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Stigma structure and receptivity in Bromeliaceae

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ABSTRACT

Morphoanatomical characterization of the stigma of different species has provided important data for the taxonomy of Bromeliaceae. Stigma receptivity is fundamental for the effectiveness of reproduction in plants, and the production of hybrids by controlled pollination. This study aimed to characterize stigma morphoanatomy of 18 Bromeliaceae species, from five genera, by means of light and scanning electron microscopy, and to determine the stigma receptivity of these species. The species investigated were: *Aechmea bicolor*, *Aechmea bromeliifolia*, *Aechmea distichantha*, *Aechmea fasciata*, *Aechmea nudicaulis*, *Ananas* sp., *Ananas ananassoides*, *Ananas bracteatus*, *Ananas lucidus*, *Ananas parguazensis*, *Alcantarea nahoumii*, *Pseudananas sagenarius*, *Vriesea carinata*, *Vriesea friburgensis*, *Vriesea michaelii*, *Vriesea paraibica*, *Vriesea simplex* and *Vriesea unilateralis*. Three methods were used comparatively to determine stigma receptivity during floral opening: hydrogen peroxide, benzidine and α-naphthyl acetate. Two stigma types were observed: conduplicate-spiral in *Aechmea*, *Ananas*, *Alcantarea* and *Pseudananas* and convolute-blade in *Vriesea*. The stigma is trifid, formed by a unistratified epidermis, parenchyma with numerous idioblasts containing raphides, and three vascular bundles, one for each carpel. In the conduplicate-spiral stigmas type, the inner epidermal cells contain dense cytoplasm and a prominent nucleus, a characteristic not observed in the convolute-blade stigmas type. The stigmatic papillae vary according to species, as well as the presence of an ornamented cuticle. The longest pistil length was observed in *A. nahoumii* (91.75 mm) and shortest in *A. bromeliifolia* (4.19 mm). Higher stigma receptivity is observed at anthesis for the species studied, with the highest receptivity at 8 am and remaining receptive until noon, with the exception of *V. unilateralis*, for which the greatest receptivity was observed at midnight and extended until dawn. The use of α-naphthyl acetate was the most efficient methodology for the detection of stigma receptivity, and to identify the receptive areas of the stigma.

1. Introduction

The Bromeliaceae family presents economical and ecological importance, both for ornamental and food purposes, and also contributes to the maintenance of biodiversity in a wide range of Neotropical habitats (Benzing, 2000). Bromeliaceae is composed of 58 genera and 3352 species (Luther, 2012), with colorful or patterned leaves, and bracts and flowers with vivid and contrasting colors, which are valued by the ornamental market.

Understanding floral morphology and biology is fundamental to evaluate the interactions between pollen grains and the stigma, flowers and pollinators, as well as for successful breeding of plant species (Lenzi and Orth, 2004). The conservation of threatened species and restoration programs is thus dependent on understanding the reproductive biology of the species of interest (Bernardello et al., 2001), including stigma morphology and receptivity. Stigma morphology can be uniform, or quite variable (Heslop-Harrison, 1981), and although some families may have more than one type of stigma, evolutionary and intermediate trends can be observed, making it possible to use stigma morphology to assist plant taxonomical classification (Heslop-Harrison, 1992). Stigma morphology of bromeliads has provided important data on their taxonomy helping to better define the generic and species boundaries (Brown and Gilmartin, 1989).

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Considering the diversity of this family, stigma morphology of a relatively small number of species has been described, such as those by Brown and Gilmartin (1984, 1989), Varadarajan and Brown (1988), Gortan and Till (1998), Vervaeke et al. (2003) and Versieux and Wanderley (2015). These authors characterized the stigma of Bromeliaceae as trifid, with differences in stigma branches or lobes, classifying them in five morphological categories: conduplicate-spiral, convolute-blade, simple-erect, cupulate and coralliform.

Stigma receptivity directly affects the plant life cycle, a detailed knowledge of these features will determine the best moment for pollination and for gametophytic selection, to enable successful controlled pollination (Galen and Plowright, 1987) in breeding programs. Wet or dry stigma may be observed in Bromeliaceae. Among the Bromeliaceae studied by Heslop-Harrison and Shivanna (1977), *Abromeitella*, *Dyckia* and *Neoregelia* presented a dry stigma with multiseriate papillae, and *Aechmea*, *Bilbergia*, *Bromelia*, *Canistrum*, *Fosterella*, *Nidularium*, *Pitcairnia*, *Portea*, *Quesnelia*, *Streptocalyx* and *Vriesea* presented a wet stigma with low to medium papillae in the receptive surface.

Stigma receptivity is related to the activity of enzymes such as peroxidase, esterase and dehydrogenase (Heslop-Heslop-Harrison and Shivanna, 1977; Galen and Plowright, 1987; Dafni and Maués, 1998). Receptive stigmas have high enzyme activity, which can occur in different phases of flower development (Knox, 1984; Shivana and Rangaswamy, 1992). The observation of the activity of these enzymes can be used to characterize stigma receptivity (Knox et al., 1986; Dafni, 1992; Kearns and Inouye, 1993). These enzymes play a key role in pollen grain germination, penetration of the pollen tube in the stigma and probably the incompatibility responses (Heslop-Harrison et al., 1975; Kulloli et al., 2010). The method used for detecting stigma receptivity may vary according to plant species and should also consider an evaluation of pollen grain germination on the stigma (Stone et al., 1995). Stigma receptivity occurs during a very short period during the life time of a flower, varying from minutes to a few days, making it the shortest-lived structure in a plant (Heslop-Harrison, 1992), thus important for cross pollination.

The aims of this study were to characterize pistil morphoanatomy of 18 Bromeliaceae species, belonging to five genera, classifying them among the different stigma types, and to determine the best period of stigma receptivity, evaluating different methods.

