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Floral, reproductive, and pollination biology of *Eugenia myrcianthes Nied.*

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Abstract

Studies on species of the family Myrtaceae are mostly related to floristic surveys, reproduction involving large plant communities, and family taxonomy. Thus, the objective of this study was to elucidate aspects of the floral and reproductive biology, as well as floral visitors of ubajai tree (*Eugenia myrcianthes Nied.*). Studies were conducted on floral morphology and morphometry, identification of nectaries and structures attractive to floral visitors, characterization of floral visitors, stigma receptivity, and androecium maturation, pollen storage, in vitro viability testing, and characterization of the reproductive system. Ubajai flowers open at approximately 6:00 a.m., and their anthers are the main attractive structure to floral visitors. The main floral visitors and effective pollinators of ubajai are honeybees (Apis mellifera). The addition of 40% sucrose to the culture medium, using fresh pre-anthesis pollen, allows for a 90% germination rate. Ubajai pollen is recalcitrant, thus, it loses viability before 30 days of storage, even when stored in a refrigerator, freezer, liquid nitrogen, or natural environment. Ubajai tree can be considered a self-compatible plant; however, fertilization of flowers through cross-pollination also occurs, and apomixis does not occur.

Keywords: floral visitors, Myrtaceae, pollen germination, ubajai, vibration pollination

Introduction

Brazil is one of the world's main hotspots for high diversity of native fruit trees; however, little is known about most existing species. Native fruit trees from the family Myrtaceae are widely distributed throughout Brazil and are found in several biomes.

These species constitute a genetic heritage of great value, economic potential, and ecological expression. However, basic studies are still scarce, despite their importance, especially those linked to lesser-known species such as ubajai tree (*Eugenia myrcianthes* Nied.), which possesses antioxidant and anti-inflammatory activities (Infante et al., 2016) and is important for producing food supplements with health-promoting properties and other products that can be options for some niche markets.

Genetic improvement is the main method for transforming species by using components of biodiversity as genetic resources and producing economically valuable products for modern markets. Thus, new varieties are developed and incorporated into production systems, resulting in superior cultivars through selection and recombination.

Studies on floral and reproductive biology contribute to protection and restructuring of populations and are important because information on these aspects are essential for developing genetic improvement programs, understanding ecological relationships among different species and biotic and abiotic influences on the establishment and phenology patterns of species, germplasm conservation, and the development of culture management protocols (Danner et al., 2011b; Torres & Galetto, 2011; Kuaraksa et al., 2011; Françoso et al., 2014).

Studies on floral, reproductive, and pollination biology of Myrtaceae species are mostly related to

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floristic surveys, reproductive studies involving large plant communities, and family taxonomy. Considering this context and the scarcity of studies on native species of the family Myrtaceae, the objective of this study was to elucidated unknown floral aspects, reproductive biology, and floral visitors of ubajai tree (*E. myrcianthes*), a neglected species.

Material and Methods

The study was conducted at the Laboratory of Plant Physiology and the native fruit orchard at the Experimental Station of the Universidade Tecnológica Federal do Paraná (UTFPR), Dois Vizinhos, Paraná, Brazil.

Anthesis

One hundred marked flower buds from two mature ubajai trees were observed during five non-consecutive days, from 5:00 a.m. to 8:00 p.m., to identify the moment of complete anthesis and senescence. Some branches with flowers at pre-anthesis were fixed in phenolic foam, kept under 25 °C, and monitored until full anthesis for analysis of anthesis under controlled environmental conditions.

Identification of nectaries and structures attractive to flower visitors

The presence of substances such oil or nectar in fresh flowers was evaluated using stereoscope or manual magnifying glass and microcapillary tubes, which were arranged at the base of sepals, petals, stamens, and carpels to identify the presence or absence of such substances.

The recognition of flowers by floral visitors is done through the reflection of ultraviolet rays, as some flower pigments reflect this wavelength, which is perceptible to insects. In this sense, the test was performed shortly after anthesis, with direct observation in an adapted chamber provided with ultraviolet light (luminescence).

