ($F_{(1,15)}=4.14$; $p=0.06$).

Proportion of species with lipids, resins, phenolic compounds, and alkaloids in colleters from forest and savanna

The proportion of species with colleters containing lipids, resins, alkaloids, and phenolic compounds was variable between forest and savanna species (Fig. 7). Lipids were present almost exclusively in savanna species, differing significantly from the forest ones ($\chi2=7.1$; $df=1$, $p<0.01$), while the proportion of species with other compounds did not differ significantly between the two vegetation types (resin: $\chi2=0.5$; $df=1$; $p=0.47$; phenols: $\chi2=0.8$; $df=1$; $p=0.36$; alkaloids: $\chi2=0$, $df=1$, $p=1$).

Table 3   Main classes of compounds present in the colleters' secretory epithelium in 17 Rubiaceae species from forest and savanna in central-western São Paulo state, Brazil

Vegetation	Species	Lipids	Alkaloids	Phenolic substances	Resin	Polysaccharides	Proteins	Pectic substances
Forest	*Cordiera concolor*	+	−	+	+	+	+	+
	Ixora gardneriana	−	−	−	+	+	+	+
	Ixora heterodoxa	−	−	−	+	+	+	+
	Psychotria hoffmannseggiana	−	+	+	−	+	+	+
	Palicourea marcgravii	−	+	+	−	+	+	+
	Randia sp.	−	+	+	−	+	+	+
	Richardia grandiflora	−	+	−	−	+	+	+
	Simira corumbensis	−	+	+	−	+	+	+
	Warzewiczia sp.	−	−	−	−	+	+	+
Savanna	*Cordiera sessilis*	+	−	+	+	+	+	+
	Coccocypselum lanceolatum	+	−	+	−	+	+	+
	Coussarea hydrangeifolia	+	+	+	+	+	+	+
	Guettarda viburnoides	+	−	−	+	+	+	+
	Palicourea sp.	+	+	+	+	+	+	+
	Palicourea rigida	+	−	+	+	+	+	+
	Psychotria sp.	−	+	+	−	+	+	+
	Tocoyena formosa	+	+	+	−	+	+	+

+ denotes presence; − denotes absence

Relationship among the proportion of damaged apices and the vegetation type, microclimate variables around plants, and colleter secretion

The proportion of damaged apices ranged from 0 to approximately 50 % among the Rubiaceae species. The proportion of damaged apices showed a positive correlation with maximum RH (Fig. 8a; $F_{(1,15)}=10.9$, $p=0.005$; $R^2=0.38$), regardless of the vegetation type. The other microclimate variables measured around plants did not explain the variation of damaged apices, including the minimum RH ($F_{(1,15)}=0.03$, $p=0.86$;

$R^2=0.06$), maximum ($F_{(1,15)}=2.52$, $p=0.13$; $R^2=0.09$) and minimum ($F_{(1,15)}=0.36$, $p=0.56$; $R^2=0.04$) temperatures, and maximum ($F_{(1,15)}=0.13$, $p=0.72$; $R^2=0.06$) and minimum ($F_{(1,15)}=0.40$, $p=0.53$; $R^2=0.04$) luminosity. This analysis was repeated excluding the outlier detected in the upper right corner of the graph, and the result remained the same (continuous line with the complete dataset and dashed line without the outlier—Fig. 8a). On average, forest species had a higher proportion of damaged apices as compared to the savanna species (Fig. 8b; $f_{(1,14)}=4.7$, $p=0.047$). Additionally, species that have lipids in colleters had, on average, a lower

