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Floral and reproductive biology of *Alpinia purpurata* (Vieill.) K. Schum. (Zingiberaceae): An important tropical ornamental plant

Biología floral y reproductiva de *Alpinia purpurata* (Vieill.) K. Schum. (Zingiberaceae): Una importante planta ornamental tropical

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Alpinia purpurata cultivars.
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ABSTRACT

Alpinia spp. play an important ecological role as a source of nectar in the understory of tropical and subtropical forests, where many of these species are common. The perennial species *Alpinia purpurata* (Vieill.) K. Schum. is native to the tropical rainforests of Asia and has been used as an ornamental cut plant due to its lush inflorescences and post-harvest durability. The internal and external morphology, floral biology, and reproductive system of four *A. purpurata* cultivars were investigated in order to contribute to studies of genetic improvement of the species. The inflorescence of *A. purpurata* is terminal, globose, and has flowers protected by colorful and showy bracts. The white flowers are tubular bisexual. The floral anthesis of the four cultivars lasted 9 to 12 h and pollen viability was above 80% throughout the pre-flowering period in all cultivars evaluated. During anthesis, stigma and anther crest were receptive due to the presence of stigmatic fluid. Fruiting of the cultivars was observed after hand cross-pollination, whereas no fruiting was recorded in treatments involving spontaneous self-pollination, hand self-pollination or geitonogamy. Natural pollination induced low fruiting, suggesting low pollinator availability in the studied area. The obligatory xenogamy of *A. purpurata* is a facilitator for the development of new hybrids for the ornamental plant market.

Additional key words: breeding system; floral structure; stigmatic fluid; stigmatic receptivity.

RESUMEN

Alpinia spp. desempeñan un importante papel ecológico, como fuente de néctar, en el sotobosque de los bosques tropicales y subtropicales, donde muchas de estas especies son comunes. La especie perenne *Alpinia purpurata* (Vieill.) K. Schum. es nativa de las selvas tropicales de Asia y se ha utilizado como planta de corte ornamental debido a las exuberantes inflorescencias y la durabilidad posterior a la cosecha. Se investigó la morfología interna y externa, la biología floral y el sistema reproductivo de cuatro cultivares de *A. purpurata*, para apoyar los estudios de mejora genética de la especie. La inflorescencia de *A. purpurata* es terminal, globosa y tiene flores protegidas por brácteas coloridas y vistosas. Las flores blancas son tubulares bisexuales. La antesis floral de los cuatro cultivares duró entre 9 y 12 h. La viabilidad del polen fue superior al 80% durante todo el período de pre-floración en todos los cultivares evaluados. Durante la antesis, el estigma y la cresta de la antera fueron receptivos. La fructificación de los cultivares se observó después de la polinización cruzada manual. No se registró fructificación en los tratamientos de autopolinización espontánea, autopolinización manual y geitonogamia. La polinización natural indujo baja fructificación, lo que sugiere una baja disponibilidad de polinizadores en el área estudiada. La xenogamia obligatoria de *A. purpurata* es un facilitador para el desarrollo de nuevos híbridos para el mercado de plantas ornamentales.

Palabras clave adicionales: sistema de creación; estructura floral; fluido estigmático; receptividad estigmática.

INTRODUCTION

Zingiberaceae Martinov is the largest family of the order Zingiberales, consisting of 52 genera and about 1,600 species (WFO, 2019). The family has a pantropical distribution, with only one genus (*Renealmia* L.f.) distributed in the Neotropics and Africa, while four others (*Alpinia* Roxb., *Curcuma* L., *Hedychium* J. Koenig and *Zingiber* Mill.) were introduced into and naturalized in the Neotropics (Kress, 1990; Stevenson and Stevenson, 2004; Hamidou *et al*., 2012). In Brazil, the family is represented by eight genera, 32 species and one subspecies (BFG, 2015, JBRJ, 2020).