2. Material and methods

2.1. Plant materials and growth conditions

Plants of 18 species and/or botanical varieties of Bromeliaceae with ornamental potential were cultivated in a greenhouse, in plastic pots (13 cm height by 10 cm diameter), with Basaplant® substrate, at ambient temperature and relative humidity of 70%, in the municipality of Piracicaba, São Paulo state, Brazil. A pilot study was carried to certify the time of opening.

One exemplar of each was deposited in the ESA herbarium ('Luiz de Queiroz' School of Agriculture/University of São Paulo — Esalq/USP): *Aechmea bicolor* L. B. Sm. (voucher ESA 120990), *Aechmea bromeliifolia* Baker ex Benth. & Hook. f. (ESA 121275), *Aechmea distichantha* Lem. (ESA 121281), *Aechmea fasciata* Baker (ESA 120987), *Aechmea nudicaulis* Griseb. (ESA 120991), *Ananas* sp. (L.) Merr. (ESA 120988), *Ananas ananassoides* (Baker) L. B. Smith (ESA 121274), *Ananas bracteatus* (Lindley) Schultes f. (ESA 121284), *Ananas lucidus* Miller (ESA 121285), *Ananas parguazensis* Camargo & L. B. Smith (ESA 121405), *Alcantarea nahoumii* (Leme) J. R. Grant (ESA 120986), *Pseudananas sagenarius* (Arruda da Camara) Camargo (ESA 121286), *Vriesea carinata* Wawra (ESA 121404), *Vriesea friburgensis* Mez (ESA 121282), *Vriesea michaelii* W. Weber (ESA 121280), *Vrieseaparaibica* Wawra (ESA 121276), *Vriesea simplex* Beer (ESA 120989) and *Vriesea unilateralis* Mez (ESA 121283).

2.2. Morphoanatomy of the stigma

For morphological characterization, the stigmas/styles, collected at anthesis, were fixed in a modified Karnovsky solution (Karnovsky, 1965) [glutaraldehyde (2%), paraformaldehyde (2%), $CaCl_2$ (0.001 M), sodium cacodylate buffer (0.05 M), at pH 7.2], for 48 h, and then dehydrated in an ethyl alcohol series (35–100%). The samples were then critical point dried with liquid CO_2, mounted on metal stubs and sputter coated with gold and analysed under a LEO 435 scanning electron microscope (Carl Zeiss, Jena, Germany) and digital images recorded.

For anatomical analyses, the stigmas/styles were fixed in the same modified Karnovsky solution for one week, dehydrated in an ethyl alcohol series (35–100%) for 6 h, infiltrated and embedded using the Historesin kit (hydroxymethacrylate, Leica, Heidelberg, Germany). The resin was polymerized at room temperature for 48 h. Serial histological sections (4–5 μm) were obtained with a Leica RM 2155 rotary microtome (Leica, Nussloch, Germany). The sections were placed on histological slides, stained with acid fuchsin (1%), followed by toluidine blue (0.05%) (Feder and O'Brien, 1968), covered with Entellan and coverslipped. The sections were then analysed and digital images obtained with an Axioskop 2 photomicroscope (Carl Zeiss, Jena, Germany).

Ten stigmas of each species, collected from different plants, were observed and micrographs obtained under the scanning electron microscope for measurements of stigma, style and papillae length and diameter, using the ImageJ 1.46r software (Rasband, 1997–2012). For morphological and anatomical characterization, three stigmas from different plants of each species were used.

2.3. Stigma receptivity

Stigma receptivity was determined at: pre-anthesis (floral bud — 6 pm); anthesis (8 am); post-anthesis (noon and 6 pm) and shortly after flower closing (8 am), with three replicates. In *V. unilateralis*, in which anthesis occurs at night, the evaluation times were adjusted: pre-anthesis (floral bud — 12 noon; 6 pm); anthesis (midnight); post-anthesis (8 am) and flower closing (noon).

Three methods were used to evaluate stigma receptivity, as described below: (1) The stigmas were immersed in hydrogen peroxide (3%) for 3 min to observe the release of air bubbles, since the reaction of hydrogen peroxide with the enzyme peroxidase indicates the stigma is receptive (Zeisler, 1933); (2) The stigmas were immersed in a solution of benzidine (1%), ethanol (60%) and hydrogen peroxide (3%) for 3 min, in which receptivity is indicated by the presence of peroxidase bubbles and oxidation of benzidine, which causes a blue coloration (Dafni, 1992); (3) The stigmas were immersed in a solution of α-naphthyl acetate in phosphate buffer, acetone and fast blue B salt for 5 min, rinsed with distilled water. The esterase activity was observed at the surface of the stigma and/or papillae (Pearse, 1972; Dafni, 1992).

Stigma receptivity was estimated by assigning degrees of receptivity (adapted from Dafni and Maués, (1998)): (−) no reaction; (+) weak positive reaction; (++) strong positive reaction; and (+++) very strong positive reaction.

3. Results and discussion

3.1. Morphoanatomy of the stigma

Two stigma types were observed in the species studied, a conduplicate-spiral stigma was observed in *Aechmea*, *Ananas*,