The staining test, used to identify metabolic activity or presence of osmophores, was performed using fresh flowers kept for 60 minutes in a 1% neutral red solution, with observations made using a stereoscope.

Ammonium hydroxide test was conducted to complement the staining test and identify floral resource guides, by keeping a cotton soaked in ammonium hydroxide for 10 minutes in a closed glass container until atmosphere saturation, and then adding fresh flowers for an additional 10 minutes. Flavonoid-like pigments absorb ultraviolet light while others reflect it; this contrast between the two regions (with and without flavonoids) guides flower visitors to the resource location.

Odor-releasing floral structures were identified by dissecting flowers into sepals, petals, stamens, and pistils and placing them in sealed glass tubes with plastic film for five hours, followed by evaluation by ten volunteers, who identified which floral part contained odor-releasing structures and described the type of odor emitted (sweet, floral, citrus, woody, or other).

The moment when flowers emitted odor was identified in an olfactory bioassay with post-anthesis flowers, which were placed in sealed glass tubes with plastic film and evaluated hourly by volunteers for 10 hours.

Characterization of pollinators and floral visitors

Observations on non-consecutive days, from 5:00 a.m. to 8:00 p.m. and in hours with the most frequent visitors, were carried out to search for floral visitors (Kiill & Simão-Bianchini, 2011). The importance of each floral visitor was determined based on their behavior and relative visitation frequency. Species with lower visitation frequency were considered occasional pollinators, even if they were legitimate visitors, whereas those that did not contact reproductive structures were considered pillagers (Matias & Consolaro, 2014).

Photographic records were taken. Some visitors were captured using an entomological net or aspirator and stored at FAA (formaldehyde; acetic acid; 70% ethanol [10:5:85 v/v)] for subsequent identification using identification guides (Fujihara et al., 2016).

Stigma receptivity and androecium maturity

Stigma receptivity was identified using a 3% hydrogen peroxide solution, which promoted bubbling on the stigma through peroxidase activity (Mariot et al., 2014; Matias & Consolaro, 2014). A 1% neutral red solution was also used, with immersion for 60 minutes and subsequent analysis using a stereoscope, in which tissue staining indicates metabolic activity and receptivity. In both tests, branches with flowers were kept in phenolic sponge at room temperature, and 25 flowers were used for each floral stage: balloon or pre-anthesis; beginning of anthesis; full anthesis; beginning of senescence.

Androecium maturity was evaluated through stereoscopic observation to identify pollinium release in pre- and post-anthesis materials.

Storage and in vitro pollen germination

The in vitro pollen germination test was conducted using anthers from pre- and post-anthesis flowers, which were detached and placed on paper trays in a silica chamber at room temperature (approximately 25 °C) for

8, 16, and 24 hours to promote anther dehiscence and pollinium release; fresh anthers were used as control.

A completely randomized design was used, in a factorial arrangement, in which Factor A consisted of two floral development stages (pre- and post-anthesis) and Factor B consisted of dehydration times (0, 8, 16, and 24 hours), totalizing eight treatments. A culture medium with 10% sucrose was used, as well as 1% agar, dissolved in distilled water heated in a microwave oven until boiling.

The heated culture medium was poured in Petri dishes; after cooling and solidification, it was sectioned, using a spatula, into small blocks that were arranged on slides to receive pollen, which was sprinkled using n° 4 brushes. After obtaining the conditions that provided the best germination, the sucrose concentration in the culture medium was tested, using with five concentration levels (0, 5%, 10%, 20%, and 30%).

The slides were placed in boxes (Gerbox®) with lids containing a moistened paper (simulated moist chamber) and incubated in a BOD (Biological Oxygen Demand) chamber at controlled temperature (25 °C) for 24 hours. Four replications were used for each treatment.

The number of germinated pollen grains was counted in a binocular microscope; pollen tube grains equal to or larger than the pollen grain diameter were considered germinated.

Pollen viability over time was evaluated in pollen grains stored in refrigerator (5 °C), freezer (-17 °C), liquid nitrogen (-147 °C), and room temperature (approximately 25 °C), with monthly evaluations until total viability loss.