The floral morphology of the family invariably consists of bisexual and zygomorphic flowers with a normally tubular calyx formed by two sepals; a corolla with three petals and two fused petaloid staminodes forming an abaxial labellum, and a functional stamen with two thecae (Kirchoff, 1988; Endress, 1994). Additionally, more than 25 genera of the family have a crest at the anther apex, with wide variations in shape and size (Fan and Li, 2012; 2016).

The anther crest is a specialized anther appendage that extends up from the top of the anther to form a tail-like structure, with wide variation in shape and size between the different genera, and is widely distributed in the Zingiberaceae family. The function of the anther crest is to boost the male and female functions of plants, directing pollinators to adopt an ideal position for removing and receiving pollen (Fan and Li, 2016).

The style is thin, and the anther fillet is contained within a ventral groove between the thecae. The fusion of the lateral staminodes into a labellum, the presence of a pair of nectariferous glands at the style base, and cells containing essential or ethereal oils are autapomorphies of this family (Kress, 1990).

The peculiar floral morphology of the family Zingiberaceae may be related to a specialized floral system. Flexistyly is a sexual dimorphism consisting of two morphs that differ in the temporal expression of sexual function and that also involve reciprocal movement of the stigmatic surface through a vertical axis during the flowering period, which combines both reciprocal herkogamy and dichogamy, and promotes outcrossing (Li *et al.*, 2001; Cardoso *et al.*, 2018). This strategy has been documented for 24 species of Zingiberaceae, including those within the genus *Alpinia* (Kress *et al.*, 2001).

Alpinia is the largest genus of the family Zingiberaceae, with 346 widely distributed species in tropical Asia (Smith, 1990; Kress *et al.*, 2001). The following three species are frequently cultivated in Brazil: *A. purpurata* (Vieill.) K. Schum., *A. vittata* W. Bull, and *A. zerumbet* (Pers.) B.L. Burtt & R.M. Sm. (JBRJ, 2020). Studies based on molecular data have suggested that the genus is highly polyphyletic (Kress *et al.*, 2001), and most authors consider it to be taxonomically difficult and complex (Smith, 1990; Kress *et al.*, 2001).

Apart from the important ecological role in the understory of tropical and subtropical forests, *Alpinia* species can be used as medicinal plants (e.g., *A. officinarum* Hance), as sources of food (*A. galanda* (L.) Willd.) or for landscaping purposes (*A. zerumbet*) and mainly as cut flowers, as is the case for *A. purpurata* (Althaus-Ottmann *et al.*, 2011), which has varied color bracts, of great ornamental value for decorative purposes in gardens and landscapes, due to the durability and exuberance of its inflorescences with year-round flowering.

Reproductive biological studies are essential for the cultivation, conservation, and genetic development of plants (Baskorowati *et al.*, 2010). Knowledge of the reproductive barriers of *Alpinia* is of help for the development of research regarding this promising ornamental plant. The main goal of the present study was to examine the floral morphology and morphometry of four cultivars of *A. purpurata* and to analyze their reproductive system, with a particular focus on pollen and stigma viability.

MATERIALS AND METHODS

Study location

The active germplasm bank (BAG) of Universidade do Estado de Mato Grosso, in the municipality of Tangará da Serra, MT-Brazil (14º39 'S, 57º25' W; 321 m a.s.l.) was the site of study of four cultivars of *A. purpurata* (Jungle King, Kimi, Pink Ginger and Red Ginger) planted in August, 2015. The regional climate is tropical, with a dry season from May to September and a rainy season from October to April, and an average annual rainfall of 1,830 mm (Dallacort *et al.*, 2011). The soil was classified as Latossolo Vermelho Distroférrico, clayey, with a flat to slightly undulating relief (Embrapa, 2018).

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Floral morphology and morphometry

The morphological characteristics were evaluated and illustrated with drawings of flowers during the anthesis period showing the main details of the external morphological characteristics of *A. purpurata* flowers. Photomicrographs were taken with a Leica DMLB photomicroscope equipped with a Zeiss Axio Cam digital camera.