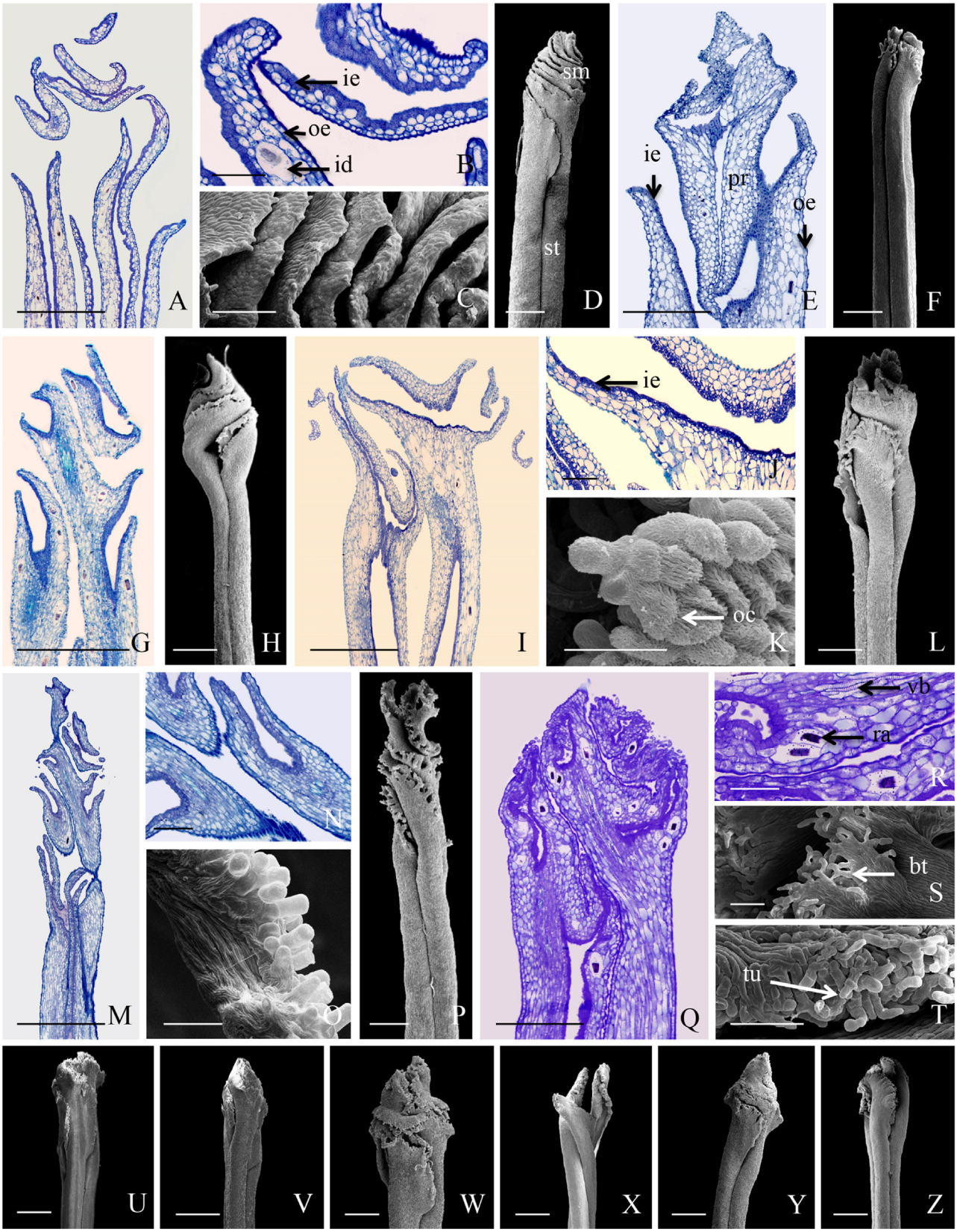

Fig. 1. Morphoanatomy of the conduplicate-spiral stigma of *Aechmea*, *Ananas* and *Pseudananas* species, subfamily Bromelioideae: *A. bicolor* (A–D); *A. bromeliifolia* (E–F); *A. distichantha* (G–H); *A. fasciata* (I–L); *A. nudicaulis* (M-P); *Ananas* sp. (Q, R, T, V); *Pseudananas sagenarius* (S,U); *A. ananassoides* (W); *A. bracteatus* (X); *A. lucidus* (Y); and *A. parguazensis* (Z). Longitudinal sections observed by light microscopy (A, B, E, G, I, J, M, N, Q, R) and morphological characteristics by scanning electron microscopy (C, D, F, H, K, L, O, P, S–Z). bt = branched tubular, pr = parenchyma, id = idioblast, ie = inner epidermis, oc = ornamented cuticle, oe = outer epidermis, ra = raphides, sm = stigma, st = style, tu = tubular, vb = vascular bundle. Bars: A, D, E–I, L–M, P–Q, U–Z = 500 μm; B–C, J, N, R–T = 100 μm; K, O = 30 μm.

