



## Interspecific and intergeneric hybridization in Bromeliaceae and their relationships to breeding systems



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### ABSTRACT

Plant hybridization allows for the creation of new genotypes playing a strategic role in the development of new cultivars of interest for the horticultural trade, and is a central phenomenon in plant speciation processes. We worked with a plant family known as a case of Neotropical adaptive radiation, characterizing the reproductive systems selected species. This study aimed to identify the reproductive systems of sixteen bromeliad species, and to evaluate potential reproductive barriers in interspecific and intergeneric crosses. A total of 931 interspecific and intergeneric hybridization crosses were conducted in 80 different combinations involving 16 species belonging to five genera *Aechmea*, *Ananas*, *Pseudananas*, *Alcantarea*, and *Vriesea*. Reproductive systems were determined by controlled pollinations, and autogamy or self-incompatibility indexes. Fluorescence microscopy was used to evaluate pollen tube growth in the pistil. Seed germination was evaluated for each treatment. Over half of the species showed evidences of self-incompatibility: *Aechmea bicolor*, *Ae. fasciata*, *Ae. nudicaulis*, *Ananas ananassoides*, *An. bracteatus*, *An. lucidus*, *An. paraguayensis*, *Vriesea carinata*, and *V. parvibica*. Among the self-compatible species, *Ae. bromeliifolia*, *Ae. distichantha*, and *Pseudananas saganarius* presented autogamy. *Alcantarea nahoumii*, *V. friburgensis*, *V. michaelii*, and *V. simplex* exhibited no autogamy thus requiring pollinators. Agamospermy was observed in *Ae. bromeliifolia*, and *Ae. distichantha*. Crosses were successful in 43% of the attempts, corresponding to 32% of the species of the *Ananas* genus. Among the intergeneric crosses, only *V. michaelii* x *Al. nahoumii* and *V. simplex* x *Al. nahoumii* succeeded. Possible causes of self-incompatibility and incongruity in these crosses are related to different impediments to the pollen tube growth, such as an irregular deposition of callose on pollen tube; curling and arrest of pollen tube growth in the style, among others. It is suggested that the self-incompatibility is gametophytic, avoiding self-fertilization.

### 1. Introduction

Bromeliaceae (58 genera, ca. 3909 species, Butcher and Gouda, 2017) is a Neotropical plant family that underwent rapid radiation, presenting different reproductive systems (Hietz et al., 2006; Vosgueritchian and Buzato, 2006; Ramírez-Morillo et al., 2009; Matallana et al., 2010; Wolowski et al., 2013). For example, in a study of 40 species of 11 genera and two subfamilies, Matallana et al. (2010) found self-incompatibility in 40% of the Bromelioideae species, and in one of the Tillandsioideae species (6%). These authors also performed an extensive review of the literature and summarized that 26% (10/39)

of the species of the subfamilies Bromelioideae (6/8), Tillandsioideae (3/22), and Pitcarnioideae (1/9) present self-incompatibility. Some authors mention gametophytic incompatibility (Brewbaker and Gorrez, 1967; Vervaeke et al., 2001; Coppens D'Eeckenbrugge et al., 2011; Wolowski et al., 2013) due to the inhibition observed in pollen tube growth through the style. Many species have allogamy as a preferential reproductive system, and are dependent on pollinators for pollination (Siqueira Filho and Machado, 2001; Cascante-Marín et al., 2005; Lenzi et al., 2006; Matallana et al., 2010). Self-incompatibility has been described as an important breeding mechanism to avoid the inbreeding depression and to promote heterozygosity or genetic diversity, thus

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Fig. 1. Flowering plants of the species: *Aechmea bicolor*, *Ae. bromeliifolia*, *Ae. distichantha*, *Ae. fasciata*, *Ae. nudicaulis*, *Ananas ananassoides*, *An. bracteatus*, *An. lucidus*, *An. paraguayensis*, *Pseudananas saganarius*, *Alcantarea nahoumii*, *Vriesea carinata*, *V. friburgensis*, *Vriesea michaelii*, *V. paraibica* and *V. simplex*.

allowing survival or adaptation of a species through time (Charlesworth, 2006). However, maintenance of selfing may explain species cohesion and isolation from sympatric congeners in high alpha-diversity environments, as previously demonstrated for sympatric bromeliads growing on rocky outcrops (Wendt et al., 2002) or inside tropical forests (Matallana et al., 2010).

From the horticultural point of view, knowledge of reproduction systems is fundamental to perform potentially beneficial hybridizations between members of the Bromeliaceae family, thus generating new genotypes having new combinations of shapes and colors (Vervaeke et al., 2001, 2002, 2003a,b, 2004), exercising a strategic role in the

development of new ornamental plant cultivars (Kuligowska et al., 2016). The search for new species of ornamental plants has been growing in recent years, especially those having originality, durability and beauty. The importance of bromeliads as ornamental plants has been increasing for many decades and currently these species are considered among the most valuable ornamental plants (Negrelle et al., 2012). The generation of new hybrids for ornamental plant trade can also reduce extractive activity and its negative impacts on natural populations of bromeliads, as has been noted in recent years.

Hybridizations in bromeliads have been described since 1879, with the first hybridization between species of the genus *Vriesea* (Samyn,

**Table 1**  
Fruit set and seed production in Bromeliaceae of genera *Aechmea*, *Ananas*, *Alcantarea*, *Pseudananas*, and *Vriesea*, after different pollination treatments.