The data were subjected to normality test (Lilliefors) and homogeneity of variance (Bartllet). Meeting the assumptions of the model, they were subjected to analysis of variance (ANOVA) to assess the significance of the factors and their interactions. When significant, Duncan's mean test ($\alpha \leq 0.05$) was applied for the qualitative factor, and regression analysis was applied for quantitative factors, using the software Genes (Cruz, 2013).

Reproductive system characterization

The reproductive system was characterized using seven selected plants due to an adequate number of flowers. A variable number of flowers per experimental unit was used, applying the following treatments: apomixis (emasculation of flower buds); spontaneous self-pollination; manual self-pollination (manual pollination with pollen from the same flower); control or open pollination (marking flower buds and leaving them accessible to floral visitors); cross-pollination or xenogamy (pre-anthesis emasculation and pollination using pollen from other plant). The treatments were applied based on the stigma receptivity test and using fresh pollen (readily available in the field). All manipulated flowers were isolated in paper bags before and after treatments, until fruit setting.

The following indices were calculated: index of self-incompatibility (ISI) (Bullock, 1985), which is the relationship between the fruiting percentages obtained from manual self-pollination and cross-pollination; and self-pollination index (ISA) (Sobrevila & Arroyo, 1982), which is the relationship between fruiting percentages obtained from spontaneous self-pollination and manual self-pollination. An ISI of up to 0.25 indicates that the species is self-incompatible (Bullock, 1985). Regarding ISA, the closer to zero, the lower the effectiveness of the reproductive strategy (Polatto & Alves-Junior, 2009).

Reproductive efficiency (RE) was obtained through the relationship between fruit set percentage with open pollination (control) and manual cross-pollination (Zapata & Arroyo 1978); the closer to zero, the higher the evidence of low pollination efficiency (Polatto & Alves-Junior, 2009).

Results and Discussion

Anthesis determination

The anthesis of ubajai tree (*Eugenia myrcianthes*) is diurnal, occurring during the morning, beginning at approximately 6:00 a.m. and extending until 9:30 a.m., with senescence occurring 48 hours after anthesis. On colder days (approximately 16 °C), anthesis begins around 11:00 a.m. and is completed around 8:00 p.m. In *E. uniflora*, *E. punicifolia*, *E. rotundifolia*, and *E. neonitida*, anthesis begins at 5:30 a.m. and extends throughout the day (Silva & Pinheiro, 2007), as observed in *Myrciaria dubia* (Maués & Couturier, 2002). During senescence, the petals and androecium fall off, remaining only the calyx and style until the beginning of fruit formation, which is a common characteristic in Myrtaceae species.

During anthesis, stamens and style expand as the petals unfurl, which then curl over the hypanthium, giving greater prominence to the androecium. This also occurs in *E. uniflora*, *E. neonitida*, *E. punicifolia*, and *E. rotundifolia* (Silva & Pinheiro, 2007). The style exhibits a slight curvature below the stigma, which promotes the adhesion of pollen grains to the stigma, as the anthers are already dehiscent, which is a common characteristic of Myrtaceae species (Proença & Gibbs, 1994).

Identification of nectaries and other structures attractive to visitors

Nectaries were not detected in ubajai flowers,

which was also found for other Myrtaceae species, including *M. dubia* (Maués & Couturier, 2002).

In the luminescence test, the tissues of anthers exhibited reflection under ultraviolet light, revealing the presence of areas responsible for emitting odors or osmophores. The presence of osmophores in the anther region was detected with neutral red solution; the odor may also be emitted by volatile substances in the pollen grains. Additionally, when subjected to the ammonium hydroxide test, the anthers showed color contrasts due to the greater presence of flavonoids that absorb ultraviolet light. Contrasts can be observed between these regions (with and without flavonoids), forming "nectar guides" or "floral resource guides", in this case, to pollen grains.

Petals, sepals, and pistils of ubajai flowers have no odor, as confirmed by the olfactory tests; it is mainly concentrated in male structures (anthers/pollen), persisting from anthesis and lasting for up to 10 hours. This result confirms the luminescence test, which identified the presence of osmophores in the anthers.