Fig. 5   Circular histogram of the monthly relative frequency of budding Rubiaceae species in **a** forest and **b** savanna vegetation located in central-western São Paulo state, Brazil
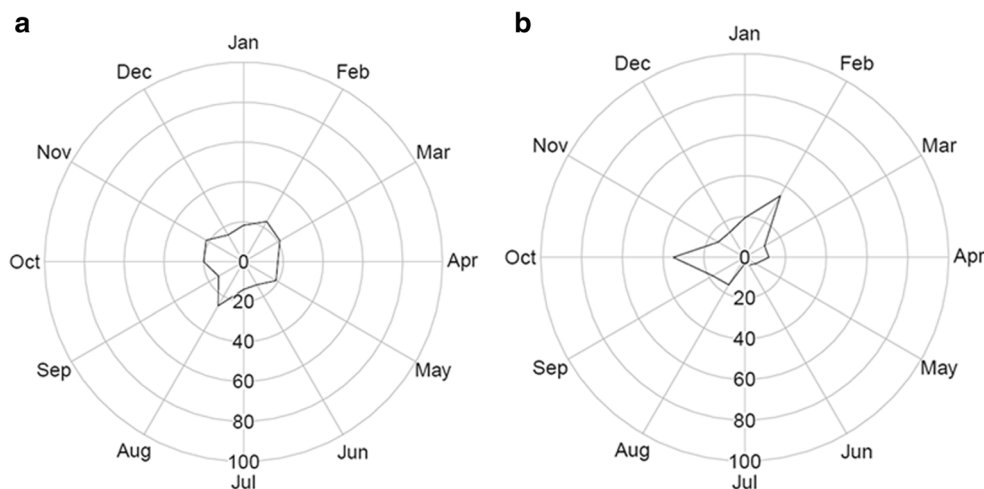
Table 4   Descriptive circular statistics for budding period in Rubiaceae species from forest and savanna in central-western São Paulo state, Brazil

Species	Median (1° and 3° quartiles)	Month of budding peak	r	Kuiper test	Watson test
Forest					
Cordiera concolor	337.52 (278.70–19.60)	September	0.634	101.40	14.624
Ixora gardneriana	139 (78.38–309.13)	May	0.281	35.58	0.575
Ixora heterodoxa	168.56 (78.38–248.33)	August	0.302	77.74	1.207
Palicourea marcgravii	49.98 (367.90–78.38)	February–April	0.479	63.12	0.907
Psychotria hoffmannseggiana	309.13 (309.13–337.52)	October	0.758	83.97	29.629
Randia sp.	93.56 (49.98–139)	February–May	0.511	82.31	0.673
Richardia grandiflora	248.33 (139–248.33)	May, August	0.537	4.64	1.747
Simira corumbensis	49 (19.60–78.38)	Jan, March	0.871	39.16	0.584
Warzewiczia sp.	278.70 (218.94–367.90)	August, November	0.226	55.64	0.557
Savanna					
Cordiera sessilis	278.70 (168.56–309.13)	February, May, October	0.117	66.85	15.256
Coccocypselum lanceolatum	248.33 (108.80–248.33)	August	0.191	61.08	18.094
Coussarea hydrangeifolia	399.60 (19.60–49.98)	January–February	0.920	75.07	13.639
Guettarda viburnoides	337.52 (309.13–19.6)	October	0.508	76.57	14.793
Palicourea rigida	323.31 (309.13–367.90)	October	0.700	82.37	0.775
Palicourea sp.	49.98 (367.90–78.38)	February–March	0.554	74.48	0.829
Psychotria sp.	19.60 (309.13–49.98)	February	0.353	57.65	1.234
Tocoyena formosa	49.98 (278.70–49.98)	February, September	0.483	43.65	11.631

All tests were significant ($p<0.05$)

proportion of damaged apices (Fig. 8c; $f_{(1,\,14)}=6.6$; $p=0.02$). The species with other compounds in the colleters showed no significant differences in the proportion of damaged apices (Fig. 8d–f; resins: $F_{(1,14)}=3.5$, $p=0.08$; phenolic: $F_{(1,14)}=0.35$; $p=0.56$; alkaloids: $F_{(1,14)}=0.91$; $p=0.35$).

Discussion

In this study, we compared 17 Rubiaceae species located in the neotropical savanna and forest focusing on colleters' secretions, using an integrative approach combining morphological, histochemical, and ecological evidences of the colleters' function. Firstly, we detailed the morphological patterns of colleters, its arrangement (descriptive and quantitative variation of colleters), and the histochemistry with focus on the nature of secretion (hydrophilic, lipophilic, or mixed). We also discussed the temporal variation of budding and exudates

on vegetative apices and its implications on the protective role of stipular colleters between the savanna and forest species. Additionally, we explored the differences between the microclimate around plants from savanna and forest, the proportion of damaged apices in both environments, and its correlation to colleters' secretions.