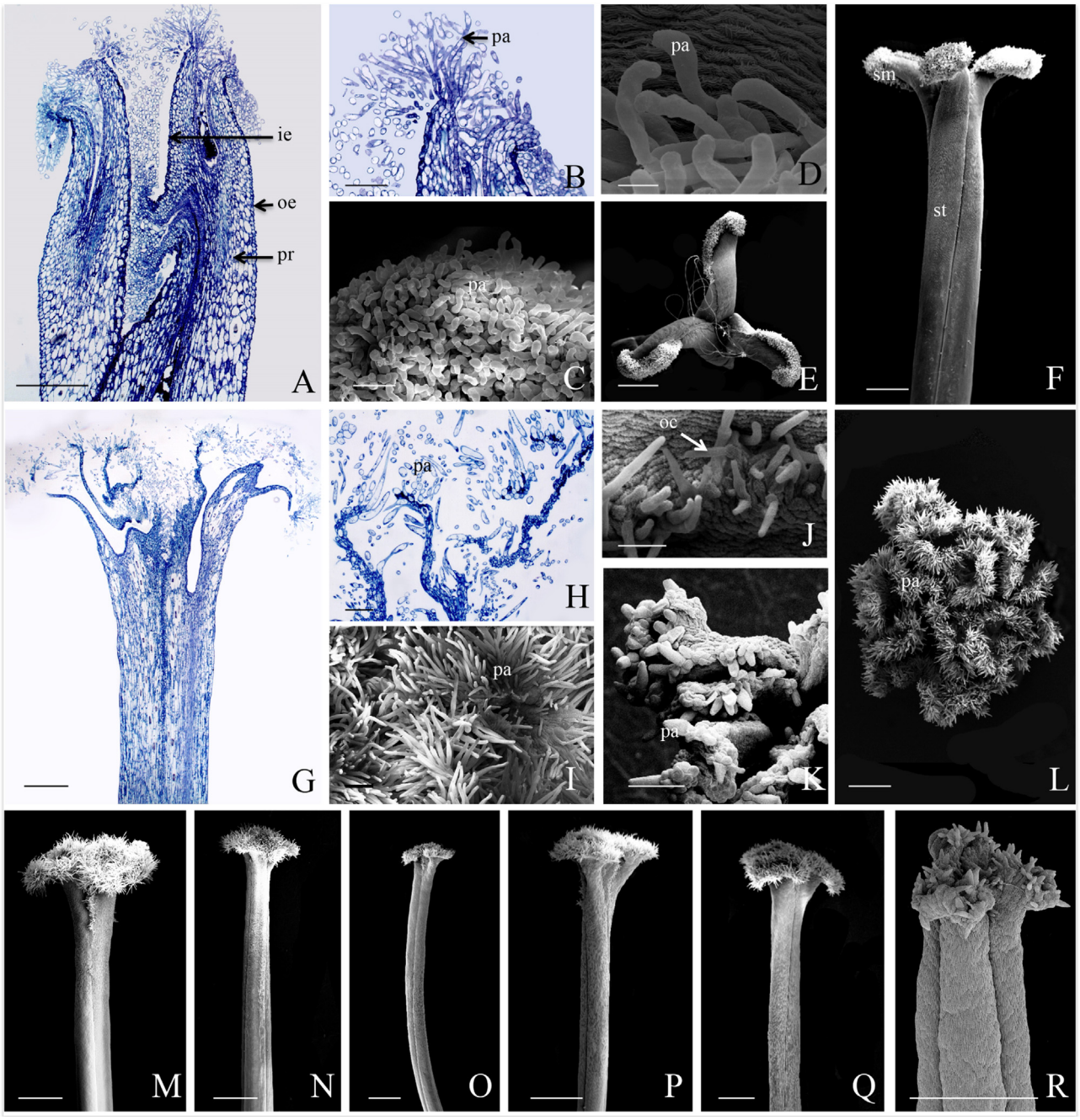

Fig. 2. Morphoanatomy of the conduplicate-spiral stigma in *Alcantarea nahoumii* and convolute-blade stigma in species of the genus *Vriesea* of the subfamily Tillandsioideae/Bromeliaceae: *Alcantarea nahoumii* (A–F); *Vriesea carinata* (G, I, M); *V. friburgensis* (N); *V. michaelii* (O); *V. paraibica* (J, P); *V. simplex* (L, Q); and *V. unilateralis* (K, R). Longitudinal sections observed by light microscopy (A–B, G–H) and morphological characteristics by scanning electron microscopy (C–F, I–R). pr = parenchyma, ie = inner epidermis, oc = ornamented cuticle, oe = outer epidermis, pa = papillae, sm = stigma, st = style. Bars: A, G, = 500 μm; B–C, H–I, K = 100 μm; D, J = 30 μm; E–F, L–R = 1 mm.

Pseudananas (Bromelioideae), and *Alcantarea* (Tillandsioideae) while *Vriesea* (Tillandsioideae) presented a convolute-blade stigma (Table 1, Figs. 1 and 2). Brown and Gilmartin (1984, 1989) reported that the conduplicate-spiral type is characteristic of the subfamily Bromelioideae with the exception of the genera *Cryptanthus* and *Orthophtum*, which have a simple-erect stigma, while in the subfamily Tillandsioideae, all five stigma types can be observed (conduplicate-spiral, convolute-blade, simple-erect, coralliform and cupulate. Morphologically, both stigma types, conduplicate-spiral and convolute-blade, are trifid, with an unistratified epidermis, a parenchyma with numerous idioblasts containing raphides, especially in the *Ananas* and *Pseudananas* genus (Fig. 1Q), and three vascular bundles, one for each carpel. In the conduplicate-spiral stigma, the cells of the inner epidermis contain dense cytoplasm and prominent nucleus (Fig. 1A,B,E,G,I,J,M,N,Q,R and Fig. 2A,B), characteristics not observed in the convolute-blade stigma (Fig. 2G,H). The presence of cells with dense cytoplasm and prominent nucleus in the inner epidermis suggests an intense metabolic activity characteristic of secretory cells, related to the adherence of the pollen grains, normally the role of the specialized papillae in the convolute-blade stigma type. Brown and Gilmartin (1988) suggested that the conduplicate-spiral stigma shape represents a plesiomorphic condition within the family and that the convolute-blade type is more specialized, having a larger surface

Table 1
Pistil characteristics in Bromeliaceae species of the genera *Aechmea*, *Ananas*, *Alcantarea*, *Pseudananas* and *Vriesea*.