Odor is also emitted by pollen grains due to the presence of volatile oils in pollen grains, which is a characteristic of Myrtaceae species (Proença & Gibbs, 1994; Maués & Couturier, 2002).

The odor of ubajai flowers can be characterized as fruity notes with a slightly sweetness. This can be attributed to the species' consistent strategy of attracting insects for pollen dispersal and placement on female organs, resulting in pollination. According to the results, pollen is the main resource offered to pollinators, which is commonly observed in flowers of Myrtaceae species (Proença & Gibbs, 1994).

Characterization of pollinators and floral visitors

Insects from the family Apidae exhibited the highest frequency of visitation and number of visitors, mainly those from the order Hymenoptera, which accounted for 56.25% of the visitors. Pollen is typically collected mainly by bees from the family Apidae through vibration, as anthers are rimous (longitudinal dehiscence) and the pollen is easily removed by clinging to the stamens and vibrating them. This behavior has been observed in other Myrtaceae species, including *E. dysenteries*, *Siphoneugena densiflora*, *Blepharocalyx salicifolius*, *Campomanesia pubeacens*, *C. velutina*, *M. linearifolia*, *M. rhodosepala*, and *P. firmum* (Proença & Gibbs, 1994).

All identified bees, especially honeybees (*Apis mellifera*), exhibited movements within and between plants while visiting their flowers, always touching reproductive structures, and were considered effective pollinators of ubajai flowers.

As pollen release begins before anthesis and is widely used as a food resource, bees prefer flowers at the beginning of anthesis, before petal curvature, moving across the reproductive system, collecting pollen to produce food resources through corbiculae and agglutinating the pollen grains into spheres on their hind leg, while part of pollen grains remains adhered to the dorsal region, which usually comes into contact with the stigma. *Apis melifera* bees remained on the reproductive structures for 3 to 7 seconds, whereas native stingless bees remained for 4 to 6 seconds.

As previously observed in the detection of attractive structures for flower visitors, the odor of ubajai flowers is specifically emitted by anthers and pollen, which are responsible for attracting visitors.

Other visitors were also identified but with lower frequency, including *Chrysomelidae* (Coleoptera) at 12.5%, and floral visitors from the families *Chrysopidae* (Neuroptera), *Syrphidae* (Diptera), *Pyrrhocoridae* (Hemiptera), *Vespidae* (Hymenoptera), and *Sphecidae* (Hymenoptera) at 6.25%.

Visitors from other families (Vespidae, Sphecidae, Pyrrhocoridae) were observed pillaging fallen pollen grains in the perianth or consuming floral structures; they are classified as pillagers because the morphology of these insects does not allow them to touch the reproductive structures of ubajai flowers, and their slight intrafloral and interplant movements do not allow them to transfer pollen grains. However, pollination by Coleoptera can occur in some Myrtaceae species, as they may act as occasional or illegitimate pollinators of *M. dubia* (Maués & Couturier, 2002).

Insects of the species *Diabrotica speciosa* (Chrysomelidae; Coleoptera), in addition to pillaging pollen, fed on several parts of ubajai flowers, including reproductive structures, as it is characterized as a polyphagous pest.

Flies (Diptera) was observed sporadically, with short visits, collecting pollen with their oral apparatus but mainly staying on the petals and leaves. This was also observed in *M. dubia* by Maués & Couturier (2002). They were rarely observed on reproductive structures; however, they performed intrafloral and interplant movements, which characterizes them as occasional pollinators.

Ants (Hymenoptera) and bedbugs (Hemiptera) visited flowers to feed on pollen residues; however, no contact with reproductive structures was observed, classifying them as pillagers, as also observed in *Stenolobium stans* (Bignoniaceae) (Dutra & Machado,

2001).

The highest intensity of floral visitors was observed from 8:00 a.m. and extended until 1:00 p.m., decreasing in intensity throughout the day. The first visitors with abundant presence were from the family Apidae, mainly honeybees. During the visits of honeybees, the presence of other insects was almost null, possibly due to a hierarchy system among them. This behavior was also observed in flowers of *E. uniflora*, *E. neonitida*, *E. punicifolia,* and *E. rotundifolia* (Silva & Pinheiro, 2007). Therefore, honeybees have an aggressive behavior with an influence on the foraging period of native species, as reported by Queiroga et al. (2014) and Castro et al. (2019).