Taxon	Fruits produced <sup>a</sup>			Seeds produced <sup>b</sup>			Indexes		Reproductive system		
	Spontaneous pollination	Manual self-pollination	Cross-pollination	Agamospermy	Spontaneous pollination	Manual self-pollination	Cross-pollination	Agamospermy		AI	SII
<i>Aechmea bicolor</i>	0 (0/162)	0 (0/96)	95.34(82/86)	0 (0/25)	0	0	133.33 (25)	0	0.00	0.00	SI
<i>Ae. bromeliifolia</i>	100 (100/100)	100 (100/100)	100 (75/75)	16.66(5/30)	18.23 (25)	20.12 (25)	21.15 (25)	6 (5)	1.00	1.00	SC(A)
<i>Ae. distichantha</i>	21.95 (18/82)	33.33 (32/96)	70.83 (51/72)	8 (2/25)	28.73 (10)	46.54 (10)	74.12 (25)	8 (2)	0.31	0.47	SC(A)
<i>Ae. fasciata</i>	0 (0/75)	5.62 (5/89)	59.55 (53/89)	0 (0/25)	0	47.68 (5)	173.22 (25)	0	0.00	0.09	SI
<i>Ae. nudicaulis</i>	0 (0/145)	0 (0/105)	96.66 (87/93)	0 (0/25)	0	0	145.15 (25)	0	0.00	0.00	SI
<i>Ananas ananassoides</i>	0 (0/46)	9.52 (4/42)	93.33 (42/45)	0 (0/33)	0	1.10 (4)	8.23 (42)	0	0.00	0.10	SI
<i>An. bracteatus</i>	0 (0/57)	2.90 (2/69)	100 (62/62)	0 (0/62)	0	1.50 (2)	11.24 (62)	0	0.00	0.03	SI
<i>An. lucidus</i>	0 (0/31)	2.63 (1/38)	100 (38/38)	0 (0/29)	0	1.00 (1)	6.21 (38)	0	0.00	0.03	SI
<i>An. parguazensis</i>	0 (0/23)	9.52 (2/21)	69.56 (16/23)	0 (0/21)	0	1.00 (2)	9.43 (16)	0	0.00	0.14	SI
<i>Pseudananas sagenarius</i>	100 (23/23)	100 (28/28)	100 (45/45)	0 (0/45)	6.74 (23)	8.23 (28)	9.18 (42)	0	1.00	1.00	SC(A)
<i>Alcantarea nahoumii</i>	0 (0/22)	53.33 (8/15)	87.50 (14/16)	0 (0/12)	0	228.76 (8)	328.18 (10)	0	0.00	0.61	SC
<i>Vriesea carinata</i>	0 (0/15)	6.66 (1/15)	81.25 (13/15)	0 (0/8)	0	106.23 (1)	302.48 (10)	0	0.00	0.08	SI
<i>V. friburgensis</i>	13.04 (3/23)	72.72 (16/22)	88.46 (23/26)	0 (0/15)	43.46 (3)	223.72 (10)	318.42 (10)	0	0.15	0.82	SC
<i>V. michaelii</i>	0 (0/12)	86.66 (13/15)	100 (15/15)	0 (0/8)	53.12 (1)	228.34 (10)	285.13 (10)	0	0.00	0.87	SC
<i>V. parabiica</i>	0 (0/12)	13.33 (2/15)	57.14 (8/14)	0 (0/5)	0	103.41 (2)	387.18 (10)	0	0.00	0.23	SI
<i>V. simplex</i>	14.28 (4/21)	38.88 (7/18)	60.00 (18/30)	0 (0/11)	104.18 (4)	204.76 (10)	672.18 (10)	0	0.23	0.65	SC

<sup>a</sup> Percentage of fruits set (fruits/pollinated flowers).

<sup>b</sup> Average seeds obtained per flower. Number of fruits produced/number of flowers used are shown in brackets. The reproductive systems are determined by two indexes (fruit production): autogamy index (SI = spontaneous/cross-pollination) and self-incompatibility index (SII = manual selfing/cross-pollination). SII is defined as self-incompatible when SII is lower than 0.30, and self-compatible or partially self compatible (SC) when SII is greater than 0.30. The letter (A) in brackets indicates an autogamy index greater than 0.30.

1995). Other reports of interspecific and intergeneric hybridizations were presented by Chevalier (1951), Richter (1976), Smith and Downs (1974), Smith (1983), Anderson (1986), Dutrie (1989a,b), Wendt et al. (2000, 2001, 2002), Vervaeke et al. (2003a), Rôças et al. (2004), Vervaeke et al. (2004), Barbará et al. (2009), Gonçalves and Azevêdo-Gonçalves (2009), Souza et al. (2009), Souza et al. (2014), Matallana et al. (2016), Zanella et al. (2016) involving species of the genera *Aechmea*, *Alcantarea*, *Ananas*, *Bilbergia*, *Canistrum*, *Cryptanthus*, *Guzmania*, *Neoregelia*, *Nidularium*, *Orthophytum*, *Pitcairnia*, *Portea*, *Quesnelia*, *Tillandsia*, and *Vriesea*. However, despite the existing records, only a small percentage among the many possible combinations in Bromeliaceae has already been tested, and among them, not all have shown success due to reproductive barriers.

Studies on possible barriers in Bromeliaceae and ways to overcome them were described by Van Tuyl and De Jeu (1997), Vervaeke et al. (2001, 2002) and Matallana et al. (2016) who observed pre and post-zygotic barriers as some abnormalities mainly in the basal region of the style; difference between style and pollen tube length; morphology and size of the pollen grain; lack of pollen grain germination on the stigma due to insufficient hydration or nutrients (especially calcium and boron); absence of chemotropic factors at the micropyle region; early degeneration of the embryo and endosperm, among other factors related to pollination, such as the stigma receptivity time and pollinators activities. Several techniques were described by Van Tuyl and De Jeu (1997), and Vervaeke et al. (2002), to overcome reproductive barriers such as cut style pollination, placental grafted style pollination, and placental pollination. Thus, it is important to know the exact moment and type of fertilization barrier, and how to overcome these barriers if one aims at the production of new cultivars.

This study aimed to identify the reproductive systems of sixteen species of bromeliads, as well as to assess the possible reproductive barriers from interspecific and intergeneric hybridizations by means of observation and evaluation of pollen grain germination and pollen tube growth in the pistil. We expect that this approach will affect both the understanding of new cultivars' production, as well as the increase in reproductive data to better understand bromeliads radiation in the neotropics.