Morphology and arrangement of colleters in Rubiaceae species

Among the 17 investigated species, 14 exhibited colleters of standard-type, except for *C. hydrangeifolia*, which had lachrymiform type, and the two species of *Cordiera* genus that had dendroid colleters. As referred by Lersten (1974, 1975), the standard type is the most commonly found, with the dendroid colleters occurring in few Rubiaceae genera (Lersten 1974). Lachrymiform colleters are rarely described

Fig. 6 Variation in microclimate variables (maximum and minimum in each graph) nearby Rubiaceae plants in forest and savanna environment. In each graph, the maximum values appeared in *gray* and minimum values in *white*. *Different letters* indicate statistically significant difference between the forest and savanna microclimates (*p*≤0.05)
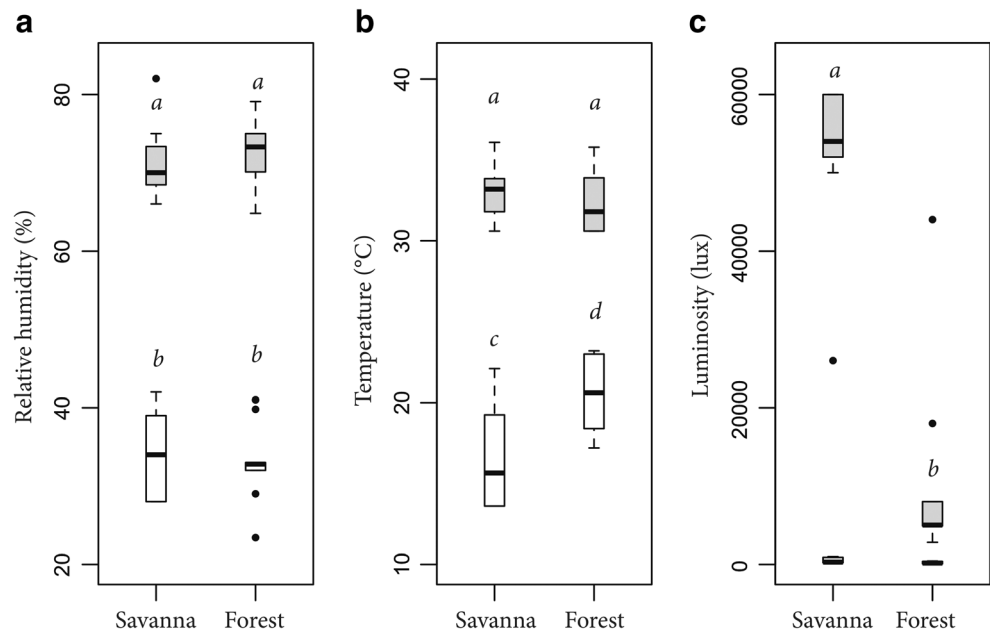
in Rubiaceae, and this colleter type was referred for the first time in *Psychotria nuda* (Miguel et al. 2009).

The arrangement of colleters of the studied species is associated with the morphological type of the stipule. Oval stipules with bifurcate apices had colleters arranged in rows, rounded stipules had colleters arranged across the entire surface or triangularly clustered, fimbriate stipules exhibited colleters across the entire surface and lanceolate stipules showed colleters triangularly clustered or arranged in rows. The colleter arrangement is an important character to identify Rubiaceae species (Aiello 1979; Thomas 1991). In agreement with these reports, more recently, Klein et al. (2004) observed interspecific variation in the arrangement of colleters as was observed in *Simira* genus, with colleters arranged in rows in *S. rubra* and *S. pikia* and triangularly arranged in *S. glaziovii*,

showing the importance of this character to distinguish species from the same genus. The presence of non-glandular trichomes distributed among the colleters has been referred as a common trait in Rubiaceae species (Lersten 1974).