Days with milder temperature (approximately 16 °C) promote visits by honeybees over the day (from approximately 8:00 a.m. to 6:00 p.m.), while the presence of other visitors was almost null, as observed by Malerbo-Souza & Silva (2011).

Stigma receptivity and androecium maturity

In the hydrogen peroxide test, 100% of pre-anthesis flowers showed bubbling in the stigma cavity until the beginning of senescence, indicating peroxidase activity and, consequently, stigma receptivity throughout the period. Similar result was found using the neutral red solution, with an intense metabolic activity in the stigma region. Ubajai flowers have dehiscent anthers, even inside the balloon, which synchronizes with stigma receptivity.

In vitro pollen germination

The interaction between the analyzed factors (floral development stage and dehydration time) was significant at 1% probability level, thus rejecting the null hypothesis *H0*. The coefficient of variation obtained can be considered low, denoting a good experimental control (7.6%). The regression equations adjusted for quantitative factors were significant at 1% probability level by the t test.

The ideal floral development stage for collecting anthers of ubajai flowers was in pre-anthesis; pollen germination from post-anthesis flowers was ineffective, reaching only 8% germination. Similar results were reported for *E. involucrata*, which presented better germination percentages in pollen grains from pre-anthesis flowers (Franzon & Raseira, 2006), as well as in flowers of loquat tree (*Eriobotrya japonica*) (Nogueira et al., 2015).

Regarding the dehydration time, pollen germination values were higher when fresh (without dehydration) (**Figure 1**). Germination can reach 31% (Point of Maximum Technical Efficiency) when pollen is collected at pre-anthesis and used without desiccation. The viability decreased when pollen grains are dehydrated, denoting a recalcitrant characteristic.

Pollen reserves are partially used to produce dehydration-resistant molecules and sustain the initial germination phase. Desiccation can occur before dehiscence, even within the anther, during anthesis or dispersion. The achieved water content and the potential to remain viable characterize pollen as orthodox or recalcitrant (Franchi et al., 2011).

Orthodox pollen grains (tolerant to desiccation) may have water contents lower than 30%, whereas recalcitrant pollen grains (sensitive to desiccation) may have 30% (Franchi et al., 2011). Changes in pollen grain water contents may be affected by the presence or absence of aquaporins, which are proteins that facilitate the transport of water and solutes across cell membranes (Soto et al., 2012).

Dehydration of pollen grains is a subject rarely addressed in the scientific literature, especially for native species, but it has a direct effect on pollen viability. Thus, more comprehensive explanatory studies exclusively focused on this factor are needed.

The effect of the culture medium with sucrose was confirmed in the experiments using fresh pollen (non-dehydrated) from pre-anthesis flowers. Sucrose had a significant effect, promoting a linear increase in germination as the sucrose concentration was increased (**Figure 2**). The use of 40% sucrose promoted approximately 90% germination of ubajai pollen grains.

The germination of ubajai pollen obtained was higher than those found in the literature for *E. involucrata* (Franzon & Raseira, 2006), *E. uniflora* L. (Franzon et al., 2007), and *Platonia insignis* (Sinimbú Neto et al., 2011; Souza et al., 2013).

The pollen viability test can be affected by many factors; however, sugars are the most important components of the culture medium (Lyra et al., 2011; García et al., 2012), as they provide metabolic energy for biosynthesis of organic compounds and are essential for cell growth and establishment of osmotic balance in the medium, facilitating the diffusion of nutrients (Figueiredo et al., 2013).

Therefore, the addition of 40% sucrose to the culture medium in the pollen germination test using fresh pollen grains from pre-anthesis flowers can result in up to 80% germination, which is a satisfactory percentage.