## 2. Materials and methods

### 2.1. Plant materials and growth conditions

Sixteen Bromeliaceae taxa (nomenclature follows Luther, 2012) were studied. Specimen of each taxon are deposited in the ESA herbarium (Escola Superior de Agricultura "Luiz de Queiroz", Universidade de São Paulo): *Aechmea bicolor* L.B. Sm. (voucher ESA 120990), *Ae. bromeliifolia* (Rudge) Baker ex Benth. & Hook.f. (ESA 121275), *Ae. distichantha* Lem. (ESA 121281), *Ae. fasciata* Baker (ESA 120987), *Ae. nudicaulis* Griseb. (ESA 120991), *Ananas ananassoides* (Baker) L.B.Sm. (ESA 121274), *An. bracteatus* (Lindl.) Schult.f. (ESA 121284), *An. lucidus* Miller (ESA 121285), *An. parguazensis* Camargo & L. B. Sm. (ESA 121405), *Pseudananas sagenarius* (Arruda da Camara) Camargo (ESA 121286), *Alcantarea nahoumii* (Leme) J.R. Grant (ESA 120986), *Vriesea carinata* Wawra (ESA 121404), *V. friburgensis* Mez (ESA 121282), *Vriesea michaelii* W. Weber (ESA 121280), *V. parabiica* Wawra (ESA 121276) and *V. simplex* (Vell.) Beer (ESA 120989) (Fig. 1). The plants were grown in a greenhouse in plastic pots (13-cm-high and 10 cm in diameter) with Basaplant® substrate, at ambient temperature and relative humidity of 70%, in Piracicaba, SP, Brazil. Flowering was induced with 30 ml of Ethrel® spray (ethephon) per plant, at 240 mg mL<sup>-1</sup>, inside the rosette.

### 2.2. Reproductive system

Controlled pollinations were performed to determine the reproductive system: spontaneous pollination (bagged flowers without emascul-

lating); manual selfing (flowers manually pollinated with its own pollen grain); cross-pollination (previously emasculated flowers pollinated with pollen from another individual of the same species), and agamospermy (emasculatation of flowers and no pollination). Souza et al. (2016) and Souza (2013) previously reported the time of flower opening – when the greater stigma receptivity and viability of pollen grains are detected – for all species in this study, indicating that flower opening occurs around 8 a.m., thus the experiments started at this time. The controlled pollinations were always conducted at 8:00 a.m. (flowering) for each treatment, and used different quantities of flowers, as shown in Table 1. The flowers were marked according to each treatment.

The autogamy index was calculated for each species, dividing the percentage of fruit set in spontaneous pollinated flowers by fruit set in cross-pollinated flowers. Species were considered autogamous or partially autogamous when the autogamy index was greater than 0.30, and a non-autogamous species had autogamy index lower than 0.30 (Ramírez and Brito, 1990).

The self-incompatibility index (SII) was calculated by dividing the percentage of fruits formed in flowers self-pollinated manually by the fruit set percentage in cross-pollinated flowers. Compatible and partially compatible species presented a SII greater than 0.30, while self-incompatible species presented a SII lower than 0.30 (Ramírez and Brito, 1990).

2.3. Interspecific and intergeneric crosses

A total of 931 interspecific and intergeneric hybridization crosses were conducted in 80 different combinations involving 16 species belonging to five genera *Aechmea*, *Ananas* and *Pseudananas* (Bromelioideae subfamily), *Alcantarea*, and *Vriesea* (Tillandsioideae subfamily) (Table 2). In pollen recipient plants, anthers were removed

and flowers were bagged at pre-anthesis, avoiding contamination by selfing, and from pollen of other plants. Pollen grains from the donor species were deposited on the stigma of the emasculated flower. After this process, the flowers were tagged, and again protected with cloth bags to avoid contamination. The number of fruits and seeds per fruit produced at every treatment was evaluated.

2.4. Development of the pollen tube

Ten pistils (replicates) from flowers of plants of each treatment (manual self-pollination, outcrossing between individuals of the same species, and hybridizations involving different parents) were collected 48 h after the pollination, fixed in a solution of 3:1 ethanol (95%): glacial acetic acid, for 48 h, clarified in sodium sulfite solution (10%), and autoclaved for 20 min at 120 °C, in order to soften the tissues. Staining was done overnight with aniline blue solution (0.01%) in tribasic phosphate buffer. Fluorescence microscopy with an ultraviolet filter was used to analyze the germination of pollen grains on the stigma, and development of pollen tubes through the style (Martin, 1959; Yi et al., 2006). The samples were analyzed under a light microscope (Axioskop2, Carl Zeiss, Jena, Germany).

Pre-determined regions (see Bandeira et al., 2011) were analyzed to evaluate pollen tube growth in the pistil: a) pollen tube germination on the stigma, without growth in the style; b) pollen tube growth up to the first third of the style; c) pollen tube growth up to the second third of the style; d) pollen tube up to the third of the style; e) pollen tube inside the ovary; f) pollen tube near the ovule; g) pollen tube penetrating the micropyle.

Table 2

Interspecific and intergeneric hybridization between Bromeliaceae species of genera *Aechmea*, *Alcantarea*, *Ananas*, *Pseudananas*, and *Vriesea*: number of fruits per total number of crosses from each combination (number of fruits/crosses).