Our results showed variation in the average length of colleters ranging from 1805 to 60 μm, with most species presenting average length of colleters between 590 and 415 μm, contradicting the hypothesis proposed by Lersten (1974). According to this author, the variation in the size of colleters is associated with the species geographic distribution; the larger colleter sizes was founded in neotropical Rubiaceae species (2600 to 776 μm), with average colleters presenting declines westward through Oceania (735 μm), Philippines (553 μm), East Indies, and Australia (480 μm), Asia (410 μm), and Africa (334 μm). Furthermore, the size of colleters does not

Fig. 7 Proportion of Rubiaceae species with colleters containing lipids, resins, phenolic compounds, and alkaloids in forest and savanna vegetation in central-western São Paulo state, Brazil. *Asterisk* indicates statistically significant difference (*p*≤0.05)
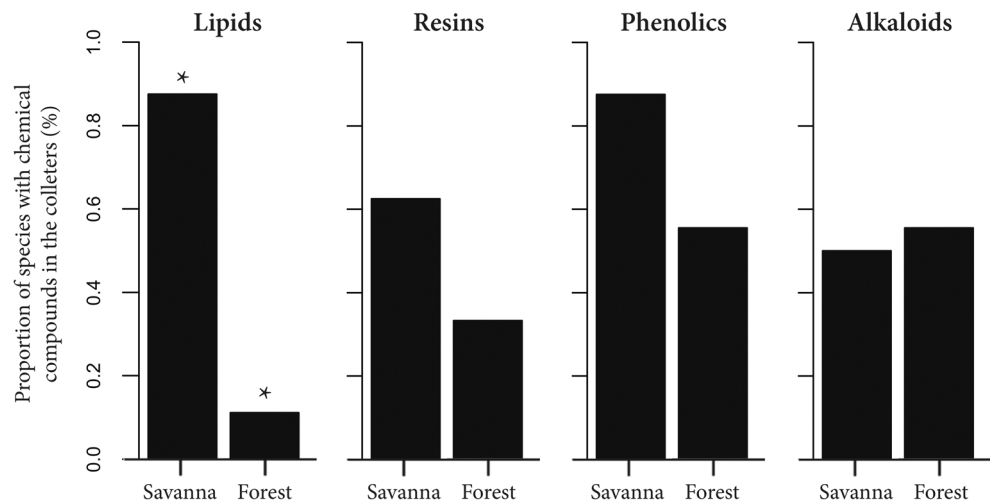
Fig. 8 Variation in the proportion of damaged apices in relation to the relative humidity (**a**) (without outlier is represented with a *dotted line*), vegetation type (**b**), and chemical compounds in colleters (**c**–**f**) in Rubiaceae species from forest and savanna vegetation in central-western São Paulo state, Brazil. *Different letters* indicate statistically significant difference ($p \leq 0.05$)
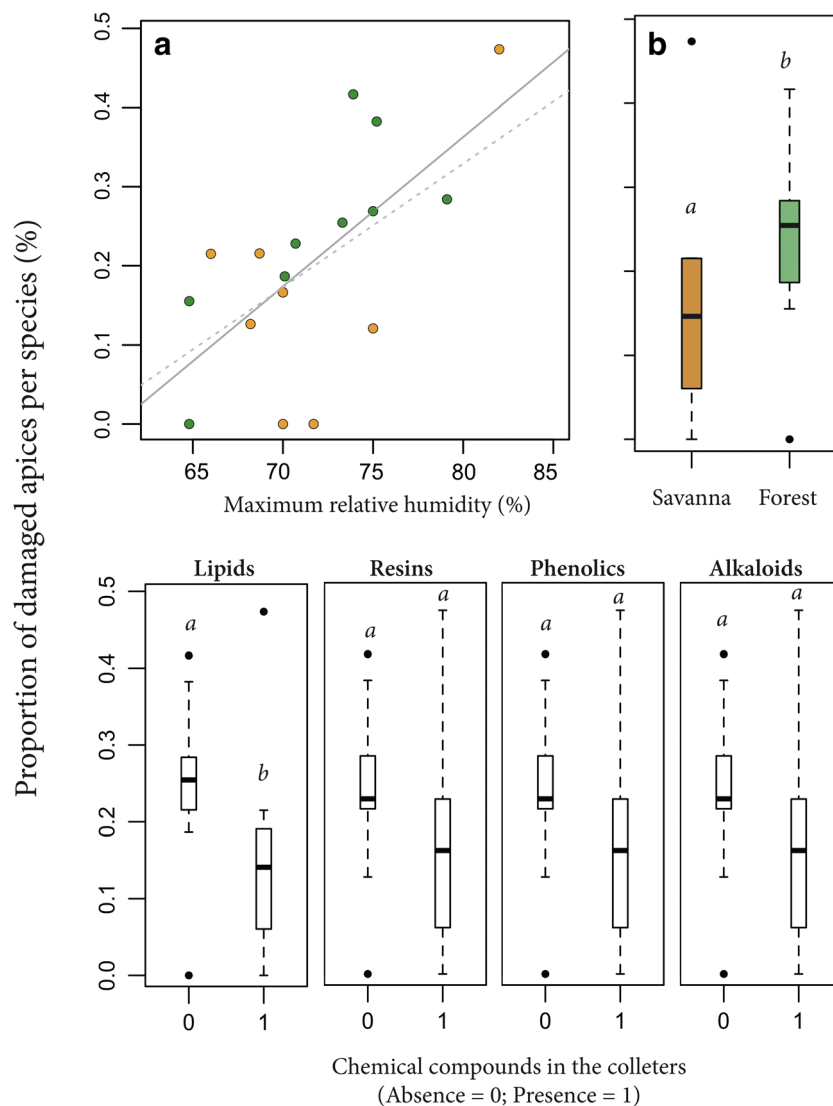
appear to be associated with the colleters' vascularization as proposed by Carlquist (1969). For example, the colleters of *I. gardneriana* were the biggest but were not vascularized, being markedly reduced in numbers (around nine per stipule). Whereas, the colleters of *P. rigida*, relatively smaller, but more abundant, were vascularized. We hypothesize that the number of colleters associated with their large size is determinant to the vascularization supply since this sink area requires a greater input of assimilates. These requirements could be provided by vascular tissue connected to the parenchyma cells of the central axis, aiding in supplying precursors to synthesize the secretion compounds, similar to what occurs in some extrafloral nectaries (Fahn 1979; Nicolson *et al.* 2007).