The floral morphometry of the four *A. purpurata* cultivars (Jungle King, Kimi, Red Ginger and Pink Ginger) was analyzed in fresh flowers in anthesis (N = 25). A pachymeter was used to measure flower length and width.

Statistical analyses were performed using the Sisvar® software (Ferreira, 2000). Means and standard deviation of the floral structures of the four cultivars of *A. purpurata* were compared by the Tukey test.

Floral biology

Floral anthesis, pollen viability and stigmatic receptivity were observed in 40 flowers of 10 plants per cultivar at flowering peak, from March to May 2016. During anthesis, the flowers were monitored from the beginning of opening until senescence, characterized by loss of glossiness and darkening of the flower.

Pollen viability was determined in previously bagged flowers in anthesis. The flowers were evaluated every two hours, from 07:00 AM to 5:00 PM. At each sampling, five slides per cultivar were mounted. The pollen grains were stained with acetic carmine and 200 units per slide were counted under an optical microscope (Kearns and Inouye, 1993). Grains with stained protoplasm were considered to be viable and those with a translucent and ruptured envelope were considered to be unviable.

The stigmatic receptivity of the same flowers used in the pollen viability tests was evaluated with two solutions: alpha naphthyl acetate, which in reaction with esterase darkens the stigma if receptive, and hydrogen peroxide (H_2O_2) which, in the presence of the enzyme peroxidase, indicates receptivity by forming air bubbles (Kearns and Inouye, 1993). In addition to the stigmatic region, the solutions were also used to test the receptivity of the anther crest located just above the stigma, since the presence of stigmatic fluid in this structure could enable it to receive pollen grains.

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Breeding system

Pollination treatments

Preliminary observations confirmed the receptivity of the anther crest of the four *A. purpurata* cultivars. Therefore, controlled pollination was carried out in three ways: (1) simultaneously on the stigma and anther crest, (2) only on the stigma and, (3) only on the anther crest.

The flowers of all cultivars, previously protected with organza bags, were hand-pollinated between 07:00 and 09:00 AM. After pollination, the inflorescences were bagged again and monitored until flower senescence or fruit formation.

The previously bagged inflorescences and floral pedicels of the four *A. purpurata* cultivars were pollinated as follows: (1) spontaneous self-pollination, bagging inflorescences with intact flowers; (2) hand self-pollination of previously bagged inflorescences by manual transfer of pollen to the stigma of the same flower; (3) geitonogamous pollination of previously bagged inflorescences by pollen transfer between flowers of the same plant; (4) cross-pollination of inflorescences by pollen transfer between flowers of different cultivars; (5) natural pollination (control) of inflorescences which were exposed to pollinators and monitoring of fruit formation.

The criteria for the selection of cultivars for cross-pollination were chosen in order to serve the ornamental plant market, such as variations in the color of the bract and productivity among cultivars.

The lower number of flowers of the Red Ginger and Pink Ginger cultivars was possibly due to the investment of these cultivars in above-ground plant parts (bulblets) in the bract axils.

Pollen tube growth

Pollen tube growth was analyzed in the ventral region of the anther crest. To this end, the flowers were collected one day (approximately 24 h) after manual and natural pollination and fixed in FAA for approximately 36 h. For slide preparation, the flowers (N=25 flowers per cultivar) were rinsed three times in distilled water and treated with sodium hydroxide (8N NaOH). The pistils were then mounted on slides and stained with 0.1% aniline blue solution at 0.1 mol/L K_3PO_3 (potassium phosphite). Pollen tube growth was observed under an Olympus BX 51 epifluorescence microscope.