Species/varieties	Type[a]	Stigma (mm)		Style (mm)		Color
		Length	Diameter	Length	Diameter	
Aechmea bicolor	Conduplicate-spiral	1.07 ± 0.12	0.70 ± 0.06	4.06 ± 0.05	0.72 ± 0.02	White
A. bromeliifolia	Conduplicate-spiral	0.61 ± 0.07	0.52 ± 0.02	3.58 ± 0.07	0.50 ± 0.03	White
A. distichantha	Conduplicate-spiral	1.40 ± 0.09	0.84 ± 0.02	6.26 ± 0.18	0.46 ± 0.02	White
A. fasciata	Conduplicate-spiral	1.63 ± 0.10	1.23 ± 0.04	14.03 ± 0.15	0.49 ± 0.05	Light lilac
A. nudicaulis	Conduplicate-spiral	1.13 ± 0.07	0.42 ± 0.04	9.12 ± 0.16	0.40 ± 0.04	Light yellow
Ananas sp.	Conduplicate-spiral	1.17 ± 0.07	0.87 ± 0.03	4.80 ± 0.34	0.52 ± 0.03	White
A. ananassoides	Conduplicate-spiral	1.48 ± 0.19	1.71 ± 0.07	5.08 ± 0.10	0.92 ± 0.05	White
A. bracteatus	Conduplicate-spiral	1.28 ± 0.18	1.45 ± 0.13	6.98 ± 0.17	0.63 ± 0.07	White
A. lucidus	Conduplicate-spiral	1.38 ± 0.23	1.63 ± 0.05	5.30 ± 0.06	0.89 ± 0.11	White
A. parguazensis	Conduplicate-spiral	1.21 ± 0.12	1.05 ± 0.08	5.11 ± 0.05	0.65 ± 0.08	White
Alcantarea nahoumii	Conduplicate-spiral	1.75 ± 0.25	4.59 ± 0.09	89.99 ± 0.71	1.49 ± 0.05	White
Pseudananas sagenarius	Conduplicate-spiral	1.12 ± 0.12	1.02 ± 0.13	7.17 ± 0.14	0.85 ± 0.08	White
Vriesea carinata	Convolute-blade	1.61 ± 0.17	2.55 ± 0.09	27.97 ± 0.24	0.69 ± 0.03	Light yellow
V. friburgensis	Convolute-blade	0.83 ± 0.07	1.76 ± 0.04	22.38 ± 0.45	0.50 ± 0.03	Light yellow
V. michaelii	Convolute-blade	1.05 ± 0.14	1.47 ± 0.05	20.56 ± 0.15	0.44 ± 0.02	Light yellow
V. paraibica	Convolute-blade	1.81 ± 0.16	2.75 ± 0.07	25.99 ± 0.25	0.74 ± 0.03	Light yellow
V. simplex	Convolute-blade	1.98 ± 0.14	3.42 ± 0.08	31.90 ± 0.38	0.84 ± 0.02	Light green
V. unilateralis	Convolute-blade	0.57 ± 0.08	1.84 ± 0.20	18.85 ± 0.41	1.39 ± 0.22	Light yellow

[a] According to the terminology of Brown and Gilmartin (1984, 1989). Measurements represent the average of 10 samples ± standard deviation.

Table 2
Stigma receptivity in Bromeliaceae of the genera *Aechmea, Ananas, Alcantarea, Pseudananas* and *Vriesea* evaluated by three methods at different times related to anthesis.

Species/varieties	HP	B + HP	∝-NA + FB	HP	B + HP	∝-NA + FB	HP	B + HP	∝-NA + FB	HP	B + HP	∝-NA + FB	HP	B + HP	∝-NA + FB
	6 pm (pre-anthesis)			8 am (anthesis)			noon (post-anthesis)			6 pm (post-anthesis)			8 am (post-anthesis)		
Aechmea bicolor	–	–	–	+++	+++	+++	+++	+++	+++	+++	+++	+	++	++	–
A. bromeliifolia	–	–	–	+++	+++	+++	+++	+++	+++	+++	++	++	++	+	+
A. distichantha	–	–	–	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	+
A. fasciata	–	–	–	+++	+++	+++	+++	+++	+++	+++	+++	+	+++	++	–
A. nudicaulis	–	–	–	+++	+++	+++	+++	+++	+++	+++	++	++	++	+	+
Ananas sp.	–	–	–	+++	+++	+++	+++	+++	+++	+++	+++	++	+++	++	+
A. ananassoides	–	–	–	+++	+++	+++	+++	+++	+++	+++	+++	++	+++	++	+
A. bracteatus	–	–	–	+++	+++	+++	+++	+++	+++	+++	+++	++	+++	++	+
A. lucidus	–	–	–	+++	+++	+++	+++	+++	+++	+++	+++	++	+++	++	+
A. parguazensis	–	–	–	+++	+++	+++	+++	+++	+++	+++	+++	++	+++	++	+
Alcantarea nahoumii	–	–	–	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Pseudananas sagenarius	–	–	–	+++	+++	+++	+++	+++	+++	+++	+++	++	+++	++	+
Vriesea carinata	–	–	–	+++	+++	+++	+++	+++	+++	+++	++	++	++	+	–
V. friburgensis	–	–	–	+++	+++	+++	+++	+++	+++	+++	+++	++	+++	++	+
V. michaelii	–	–	–	+++	+++	+++	+++	+++	+++	+++	+++	++	+++	++	–
V. paraibica	–	–	–	+++	+++	+++	+++	+++	+++	+++	+++	++	+++	++	+
V. simplex	–	–	–	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++

Species/varieties	HP	B + HP	∝-NA + FB	HP	B + HP	∝-NA + FB	HP	B + HP	∝-NA + FB	HP	B + HP	∝-NA + FB	HP	B + HP	∝-NA + FB
	noon (pre-anthesis)			6 pm (pre-anthesis)			midnight (anthesis)			8 am (post-anthesis)			noon (post-anthesis)		
V. unilateralis	–	–	–	++	++	+	+++	+++	+++	+++	+++	+++	+++	++	+

(–) no reaction; (+) weak positive reaction; (++) strong positive reaction; (+++) very strong positive reaction. Methods adapted from Dafni and Maués (1998); ∝-NA + FB = solution of ∝-naphthyl acetate and fast blue B salt; B + HP = solution of benzidine and hydrogen peroxide; HP = hydrogen peroxide.

area due to the presence of papillae, allowing more efficient pollen adherence. The presence of idioblasts with raphides are commonly observed in nectary tissue of *A. ananassoides* (Stahl et al., 2012), and when associated with bromelain has been shown to have an effect against herbivores (Konno et al., 2014).