	<i>Ae. bicolor</i>	<i>Ae. bromeliifolia</i>	<i>Ae. distichantha</i>	<i>Ae. fasciata</i>	<i>Ae. nudicaulis</i>	<i>P. sagenarius</i>	<i>An. ananassoides</i>	<i>An. bracteatus</i>	<i>An. lucidus</i>	<i>An. parguazensis</i>	<i>Al. nahoumii</i>	<i>V. carinata</i>	<i>V. friburgensis</i>	<i>V. michaelii</i>	<i>V. paraibica</i>	<i>V. simplex</i>
<i>Aechmea bicolor</i> (SI)	* 0/8	0/4	2/8	2/65	-	0/5	-	-	-	0/4	0/8	-	0/8	-	0/2	
<i>Ae. bromeliifolia</i> (SC)A	-	*	-	-	-	-	-	-	-	0/4	0/2	-	0/5	-	-	
<i>Ae. distichantha</i> (SC)A	0/16	-	*	-	0/4	0/12	0/8	-	-	0/5	-	0/7	0/5	-	-	
<i>Ae. fasciata</i> (SI)	-	0/28	0/23	*	0/4	-	-	-	-	0/12	0/14	0/23	-	0/7		
<i>Ae. nudicaulis</i> (SI)	14/20	-	-	7/18	*	-	-	-	-	0/5	-	0/6	0/3	0/2	0/10	
<i>Pseudananas sagenarius</i> (SI)	-	-	0/3	-	-	*	-	-	-	0/5	-	0/3	-	0/2		
<i>Ananas ananassoides</i> (SI)	0/8	-	0/5	-	0/8	-	*	24/24	-	-	-	-	-	-	0/4	
<i>An. bracteatus</i> (SI)	-	-	-	-	-	-	99/99	*	52/52	-	-	-	-	-	-	
<i>An. lucidus</i> (SI)	-	-	-	-	-	-	35/35	27/27	*	-	-	-	-	-	-	
<i>An. parguazensis</i> (SI)	-	-	-	-	-	-	23/23	-	39/39	*	-	-	-	-	-	
<i>Alcantarea nahoumii</i> (SC)A	-	-	-	0/8	-	-	-	-	-	-	*	-	0/5	0/3	-	0/5
<i>Vriesea carinata</i> (SI)	0/10	-	-	0/5	0/4	-	-	-	-	-	-	*	0/3	-	15/15	-
<i>V. friburgensis</i> (SC) (SC)	0/5	-	0/4	0/8	-	0/4	0/6	-	-	-	0/11	-	*	16/16	-	14/16
<i>V. michaelii</i> (SC)	0/6	-	-	0/15	0/4	-	0/3	-	-	-	2/6	-	8/10	*	-	3/6
<i>V. paraibica</i> (SI)	-	-	-	-	-	-	-	-	-	-	-	14/14	-	-	*	-
<i>V. simplex</i> (SC)	0/8	-	-	0/7	0/8	-	0/5	-	-	-	3/7	-	4/6	2/6	-	*

Successful hybridizations are marked in black.

## 2.5. Seed germination

All seeds produced from hybridizations involving *Aechmea*, *Alcantarea*, and *Vriesea* were tested for germination *in vitro* in culture medium containing half the concentration of MS salts (Murashige and Skoog, 1962), according to Souza et al. (2012). Seeds from the *Ananas* crosses were sown in 96-cell-trays filled with Basaplant® substrate.

## 3. Results

### 3.1. Reproductive system

Almost half of the species (56%) showed self-incompatibility (SII < 0.30). Among these, *Ae. bicolor*, and *Ae. nudicaulis* produced no fruit or seeds (Table 1). The *Ananas* species, *An. ananassoides*, *An. bracteatus*, *An. lucidus*, and *An. paraguayensis* produced few seeds. By contrast, *P. sagenarius* showed 100% fruit formation and the number of seeds was higher than on the other *Ananas* species in all reproductive systems (Table 1).

Among the self-compatible species, *Ae. bromeliifolia*, *Ae. distichantha*, and *P. sagenarius* presented autogamy (spontaneous self-pollination) (AI > 0.30), which indicates that these species do not require pollinators for fertilization. On the other hand, *Al. nahoumii*, *V. friburgensis*, *V. michaelii*, and *V. simplex* require pollinators for fertilization, even for self-pollination.

Cross-pollination led to fruit set in 94.11% of the species, and to a higher number of seeds when compared to other reproductive systems evaluated (Table 1). Agamospermy was observed in only two species, *Ae. bromeliifolia* and *Ae. distichantha*, with a small percentage of fruit set as well as a low number of seeds formed (Table 1).

Different events in the development of the pollen tube in the pistil characterized the self-incompatibility in the different species studied (Fig. 2). All species showed good germination of pollen grains and early development of the pollen tube in the style. *Aechmea bicolor*, *Ae. fasciata*, *Ae. nudicaulis* and *Al. nahoumii* showed germination of pollen grains on the stigma, and subsequent curling of the pollen tube in the first third of the style, often growing backwards. In *V. paraibica* pollen tube curling occurred at the end of the second third of the style. For *An. ananassoides*, *An. bracteatus*, *An. lucidus* and *V. carinata*, it was observed that pollen grains germinated and the pollen tube lost orientation, and growth was arrested at the end of the first third of the style (Fig. 2).

In *V. friburgensis* and *V. simplex* we observed that the pollen tube growth was arrested, losing orientation at the end of the second third of the style in 70% of pistils analyzed, while 30% reached the ovary, but did not penetrate through the micropyle (Fig. 2). These results are consistent with studies of their reproductive system (Table 1), demonstrating that these species are self-incompatible but may produce a small number of seeds by self-pollination.

Irregular deposition of callose deposition on pollen tubes was observed in self-incompatible species (Fig. 2d) whereas normal deposition was observed in self-compatible taxa (Fig. 2c). In all varieties of *Ananas* a callose plug was observed at the distal end of the pollen tube.

### 3.2. Interspecific and intergeneric hybridization

Among 256 possible combinations of interspecific and intergeneric crosses, 80 were performed and 21 succeeded (2 intergeneric and 19 interspecific). The success obtained in hybridizations corresponds to 43.5% (405 out of 931), with 32.1% (299 out of 931) involving the *Ananas* species. Four interspecific hybridizations were recorded in the genus *Aechmea*: *Ae. bicolor* x *Ae. nudicaulis* with 3% success (2 out of 65); *Ae. nudicaulis* x *Ae. bicolor* with 70% success (14 out of 20); *Ae. bicolor* x *Ae. fasciata* with 25% success (2 out of 8), and *Ae. nudicaulis* x *Ae. fasciata* with 38.8% success (7 out of 18).