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RESULTS AND DISCUSSION

The *A. purpurata* inflorescence is terminal, globose or spiciform, and the flowers are protected by colorful and conspicuous bracts (Fig. 1A, 2A). Five to seven flowers or vegetative propagules (bublets) emerge from each bract. The flowers are tubular, bisexual, zygomorphic, and white (Fig. 1B-C, Fig. 2 A-B), have a fused calyx; a tubular corolla with three petals fused with the curved distal lobes; one fertile stamen with a conspicuous anther grooved ventrally with an apex crest (Fig. 1D), and four external lateral staminodes, two on either side, forming a petaloid labellum (Fig. 1E). The stigma is exserted above the anther, flattened (claviform) and moist, with a ciliate margin (Fig. 1E). The upper part of the ovary has two epigynous nectaries surrounding the style base (Fig. 1F). The ovary is tricarpellary, trilocular and plurilovular. The fruit consists of a 3-valved dry or fleshy capsule, dehiscent from apex to base, containing numerous toothed seeds.

The morphometric calyx and corolla characteristics of the Red Ginger and Pink Ginger cultivars were similar, while the other cultivars differed significantly from each other in this regard (Tab. 1).

Table 1. Flower morphometry of four *Alpinia purpurata* cultivars.

Cultivars	**Jungle King**	**Kimi**	**Red Ginger**	**Pink Ginger**
Floral structure length (cm)	$\bar{X}$±SD	$\bar{X}$±SD	$\bar{X}$±SD	$\bar{X}$±SD
Calyx	2.38 ± 0.11 a	2.16 ± 0.14 b	1.65 ± 0.06 c	1.88 ± 0.08 c
Corolla	4.68 ± 0.15 a	4.30 ± 0.16 b	3.10 ± 0.17 c	3.48 ± 0.20 c
Lacinia of the corolla	1.42 ± 0.10 a	1.38 ± 0.09 a	1.00 ± 0.06 b	1.16 ± 0.04 b
Labellum	1.32 ± 0.05 a	1.22 ± 0.08 a	0.96 ± 0.02 b	1.04 ± 0.07 b
Stigma	4.12 ± 0.12 a	3.47± 0.16 b	2.53 ± 0.20 c	2.93 ± 0.20 b
Anther + crest	0.97± 0.02 a	0.88 ± 0.02 b	0.68 ± 0.03 c	0.75 ± 0.02 d
Ovary	0.60 ± 0.04 a	0.65 ± 0.06 a	0.57 ± 0.09 a	0.58 ± 0.02 a

Means followed by the same letter in a row do not differ from each other by the Tukey test at 5% probability. N = 25 flowers.