A. bicolor, *A. distichantha*, *A. fasciata* and *A. nudicaulis* present highly contorted stigmatic lobes (Fig. 1D,H,L,P) covered by an ornamented cuticle (Fig. 1C,H,K,O), characteristics less evident in *A. bromeliifolia* (Fig. 1F). The edges of the stigmatic blades vary from intensely lobed in *A. bromeliifolia* (Fig. 1F), *A. distichantha* (Fig. 1H), *A. fasciata* (Fig. 1K,L) and *A. nudicaulis* (Fig. 1O,P) to partially lobed in *A. bicolor* (Fig. 1C,D). The papillae of all the species are restricted to the edge of the stigmatic blade, and are tubular in *A. bicolor* and *A. bromeliifolia,* and bulbous in *A. distichantha*, *A. fasciata* and *A. nudicaulis*. Papillae number ranges from a few in *A. bicolor* (Fig. 1C), to many in *A. nudicaulis,* without cuticle ornamentation (Fig. 1O). The stigma color varies from white in *A. bicolor*, *A. bromeliifolia, A. distichantha* and *A. nudicaulis*, to light lilac in *A. fasciata* and light yellow in *A. nudicaulis* (Table 1).

In *Ananas* and *Pseudananas* the stigma is white, with highly contorted lobes covered with an ornamented cuticle (Fig. 1U–Z). The blade edges are lobed and papillose, varying from branched tubular in *P. sagenarius* (Fig. 1S) to unbranched tubular in the all species of *Ananas*, without cuticle ornamentation (Fig. 1T).

A. nahoumii has a white conduplicate stigma (Fig. 2A–F), with slightly contorted lobes and curved apices, with densely distributed short papillae (99.26 ± 29.18 μm), uni or multicellular, with smooth cuticle (Fig. 2C). This genus, previously classified in the *Vriesea* sub-genus, was reported by Grant (1995) to have a convolute-blade stigma, but Leme (2007) and Versieux and Wanderley (2015) suggests a conduplicate stigma for all species of this genus, with variations in the stigma shape among species.

The species of *Vriesea* have stigmatic lobes with unicellular tubular papillae (Fig. 2H), covered by an ornamental

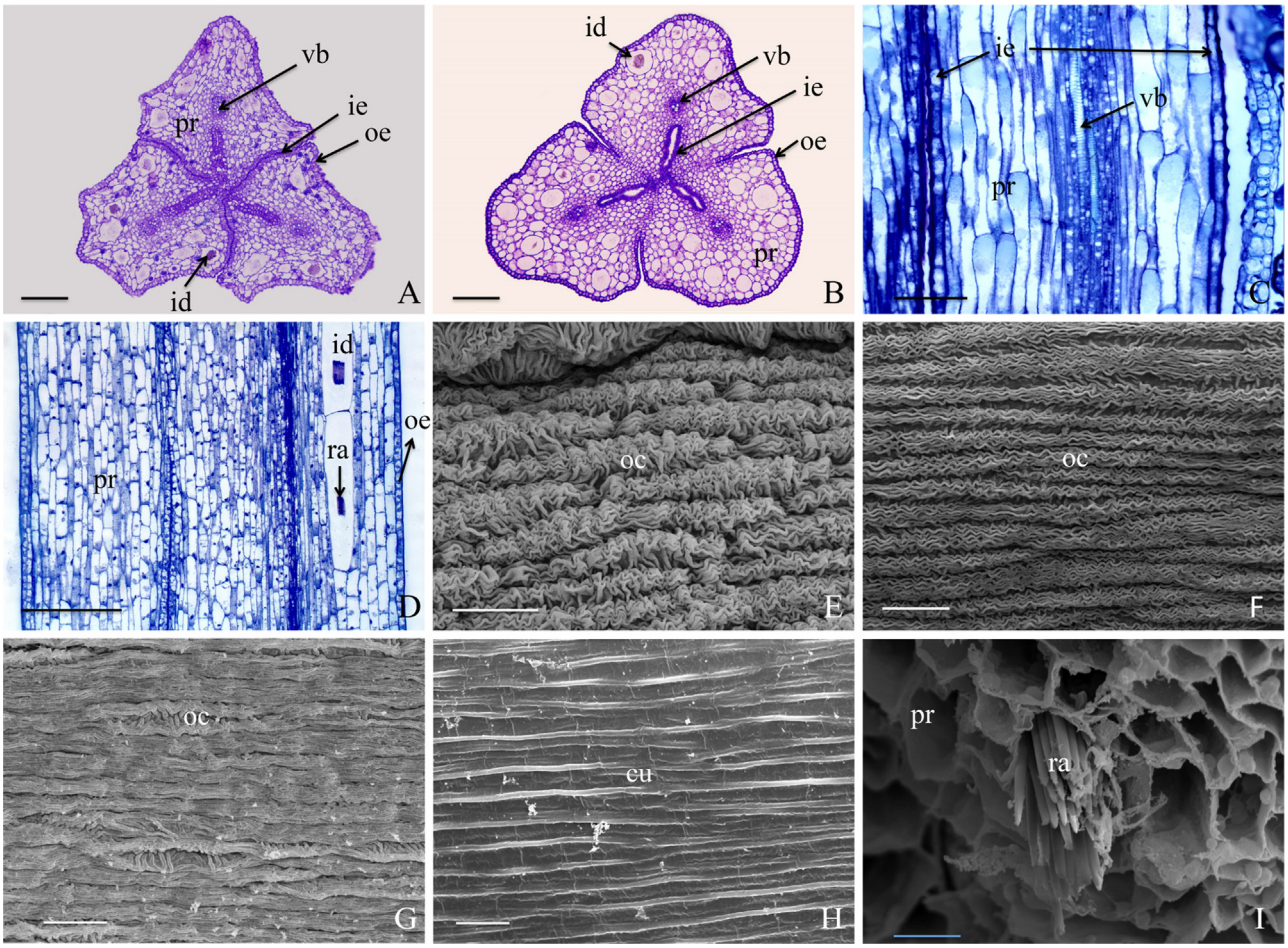

Fig. 3. Morphoanatomy of the style in Bromeliaceae. (A–B) Cross sections of the style of *Aechmea bicolor* (A) and *A. nudicaulis* (B) observed by light microscopy (LM); (C–D) longitudinal sections (LM) of the style of *Ananas* sp., showing the vascular bundle (C) and *Pseudananas sagenarius*, showing idioblasts and raphides (D); different morphologies of the striae in the cuticle of the style of *V. carinata* (E), *V. michaelii* (F), *A. fasciata* (G) and *A. nahoumii* (H), observed by scanning electron microscopy (SEM); raphides in *Vriesea simplex* observed by SEM (I). cu = cuticle, pr = parenchyma, id = idioblast, ie = inner epidermis, oc = ornamented cuticle, oe = outer epidermis, ra = raphides, vb = vascular bundle. Bars: A–C = 100 μm; D = 500 μm; E–I = 20 μm.