The other interspecific combinations within *Aechmea* genus were incongruous, with abnormalities in pollen tube growth confirmed by

fluorescence microscopy: *Ae. distichantha* x *Ae. bicolor*, *Ae. fasciata* x *Ae. bromeliifolia* and *Ae. fasciata* x *Ae. nudicaulis* showed pollen tube curling in the upper third of the style (Fig. 3a); *Ae. bicolor* x *Ae. bromeliifolia* and *Ae. bicolor* x *Ae. distichantha* showed germination of pollen grains followed by pollen tube growth arrest already in the stigma (Fig. 3b); *Ae. fasciata* x *Ae. distichantha* pollen tubes grew towards the ovary but did not penetrate the micropyle. Abnormalities detected were prezygotic barriers preventing hybridization between these pairs of species.

Among the nine combinations of *Vriesea* species, eight were congruent, producing large numbers of seeds in all hybridizations. Crosses between *V. carinata* and *V. paraibica* registered a 100% success rate in unilateral hybridizations, with an average of 298 seeds. Other unilateral hybridizations were recorded with *V. friburgensis* and *V. michaelii*, with a success above 80%, and an average of 277 seeds. *V. friburgensis* and *V. simplex* accomplished 66.6% to 87.5% success and in *V. michaelii* and *V. simplex* success was below 50%. The number of seeds produced was similar to results from cross-pollination.

There was 100% success of pollination in 299 flowers among the seven combinations of *Ananas*, with an average of 5.11 seeds per flower in *An. lucidus* x *An. bracteatus* and 18.63 seeds per flower in the reciprocal combination. Most of the seeds produced from hybridizations involving *Ananas* were fertile, with germination rate higher than 93%, in the greenhouse.

Among the intergeneric hybridizations, only two combinations were successful: *V. michaelii* x *Al. nahoumii*, and *V. simplex* x *Al. nahoumii*, with 33.3% (2 out of 6) and 42.8% (3 out of 7) success rate, producing an average of 168 and 418 seeds, respectively.

Abnormalities were observed in pollen tube development in intergeneric hybridizations such as irregular deposition of callose on pollen tube, pollen tube curling in the style, pollen tube growth arrest in specific regions of the style (Fig. 3d and e), and low germination of pollen grains in hybridizations involving *Aechmea* and *Vriesea* as pollen donors (Fig. 3f).

Seeds generated in interspecific and intergeneric hybridizations involving *Aechmea*, *Alcantarea* and *Vriesea* presented germination higher than 87%, with seedlings being transferred after 45 days to vials containing MS medium, until an ideal size (Fig. 3g) for later transplanting to substrate (Fig. 3j).

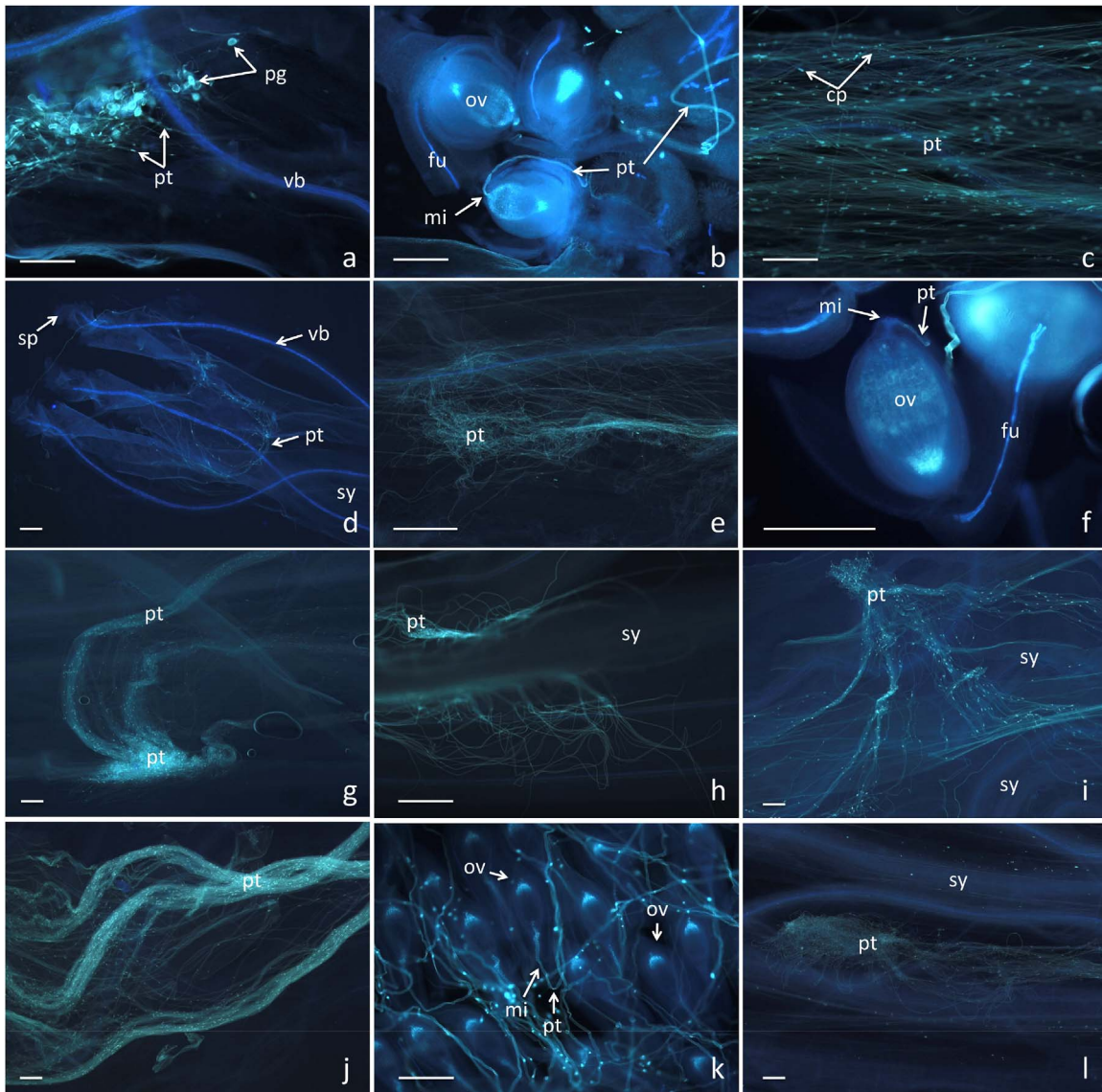
## 4. Discussion

### 4.1. Reproductive system

Different reproductive mechanisms were observed in different species studied. Coppens D'Eeckenbrugge et al. (2011) described the reproductive system in *Ananas* as a gametophytic pseudo self-incompatible type, with inhibition of pollen tube growth in the upper third of the style. These authors reported that the cultivars of *An. comosus* are strongly self-incompatible. However, other species of *Ananas* have reduced self-incompatibility, producing a few seeds.