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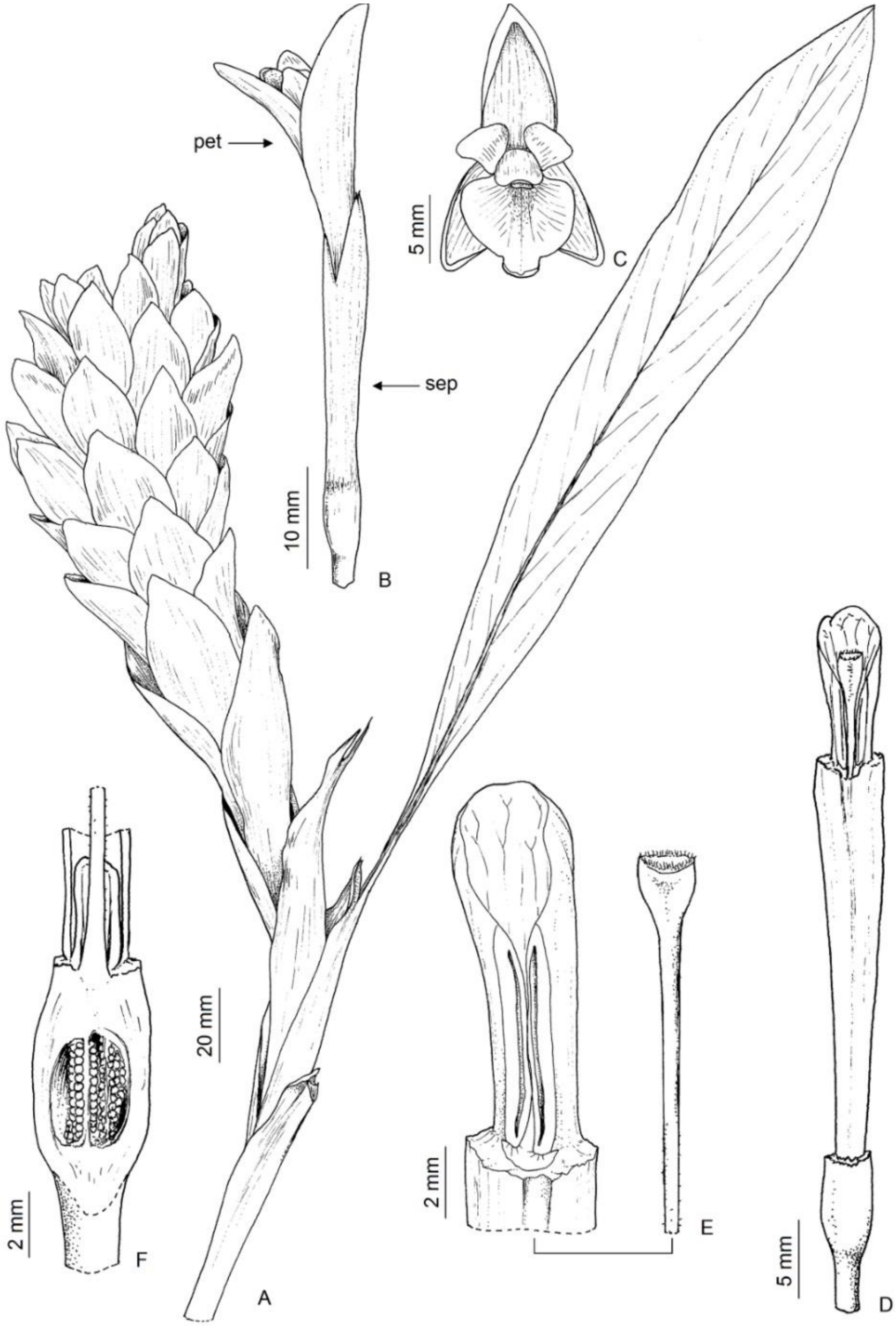

Figure 1. Terminal inflorescence of *A. purpurata*; A) Stem apex with inflorescence; B) Side view of the flower; C) Front view of the flower; D) Detail of the tubular corolla; E) Detail of stigma and anther; F) Inferior ovary with nectariferous glands at the fillet base. Illustration: Reinaldo Pinto.

Comparisons of the lengths of floral structures can be observed in Table 1. The length and labellum of the corolla lobes are proportional to the calyx length and corolla of the Jungle King and Kimi cultivars. The anther length and crest differed statistically between cultivars and were also related to corolla length. The ovary length did not differ significantly between cultivars.

The different flower sizes observed in the four *A. purpurata* cultivars may be the result of environmental differences such as temperature and relative humidity, as recorded for other *Alpinia* species (Takano *et al.*, 2013).

As observed for *A. purpurata*, there are many other plants in which all or some flowers of an inflorescence are converted into asexual bulbs: *Polygonum viviparum* L. (Polygonaceae) (Diggle, 1997), *Globba* spp. (Zingiberaceae) (Box and Rudall, 2006), *Calathea marantifolia* Standl. (Marantaceae) (Matlaga and Horvitz, 2009). According to Box and Rudall (2006), the production of bulblets allows the herbaceous plants to grow vigorously, as well as ensuring a fast establishment.

The anthesis period, characterized by the opening of the corolla lacinia of the four *A. purpurata* cultivars lasted 9 to 12 h. The anthesis of the Jungle King, Red Ginger, and Pink Ginger cultivars started at 06:00 AM and that of the Kimi cultivar at about 09:00 AM. Anthesis ended between 05:00 and 06:00 PM on the same day. At the beginning of anthesis, the stigma was positioned above the anther, which was dehiscent. Unfertilized flowers were not abscised during the evaluation period. The ovary development of the fertilized flowers began about four days after pollination. The bracts remained attached to the inflorescence throughout fruit maturation. Style movements such as those of the so-called "anaflexistylous" and "cataflexistylous" phenotypes were not observed.