cuticle. The papillae are long (169.25 ± 77.93 μm) and thin (8.42 ± 1.21 μm) (Fig. 2I–J), except for *V. unilateralis,* which has shorter (59.29 ± 20.42 μm) and thicker (21.32 ± 6.37 μm) papillae (Fig. 2K,R). The color varies from light green in *V. simplex* to light yellow in the other species studied (Table 1).

The stigmas of all the species studied remain wet during the entire period that the flower remains open. Heslop-Harrison and Shivanna (1977), who classified the stigmas of bromeliads as wet for the genera *Aechmea*, *Bilbergia*, *Bromelia*, *Canistrum*, *Fosterella*, *Nidularium*, *Pitcairnia*, *Portea*, *Quesnelia* and *Vriesea*, and dry for the species of the genera *Abromeitiella* (*Deuterocohnia*), *Dyckia* and *Neoregelia*, here we include detailed information for species/varieties of *Alcantarea* and *Ananas*.

The largest values for length and diameter of the stigma and style were observed in *A. nahoumii* (stigma/style with length of 91.75 mm and diameter of 4.59 mm). This genus is characterized by large flowers, which are pollinated by bat, hummingbird or moth (Versieux and Wandereley 2015). For the species of *Vriesea*, the lengths varied from 19.42 mm in *V. unilateralis* to 33.88 mm in *V. simplex*, while for the species of *Aechmea* these measures were 4.19 mm in *A. bromeliifolia,* and 15.65 mm in *A. fasciata* (Table 1). The morphometric characteristics of the pistil of different species can interfere directly in their ability to cross, due to the direct correspondence with growth of the pollen tube (Gopinathan et al., 1986; Parton et al., 2001).

The styles of all the species studied are formed by an unistratified epidermis (Fig. 3A–D), in the outer epidermis cells present periclinal cell walls that are slightly convex externally, and covered by a striated cuticle. The inner epidermal cells have a dense cytoplasm, prominent nucleus and smooth cuticle (Fig. 3A–B). The styles have vascular bundles, one for each carpel, surrounded by parenchyma cells (Fig. 3A–C). The cuticle varies from smooth to rough, according to the species, with more pronounced roughness or in the species of the *Vriesea* genus (Fig. 3E–F), less pronounced in the species of *Aechmea* and *Ananas* (Fig. 3G), and almost smooth in *A. nahoumii* (Fig. 3H). Numerous idioblasts containing raphides were observed in the parenchymatic tissue of the style, in all the species studied (Fig. 3D–I).

3.2. Stigma receptivity

There was a very strong positive reaction from anthesis (8 am) until four hours later, after which a decline in receptivity was observed, reaching a minimum at a different time according to the species (Table 2). *V. unilateralis* was the only species studied that presented a very strong positive reaction at midnight, which lasted until dawn. However this is related to the floral opening, with *V. unilateralis* presenting a nocturnal, around 9 pm, while in the others it occurs in the early morning hours. This species is placed in section Xiphion that is characterized by bat-pollination (Benzing, 2000).