The literature reports that in Bromeliaceae the prevailing reproductive system is self compatibility (Martinelli, 1994; Wendt et al., 2001, 2002; Matallana et al., 2010). According to Matallana et al. (2010), current research points to this trend, which is also contrary to the hypothesis that out-crossing prevails for herbaceous angiosperms (Bawa et al., 1985). The most abundant reproductive system in the species studied here was self-incompatibility (9; 56%) versus self-compatibility (7; 44%). However, the proximity between these two values, without a significant difference, allows us to conclude only that there was no clear preference for each of the systems. Differences reported among the reproductive systems of bromeliads may be explained by the relatively reduced number of species studied so far, approximately 2.5% of 3494 species.

The self-compatible species that do not spontaneously self-pollinate showed the stigma higher than the stamens, preventing the deposition



**Fig. 2.** Pollen tube growth in bromeliads of genera *Aechmea*, *Ananas*, *Alcantarea*, *Pseudananas* and *Vriesea* after manual self-pollination, observed by fluorescence microscopy in pistils treated with aniline blue. a) Pollen tube growth arrest at the beginning of the first third of the style in *Ae. bicolor*. b) Pollen tube penetration through the micropyle in *Ae. bromeliifolia*, indicating the occurrence of fertilization. c) Pollen tube bundles and a regular distribution of callose plugs in *Ae. distichantha*. d) Pollen tube growth and curling in the first third of the style in *Ae. nudicaulis*. e) Curling of pollen tubes at the end of the second third of the style in *V. friburgensis*. f) Penetration of pollen tube in the micropyle in *P. sagenarius*, indicating the occurrence of fertilization. g) Pollen tube arrest and curling at the end of the first third of the style, in *Al. nahoumii*. h) Pollen tube growth at the end of the first third of the style, in *V. carinata*. i) Pollen tubes growing on the style, and pollen tubes curling, in *V. friburgensis*. j) Bundles of pollen tubes in *V. michaelii*. k) Ovules (arrow head) and pollen tubes, with at least one event of a pollen tube penetrating in the micropyle (arrow), indicating the occurrence of fertilization, in *V. michaelii*. l) Curling of pollen tubes at the end of the first third of the style, in *V. simplex*. Abbreviations: cp = callose plug, fu = funiculus, mi = micropyle, nv = non-viable pollen grain, ov = ovule, pg = pollen grain, pt = pollen tube, sp = stigmatic papillae, sy = style, vb = vascular bundle. Bars = 200  $\mu$ m.

of its own pollen on the stigma. In species with spontaneous self-pollination, the anthers are positioned nearly at the same height as the stigma.

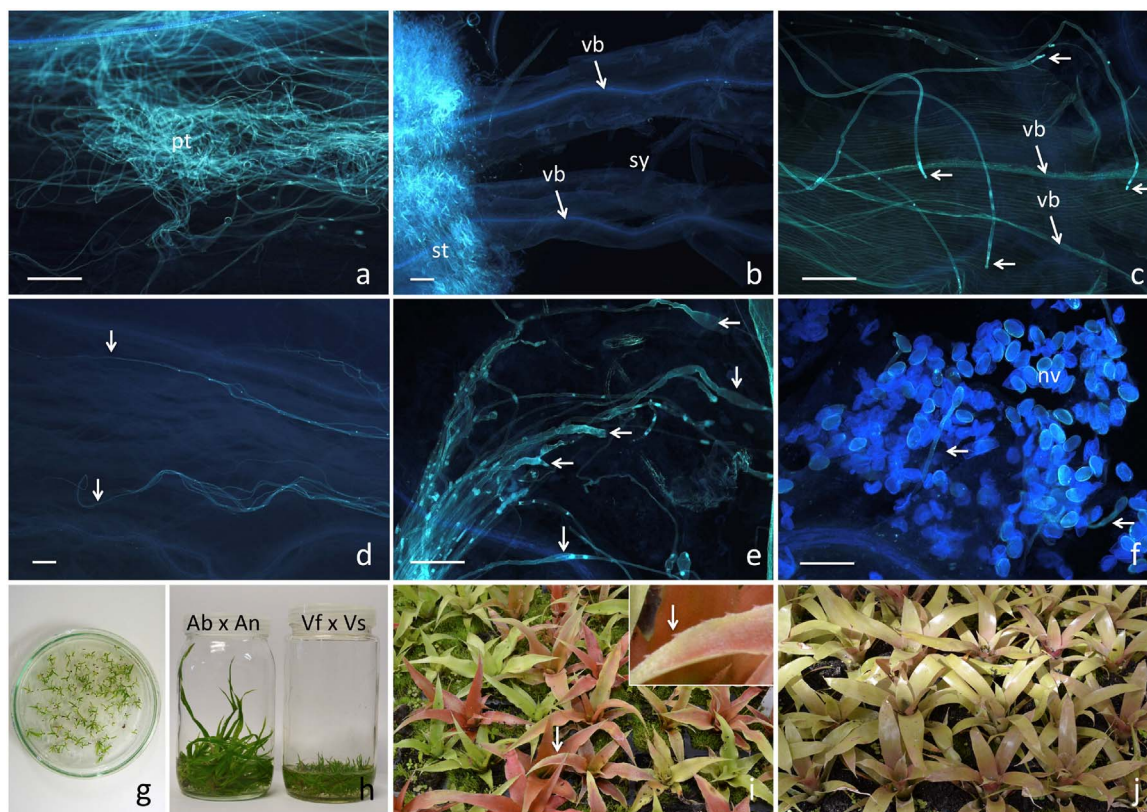
It is known that self-compatible species are derived from self-incompatible ancestors (Jarne and Charlesworth, 1993). A likely explanation for the evolution of self-compatibility is associated with a narrow distribution of their populations (Wendt et al., 2001). There are several studies supporting that narrowly distributed taxa tend to be self-compatible and have low genetic variability (Wyatt et al., 1992; Wendt et al., 2001).