In *A. purpurata* the stigmatic fluid was continuously secreted during flowering, forming a drop on the stigma, which could also contact and cover the anther crest (Fig. 2C-D). In *Roscoea debilis* (Zingiberaceae), the stigmatic fluid helps the germination of pollen grains and pollen tube elongation of the styles (Fan and Li, 2012).

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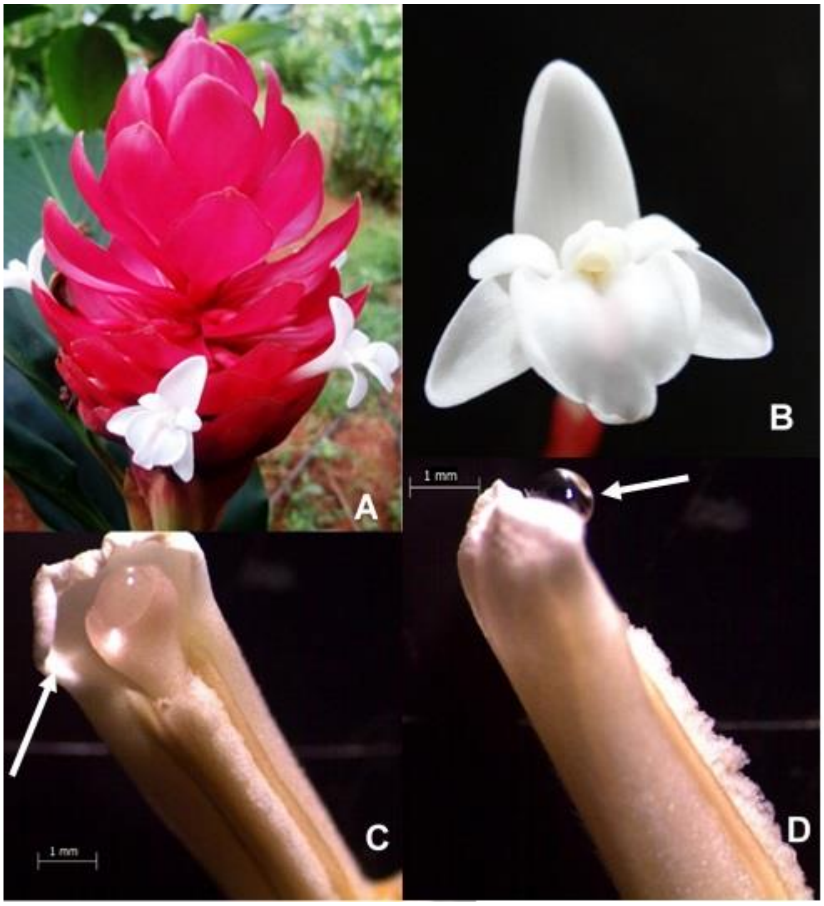

Figure 2. External morphology of *Alpinia purpurata*. A: Inflorescence with presence of flowers in the bracts; B: Front view of flower C: Anther crest (arrow) and dehiscent anthers; D: Side view of the anther crest with exudate release (arrow) at the stigma.

The receptivity of the stigma and anther crest was confirmed by the response to the alpha-naphthyl dye and to the H_2O_2 solution (Fig. 3A-B). Pollen viability of more than 86% was observed in all cultivars during the evaluation period. The viability peak was between 07:00 and 11:00 AM (Tab. 2). Although the *A. purpurata* stigma is receptive when viable pollen is released, the stigma position above the anther hampers autonomous selfing in this species. The floral herkogamy registered in *A. purpurata* prevents only pollination within the same flower, but not among plants of the same cultivar.