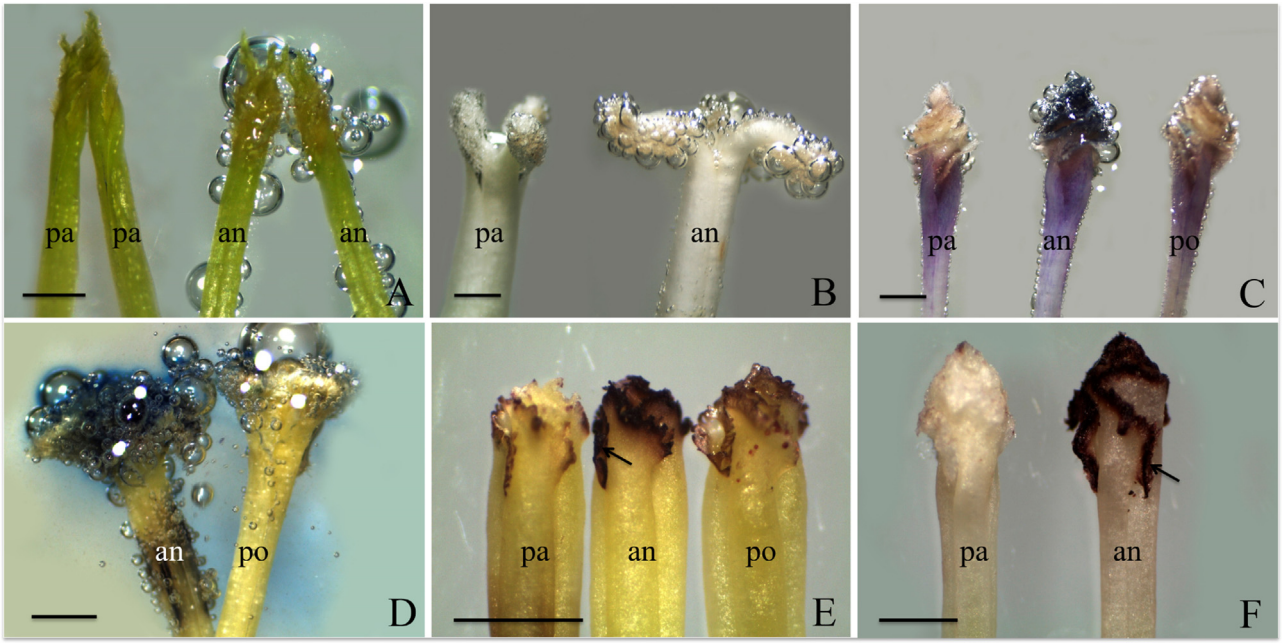

Fig. 4. Evaluation of stigma receptivity in Bromeliaceae of the genera *Aechmea*, *Ananas*, *Alcantarea*, *Pseudananas* and *Vriesea* by different methods. (A–B) Hydrogen peroxide solution: *A. nudicaulis* (A) and *A. nahoumii* (B); (C–D) Benzidine solution and hydrogen peroxide: *A. fasciata* (C) and *V. michaelii* (D); (E–F) α-naphthyl acetate solution: *A. bromeliifolia* (E) and *Ananas* sp., arrows indicate the receptive stigmatic area. pa = pre-anthesis, an = anthesis (8 h), po = post-anthesis. Bars: 1 mm.

Prior to floral opening, pre-anthesis, no reactions were observed in the stigma of any of the species, by any of the methods used for stigma receptivity analysis.

Dafni and Maués (1998), studying the stigma receptivity using four methods in 14 species from different families, observed distinct responses between species, with some methods not revealing a reaction for certain species.

In our study, the use of hydrogen peroxide identified a very strong positive response until post-anthesis for the majority of species. This method is considered inexpensive and is able to identify the reaction of the enzyme peroxidase present in the stigma by the air bubbles released during the reaction (Fig. 4A–B). However, the existence of tissue damage can lead to a false positive result. Dafni and Maués (1998) reported that the use of hydrogen peroxide produces results that are not very reliable because the substance can react differently depending on the plant age, besides not identifying the receptive area precisely. These authors recommend not using this method as the only indicator, and instead urge combining it with other techniques.

The association of benzidine with hydrogen peroxide permitted the identification of air bubbles, but also a range of color intensity, from light to dark blue, with a darker color indicating greater stigma receptivity (Fig. 4C–D). Benzidine becomes oxidized when it reacts with peroxidase, changing the color the tissue (Galen and Plowright, 1987). *A. bromeliifolia*, *A. nudicaulis*, and *V. carinata* presented a reduced reaction by this method at 6 pm, a similar result to the reaction with α-naphthyl acetate and fast blue B salt.

The reaction of α-naphthyl acetate associated with fast blue B salt revealed a dark brown coloration, mainly in the region of the stigmatic papillae (Fig. 4E–F), identifying high esterase enzyme activity, which can be related to the presence of the exudates that normally occur in the stigmatic papillae. This method produced results that identified more precisely the stigma receptive region. Kulloli et al. (2010), evaluating the esterase activity of three species of *Impatiens*, observed activity mainly in the stigmatic lobes and stigma tip, similar to our findings for the species of Bromeliaceae studied. The very strong positive response coincided with anthesis and up to 10 h after anthesis, both in all diurnal species and *V. unilateralis*, which presents nocturnal anthesis.

There was a reduction in the reaction as time progressed for all the species, indicating a decline in stigma receptivity. Ten hours after anthesis, species such as *A. bicolor*, *A. fasciata*, *V. carinata* and *V. michaelii*, presented a weak or no reaction. However, even after anthesis the responses were strong and very strong, respectively, for *V. simplex* and *A. nahoumii*. Some Tillandsioideae species may have a mixed way to get more visits of potential pollinators, with flowers remaining opened from afternoon up to the next day, so this may be the case for *A. nahoumii* that may be moth/bat pollinated (Benzing, 2000).

The stigma receptivity is also possibly related to the flower duration, since in these two species the flowers remain open for approximately 36 h, while in the other species studied the flowers stay open for approximately 24 h after anthesis. Siqueira Filho and Machado (2001), studying the reproductive biology of *Canistrum aurantiacum* E. Morren, and Canela and Sazima (2003), investigating *Aechmea pectinata* Baker, both reported that the stigma remained receptive from anthesis until closing of the flower, evaluated by hydrogen peroxide.

Parton et al. (2001), studying reproductive barriers in nine Bromeliaceae species of four genera, also observed greater stigma receptivity at anthesis. They reported that in one species of the genus *Vriesea*, the stigma was receptive until two days after anthesis and a sufficient number of pollen grains germinated, but the percentage of ovules fertilized decreased from 90% to 10% when the pollination occurred after anthesis. In another species of *Vriesea*, the authors observed sufficiently high pollen germination percentage and fertilization index from one day before until one day after anthesis.

4. Conclusion

All the species have a trifid stigma, with unistratified epidermis, numerous idioblasts containing raphides in parenchyma tissue, and an individual vascular bundle for each carpel. Two stigma

types were observed: conduplicate-spiral for species of the genera *Aechmea*, *Ananas*, *Pseudananas* and *Alcantarea* and convolute-blade for species of the genus *Vriesea*.

The greatest stigma receptivity coincided with anthesis in all the species studied, with the greatest receptivity occurring from 8 am until noon, except for *V. unilateralis*, a species with nocturnal anthesis, in which the most receptive period lasted from midnight to dawn.

Of the three methods tested, the use of α-naphthyl acetate solution associated with fast blue B presented more precise results, by allowing the identification of the receptive area of the stigma rather than the dispersed air bubbles observed with the other two methods, hydrogen peroxide and benzidine + hydrogen peroxide.

In this study detailed stigma/style micromorphology and stigma receptivity period of eighteen Bromeliaceae species belonging to five genera were characterized, providing support for controlled pollination, genetic improvement programs involving these species, as well as the taxonomy of this family.

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