Agamospermy was observed in only two species, with a small percentage of fruit set, and low number of seeds per fruit. The formation of fruits in this treatment indicates that asexual reproduction can occur in these taxa. In the literature, low fruit set and seeds were reported in agamospermy, as in *Pitcairnia* genus with an average of

17.95% of fruits produced (Wendt et al., 2001), and *Ae. distichantha* with 10% of fruits formed (Scrok and Varassin, 2011).

Although agamospermy has been reported in several species of Bromeliaceae (Wendt et al., 2001, 2002; Lenzi et al., 2006; Rogalski et al., 2009; Scrok and Varassin, 2011), the possibility of pollen grain contamination in the stigma surface before flower opening cannot be ruled out, due to the proximity of the anthers and stigma and the difficulty of emasculation, due to the reduced size of the flowers. Embryo development is needed to confirm agamospermy.

Different deposition pattern of callose have been described in interspecific and intergeneric crosses in Bromeliaceae (Vervaecke et al., 2001), but it is not known exactly if this is related to the inhibition of pollen tube growth. It is known that the deposition of callose influenced the maintenance of turgor pressure, mechanical tension, and maintenance of osmotic balance in the pollen tube and



**Fig. 3.** Interspecific and intergeneric hybridizations of Bromeliaceae taxa: pollen tube growth in the stigma observed by fluorescence microscopy of aniline blue stained pistils (a–f), *in vitro* plantlets (g–h), and greenhouse grown plants (i and j) from seeds produced from controlled crosses. a) *Ae. fasciata* x *Ae. nudicaulis*: pollen tubes curling in the upper third of the style. b) *Ae. bicolor* x *Ae. bromeliifolia*: stigma and style showing the germination of a large number of pollen grains on the stigma, where pollen tube growth is arrested (note absence of pollen tubes in the style). c) *V. carinata* x *V. friburgensis*: pollen tube growth arrest in the third of the style, with callose deposition at the tip of the pollen tube (arrows). d) *V. michaelii* x *Ae. fasciata*, indicating pollen tube growth arrest in the upper third of the style (arrows). e) *AL nahoumii* x *Ae. fasciata*: irregular deposition of callose blocking pollen tube growth (arrows). f) *Ae. fasciata* x *V. carinata*: very low percentage of germination of pollen grains on the stigma. g) Germination of seeds obtained from *Ae. bicolor* x *Ae. nudicaulis* crosses in 1/2 MS culture medium. h) *In vitro* growth of plantlets resulting from hybridization between *Ae. bicolor* x *Ae. nudicaulis* (Ab x An), and *V. friburgensis* x *V. simplex* (Vf x Vs) in MS medium. i) Progeny of *An. lucidus* x *An. bracteatus*, showing segregation of distinct phenotypes regarding color and presence of spines (arrow). j) Progeny of *V. carinata* x *V. paraibica* after 12 months of cultivation. Abbreviations: nv = non-viable pollen grains, pt = pollen tube, st = stigma, sy = style, vb = vascular bundle. Bars = 200  $\mu$ m.

aids in its penetration in the stigma. Moreover, according to Ünal et al. (2013) regular deposition of a callose plug during pollen tube growth can prevent the plasma to flow backwards from the tip of the pollen tube, and to avoid its shrinkage. Pollen tube curling, observed in this study for some species, can be a consequence of the lack of this deposition mechanism. Review on callose in plant reproduction can be found in Ünal et al. (2013).

#### 4.2. Interspecific and intergeneric hybridization

Interspecific and intergeneric hybridizations were obtained with a higher success rate on crossings involving *Ananas* species. A weak barrier to hybridization observed in *Ananas*, may explain the complex systematics of this genus, in which contrasting proposals to species recognition are available based on the lack of discontinuous morphological variation (e.g. Leal et al., 1998). Failure to produce hybrids may be the result of pre-zygotic barriers, which occur by the lack of ability of pollen grain to germinate on the stigma, or the inhibition of pollen tube growth in the style (Vervaeke et al., 2002). Post-zygotic barriers include embryo abortion in different stages of development, from the early embryo to maturity (Vervaeke et al., 2001).

Interspecific hybridization in *Vriesea* are common, and the first records date back to 1879 by Edouard Morren in Belgium, where various combinations were tested including species used in this study, such as *V. carinata* and *V. simplex* (Samyn, 1995; Matos et al., 2016). Zanella et al. (2016) reported natural hybrids between *Vriesea carinata* and *V. incurvata* based on morphological and molecular studies

conducted in southern Brazil. The only incongruity observed in interspecific hybridization involving *Vriesea* was between *V. carinata* x *V. friburgensis*. This cross led to pollen tube growth arrest at the beginning of the third of the style, with callose deposition in the pollen tube tip (Fig. 3c), leading to the incongruity. According to Ünal et al. (2013), callose has an important role in the pollen tube growth. The incompatibility of pollen tubes is also marked by swelling of pollen tube tips until they burst within the style. Another related aspect is the irregular distribution of callose deposition in the tube and the growth inhibition in the transmitting tissue in the style. All these events were also observed in the incongruent crosses studied in this work (Fig. 3a–f).

The use of different species of *Ananas* genus in hybridization processes has produced many ornamental pineapple hybrids including spineless and distinct colors (Souza et al., 2009a, 2014). Segregation characteristics have also been observed in several progenies obtained in hybridizations involving this genus (Fig. 2i).