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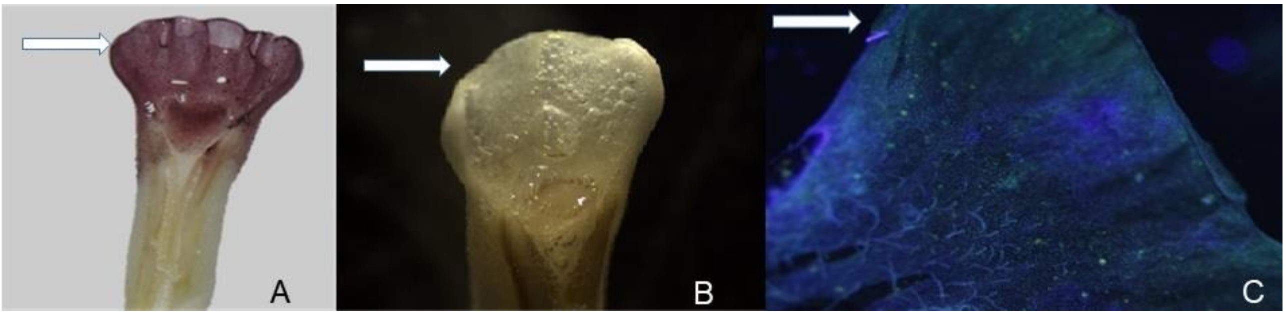

Figure 3. Receptor reaction in the anther crest and stigma of *Alpinia purpurata* (arrow). A: Receptivity using alpha naphthyl dye. B: Receptivity using hydrogen peroxide, C: Pollination in the anther crest underepifluorescence after 36 hours.

Table 2. Pollen viability (%) of four *Alpinia purpurata* cultivars.

Time of day/ Cultivars	**7:00 $\bar{X}$±SD**	**9:00 $\bar{X}$±SD**	**11:00 $\bar{X}$±SD**	**13:00 $\bar{X}$±SD**	**15:00 $\bar{X}$±SD**	**17:00 $\bar{X}$±SD**
Jungle King	94.5±2.6	94.1±3.8	94.5±2.1	93.8±2.8	89.7±3.5	87.9±6.8
Kimi	_	95.1 ±1.7	94.9±1.4	95±1.4	89.3±6.5	87.8±6.5
Red Ginger	94.9±3.6	95.3±1.3	94.9±2.9	90±4.1	88.3±4.7	86.6±6.1
Pink Ginger	93.6 ±2.1	93.4 ±3.2	93.8 ±2.0	93.1±2.8	89.9±4.0	88.1 ±4.8

N=25 flowers.

The fruiting percentage in cross-pollination treatments ranged from 60 to 83% between cultivars (Tab. 3). The number of seeds/fruit resulting from manual cross-pollination was 22% lower for the Kimi cultivar than for the other cultivars (Tab. 3).

No full pollen tube growth in the anther crest tissue was recorded in any of the hand pollination treatments (Fig. 3C), and no fruiting was recorded after manual pollen transfer in the anther crest region, indicating that this structure must act only in the reception of pollen grains, which then germinate and emit the pollen tubes that grow to the stigma through the stigmatic fluid. Simultaneous pollen transfer to the stigma and anther crest regions in the manual cross-pollination treatments caused a 45 and 52% increase of seeds/fruit in the cross for the Kimi and Jungle King cultivars, respectively, compared to manual cross-pollination performed on the stigma only (Tab. 3).

The treatments involving spontaneous self-pollination, hand self-pollination and geitonogamy did not induce fruiting, indicating obligate xenogamy. Therefore *A. purpurata* is an allogamous and self-incompatible species (Tab. 3).

The mating system of *A. purpurata* cultivars is exclusively xenogamous. The obligate xenogamy facilitates the breeding of new hybrids for the ornamental plant market (Luc-Cayol and

Fereol, 1997). Flexistyly has been interpreted to promote outcrossing by avoiding self-pollination (Li *et al.*, 2001). The absence of flexistyly in *A. purpurata* may be a result of the effectiveness of self-incompatible and herkogamy in preventing autogamy, with no need for another mechanism to promote cross-pollination. Self-compatibility with pollinator dependence has been reported for flexistylic *Alpinia* species (Li *et al.*, 2002; Zhang *et al.*, 2003; Krieck *et al.*, 2008).