The calcium oxalate crystals in colleters are one of the most important traits of the Rubioideae subfamily due to the specificity of their shape and location in the colleters (Thomas 1991). The protective role of oxalate crystals against herbivore damage is well known (Franceschi and Nakata 2005), although rarely tested experimentally. In our case, the mechanical barrier that could be caused by the oxalate crystals associated with the chemical composition of colleters' secretions might have a relevant role in the apex protection. This report represents the first record of crystals for the Ixoroideae subfamily.

Temporal variation of budding and of exudate presence in vegetative apices

Although the budding occurred all year long, there were peaks in forest and savanna. Despite the savanna species present mechanisms related to the root system to avoid restrictions on the growth during dry periods (Pinheiro and Monteiro 2010), the budding peaks occurring during the rainy season indicated that periods of higher water availability are probably

more favorable to the production of new leaves in savanna species. Budding throughout the year in both environments can be related with the hypothesis that endogenous rhythms can be responsible for the timing of phenological events in seasonal tropical environments (Reich and Borchert 1984), as observed by Wright (1991) in a study of *Psychotria* species.

Changes in secretory constituents throughout the colleter development have been described (Miguel et al. 2010, Paiva 2012), highlighting the record of the presence of lipids in the mature stages of colleters (Klein et al. 2004). During the budding peaks, the secretion that covers the apices is an additional evidence of the protective role of colleters in this phenophase. It emphasized the potential protective role of colleter secretion during the budding in the savanna species, probably protecting the apices against the high incidence of solar radiation.

Histochemistry and ecological aspects of colleters in savanna and forest species

Mucilage, consisting of polysaccharides and proteins, was detected in all Rubiaceae species. These secretion components protect apices against desiccation and lubricate the meristems and young developing organs (Fahn 1990; Thomas 1991; Evert 2006; Mayer et al. 2013).

Except for mucilage, we observed differences in the composition of secretions in the colleters, when comparing the species from the forest with those from the savanna. A predominantly hydrophilic secretion was observed in the forest species, where 65 % of the sampled species reacted positively for alkaloids and phenolic substances (not significant relationship in our results). In this environment, a positive reaction for lipids and resin was detected in 10 and 30 % of the species, respectively. In the savanna, positive reactions for lipids and resins were also recorded in approximately 90 and 60 % of the species, respectively. Lipophilic secretions, containing as the main compound lipids (and resins in some species), occurred predominantly in the savanna species (significant relationship was detected between lipids and the vegetation type). Based on the observation of the location of these secretory structures and the lipophilic nature of their secretion, it has been proposed that colleters contribute to reduce water loss due to excessive transpiration of young leaves (Paiva 2012) and can also confer protection of the vegetative apices against the desiccation (Barreiro and Machado 2007) caused by elevated temperatures and pronounced periods of drought and against the high rates of solar radiation. According to Coutinho (2002), these climate conditions are common in the opened physiognomies of the Brazilian cerrado (savanna formations) and the local microclimate measurements around savanna study plants also revealed a similar pattern. In this scenario, the high proportion of Rubiaceae species from savanna showing the apices covered with lipid secretions