Vervaeke et al. (2004), characterizing *Vriesea*, *Guzmania*, and *Tillandsia* hybrids, observed that the pollen grains were completely sterile, with a collapsed structure. Pollination using these hybrids as female parentals did not generate seeds either. In most cases, the hybrids are sterile or have some level of sterility, since the homologous chromosomes do not pair, preventing the correct meiotic division (Aparicio and Albaladejo, 2003; Larrosa et al., 2012).

Among the intergeneric hybridization, low success rate may be due to various factors such as the difference between the style length of both parentals which creates a physical barrier. Parton et al. (2001) and Vervaeke et al. (2001) also reported barriers in the style due to the

difference in length between *Aechmea* (short style), *Vriesea* and *Guzmania* (long style) species. Additionally, the viability of these intergeneric crosses may be explained by the phylogenetic proximity among the taxa involved. Brazilian *Vriesea* and *Alcantarea* are sister lineages (Versieux et al., 2012) and the latter was also treated as a subgenus of *Vriesea* during a long period (Versieux and Wanderley, 2015). The two combinations involving *Vriesea* and *Alcantarea* crosses showed unilateral incongruity, which may have been due to several factors. We highlight among them the difference between the length of the style of both parentals, which can cause the pre-zygotic mechanical isolation barrier in the style. In *Al. nahoumii* the length of the style ( $89.99 \pm 0.71$  mm) is nearly 3–4 times longer than in both *Vriesea* species ( $20.56 \pm 0.15$  mm for *V. michaelii*, and  $31.90 \pm 0.38$  mm in *V. simplex*). Interestingly, using aniline blue, it was possible to observe that in *Al. nahoumii* the growth of pollen tubes from *Vriesea* pollen was arrested at the beginning of the second third of the style (corresponding to the length of *Vriesea* species style length) which can prove the presence of this physical barrier. Different authors have report there must be compatibility between the length of the pistil and the growth of pollen tubes in order to achieve hybridization (Herrero and Hormaza, 1996; Lankinen and Skogsmyr, 2001; Williams and Rouse, 1990). Parton et al. (2001) reported that in intergeneric hybridizations, pollen tubes have more abnormalities than in interspecific hybridizations. This observation was not confirmed in our study, since similar abnormalities observed in self-incompatibility and interspecific hybridization assays were also found in intergeneric hybridizations.

Intergeneric hybridizations are reported for Bromeliaceae in the literature involving very closely related genera within a clade, such as *Guzmania*, *Tillandsia* and *Vriesea* (Tillandsioideae) (Smith and Downs, 1974; Vervaeke et al., 2004), and between *Aechmea*, *Neoregelia*, *Nidularium*, *Quesnelia* (Bromelioideae) (Smith and Downs, 1974). Smith (1983) reviewed books, catalogs, and private collections, and recorded the existence of 121 intergeneric hybrids from 23 different nothogenera, within the subfamilies Bromelioideae and Tillandsioideae. The same author reports that not even half of these are available on the market, with intergeneric hybridizations being not easily obtained due to the genetic distance between the parents. Matallana et al. (2016), after studying 13 species of different genera (*Aechmea*, *Billbergia*, *Quesnelia*, *Tillandsia* and *Vriesea*), found 28% success in crosses, including the ones using parents from different subfamilies (*Aechmea* and *Quesnelia* – Bromelioideae) and (*Tillandsia* and *Vriesea* – Tillandsioideae). Although fruit and seed setting were not verified for these intersubfamily crosses, normal pollen tube growth was observed and pollen tube penetration in the ovules was recorded for *Q. quesneliana* × *V. longicaulis*, *A. lamarchei* × *V. longicaulis*, *A. macrochlamys* × *V. longicaulis*, *A. macrochlamys* × *V. ruschii*, *T. geminiflora* × *Q. blanda*, *T. tenuifolia* × *A. lamarchei*, and *V. ruschii* × *Q. blanda*. Matallana et al. (2016) also indicated the need to conduct more studies, in particular to investigate post-zygotic barriers. Currently, intersubfamily hybrids are not available in the ornamental plant market.

Vervaeke et al. (2001, 2002), working with different species of bromeliads, observed abnormalities in the style causing pre- and post-zygotic barriers, especially in the basal region: unequal length of the style and pollen tube; morphology and size of pollen grains; absence of germination of the pollen grain on the stigma due to insufficient or uncontrolled hydration, as well as the lack of nutrients (especially calcium and boron); absence of chemotropic factors in the micropyle region, and other factors related to pollination, such as time of higher stigma receptivity, and presence of pollinators. The present work confirms several of these barriers among the distinct genera studied. However, genera such as *Aechmea* and *Vriesea*, with a rich profusion of speciation in eastern Brazil, and several complexes of species, are the ones with fewer impediments to interspecific hybridizations under our cultivation conditions.

Finally, seeds generated in interespecific and intergeneric hybridizations presented a high germination rates allowing the rescue of the

progeny. The characterization of the progeny is a following step for the identification of superior hybrids and understanding of segregation mechanisms.

## 5. Conclusion

Different reproductive mechanisms (self-incompatibility, self-compatible, autogamy and agamospermy) were observed in the different studied species. Intergeneric hybridizations were only obtained between the closely related genera *Alcantarea* and *Vriesea*, although *Vriesea* pollen tube growth was not enough to cover the long *Alcantarea* style.

Possible causes of self-incompatibility and incongruity between specific crosses are related to different events during pollen tube growth, such as irregular deposition of callose in pollen tube; curling of pollen tubes in the style; arrest of pollen tube growth in the style, among others. Among the species studied different reproductive systems were observed, such as autogamy, allogamy, and agamospermy. These data are critical in guiding hybridizations of ornamental interest. Since interspecific and intergeneric hybridization were obtained, there are many possibilities of creating new hybrids, which however, require additional morphological, cytogenetic, and molecular evaluations, as well as market analysis to demonstrate their ornamental potential.

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