The lower number of fruits resulting from natural than from artificial pollination suggests that the presence of effective *A. purpurata* pollinators in the study area is limited.

In *A. purpurata*, although hand pollination of the anther crest did not induce fruit formation, the participation of this structure located above the stigma, together with the stigma, seems to play an important role in the reproductive biology of this species. Due to its physical proximity to the stigma, the anther crest is covered by stigmatic fluid. Our data indicate that this structure helps to capture pollen grains, functioning as a stigmatic extension that must touch the pollinator's body at the time of the visit. These pollen grains that adhere to anther crest can reach the pistil, since there was an increase in seed production when pollen was deposited on such a structure, showing its participation in the pollination process. In addition, the crest can serve as protection against the viscous secretion (stigmatic fluid) produced and stored at the apex of the stigma. In Costus (Zingiberales-Costaceae), this structure has the same function contribute to pollination (Araújo and Oliveira, 2007).

In *Roscoea debilis* (Zingiberaceae) the stigmatic fluid forms a large globule and then seeps into the nearby pollen grains, inducing pollen germination and pollen tube elongation into the style. This process provides reproductive assurance when pollinator service is limited (Fan and Li, 2012), but was not observed in the present study.

Similar to other Zingiberaceae species (Sakai *et al.*, 1999), *A. purpurata* maintains the characteristics of insect-pollinated flowers, namely white, zygomorphic flowers with a landing platform and nectar rewards.

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Table 3. Reproductive system of four *Alpinia purpurata* cultivars.

Pollination on stigma + anther crest				
Treatments	**Cultivars**	**No. of flowers**	**Fruiting percentage (%)**	**No. of seed/fruit $\bar{X}$±SD**
Cross pollination	Kimi[1] x Jungle King[2]	25	60.0	200.4 ± 57.1
	Jungle King[1] x Kimi[2]	25	76.0	203.4 ± 62.2
	Red Ginger[1] x Pink Ginger[2]	8	87.5	166.0 ± 54.6
	Jungle King[1] x Pink Ginger[2]	6	83.0	158.6 ± 51.3
Pollination on anther crest				
Cross pollination	Kimi[1] x Jungle King[2]	25	0.0	0.0
	Jungle King[1] x Kimi[2]	25	0.0	0.0
Pollination on stigma				
Cross pollination	Jungle King[1] x Kimi[2]	25	80.0	105.5± 4.8
	Kimi[1] x Jungle King[2]	25	24.0	93.5 ± 20.9
Geitonogamy	Jungle King	25	0.0	0.0
	Kimi	25	0.0	0.0
Spontaneous selfing	Jungle King	25	0.0	0.0
	Kimi	25	0.0	0.0
Hand selfing	Jungle King	25	0.0	0.0
	Kimi	25	0.0	0.0
Natural pollination	Jungle King	$\bar{X}$=288*	10.0	178.9 ± 34.0
	Kimi	$\bar{X}$=280*	0.0	0.0
	Pink Ginger	-	-	0.0
	Red Ginger	-	-	0.0

[1] In the cross pollination treatments, pollen receptor; [2] X pollen donor.

* Mean number of flowers produced per inflorescence within two months counted from the scars of the floral pedicel in the inflorescence.

CONCLUSION

During the anthesis period, the anther's stigma and crest showed receptivity, indicating that both structures are covered by stigmatic fluid. *A. purpurata* flowers did not show stylet movement (flexistyly).

The cultivars showed fruiting in cross-pollination. Low fructification occurred in natural pollination and no fructification in spontaneous self-pollination, manual self-pollination or geitonogamy.

The outcrossing of *A. purpurata* facilitates obtaining new natural hybrids for the ornamental plants market.

Conflict of interests: This manuscript was prepared and reviewed with the participation of all authors, who declare that there was no conflict of interest posing a risk to the validity of the results reported.

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