produced by the colleter (and also mucilage and resin in some species) could be considered as evidence of its advantage, but future experimental studies manipulating this compounds will be necessary to properly assess this issue.

Other compounds detected in the colleters of some Rubiaceae species have been related to the anti-herbivory protection of the vegetative apices, such as alkaloids (Trigo et al. 2012), phenolic substances with organoleptic properties (Harborne et al. 1999), proteins due to entomotoxic properties (Melo and Silva-Filho 2002), and crystals, especially those of calcium oxalate, which have been cited as a type of mechanical defense (Franceschi and Nakata 2005). Although phenolic compounds and alkaloids have been verified in most of forest species and not savanna species, we did not verify a clear association between them and these environments with contrasting characteristics. A similar pattern was described for leaf herbivory by Neves et al. (2010) that reported higher abundance and richness of chewing insect in forest trees than in cerrado. We suppose that the higher proportion of damaged apices in the forest, as compared to the savanna, could be associated to the absence of lipids in the colleters' secretion of most forest species. However, only chemical defenses cannot explain the differences observed in this study. According to Neves et al. (2010), other factors should be considered at the habitat level, as soil quality, leaf sclerophylly, leaf longevity, diversity, and abundance of herbivorous insects, microclimatic environmental conditions, structural complexity, and top-down forces.

Although in general, we found characteristic patterns regarding the composition of secretions for each environment, we hypothesized that the chemical compounds associated with the colleter morphotype appear to be phylogenetically correlated and conserved in some particular groups investigated. We found that species of the same genus occurring in savanna and forest had similar patterns of morphology and histochemistry in contraposition to our general results when comparing these two contrasting environments. For example, both *Cordiera* species, one from savanna and the other from forest, exhibited dendroid colleters and secretions predominately lipophilic, while both *Psychotria* species occurring in such environments had standard-type colleters and secretion predominantly hydrophilic. Similar results were reported for other Rubiaceae species. In three species of the genus *Simira* occurring in the same environment, the standard-type colleters varied regarding their distribution in the stipules and vascularization, but not regarding the composition of secretions (Klein et al. 2004). In *Psychotria carthagenensis*, individuals occurring in contrasting habitats as tropical rain forests, sandbanks, and rheophytic environments exhibited colleters producing polysaccharide secretion (Vitarelli and Santos 2009). These findings reinforce the idea that the qualitative variation of secretion composition of colleters among Rubiaceae congeners could be a phylogenetically conserved trait, although just

two species per genus have been sampled. Additionally, we did not characterize the quantitative variation of colleter's secretion in our study (e.g., volume and chemical's concentration among species). This aspect of colleter's secretion could be quite different among congeners as suggested by the variation of the number of colleters per stipule among *Psychotria* species in our study.

In synthesis, our data showed that there is a clear relationship between the nature of a colleter's secretion, vegetation type, luminosity, and the proportion of damage apex among Rubiaceae species. We verified colleters with a prevalence of lipids in savanna species, which were distributed in environments with higher luminosity and had, on average, a lower proportion of damaged apices. In contrast, a hydrophilic secretion was prevalent in forest species growing in habitats with lower luminosity (less extreme conditions), which had higher proportion of damaged apices. The integration of this approach including data about structure, distribution, and histochemistry of colleters; phenology and microclimate of forest; and savanna environment allows a deeper knowledge on stipular colleters in Rubiaceae species from different tropical ecosystems. It is our expectation that this first view of the role of colleters in contrasting environments stimulates additional ecological and evolutionary studies exploring clearly hypothesis based on the morpho-functional variation of plant traits in a species-rich plant clade as Rubiaceae.

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