Storing ubajai pollen is unviable, as a total loss of viability was found in the first evaluation (30 days). The storage of pollen from *E. uniflora* L. was not efficient and resulted in decreased viability when stored in a freezer

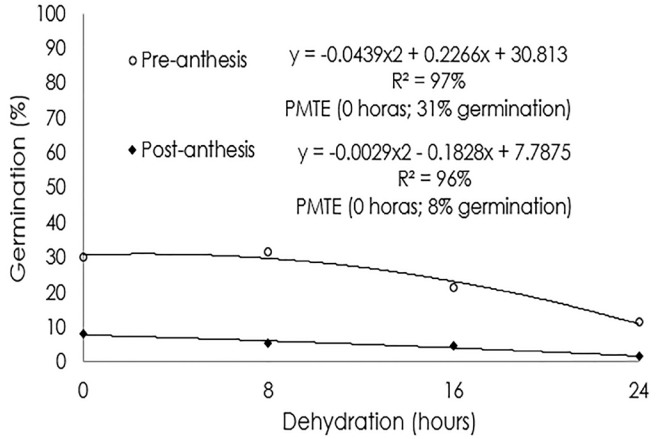

Figure 1. In vitro germination of pollen from ubajai flowers as a function of floral development stages and pollen dehydration times.

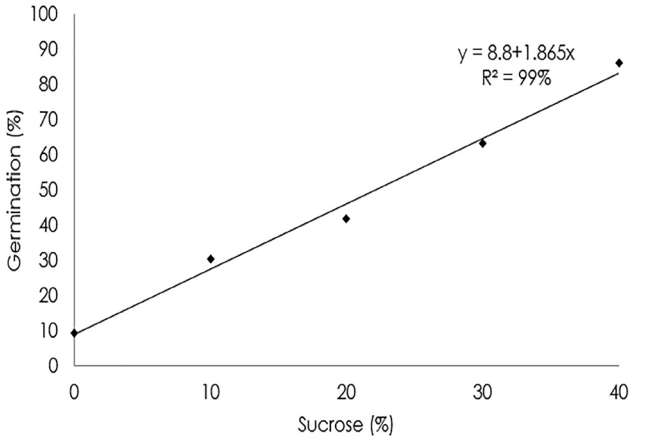

Figure 2. In vitro germination of pollen from ubajai flowers as a function of sucrose concentrations in the culture medium.

(Franzon et al., 2007); however, pollen from *P. cauliflora*, *P. trunciflora, P. jaboticaba* (Danner et al., 2011b), and *E. involucrata* (Franzon & Raseira, 2006) can be preserved for up to 90 days in a freezer at -18 °C and -16.5 °C, respectively.

Weekly evaluation is recommended for further studies, considering the recalcitrant behavior and rapid loss of viability.

Characterization of the reproductive system

Considering the crosses conducted in ubajai plants, fruit formation was mainly found through manual self-pollination, and late self-incompatibility was not observed. However, the percentage of fertilized flowers and fruit set did not exceed 12%. The lowest values of late abortion found for natural pollination (control), spontaneous self-pollination, and cross-pollination were 1%. In the apomictic treatment, all flowers were aborted 48 hours after the procedure (**Table 1**).

Similar results were found for *C. adamantium*, with 10% of fruits obtained with manual self-pollination and spontaneous self-pollination, as well as abortion in the apomictic treatment (Nucci & Alves-Junior, 2017). Although, according to Fidalgo & Kleinert (2009), the reproductive system of many Myrtaceae species varies, showing complete self-sterility or an apomictic system.

Table 1. Pollination test, percentage of fertilized flowers, fruit setting, index of self-incompatibility (ISI), index of self-pollination (ISA), and reproductive efficiency (RE), in ubajai plants

Pollination test	Number of flowers used	Fertilized flowers (%)	Fruit set (%)
Manual Self-Pollination	100	12	12
Natural Pollination (control)	440	10	9
Spontaneous Self-pollination	200	4	3
Cross Pollination (Xenogamy)	100	9	8
Apomictic treatment	100	0	0
ISI		1.20	
ISA		0.25	

Ubajai tree was considered self-compatible because the obtained index of self-incompatibility was 1.2 (Tab. 2), and values lower than 0.25 characterize self-incompatibility. Self-incompatibility can be lost and cross-fertilization may occur at low rates during the evolutionary process. Contrastingly, it increases homozygosity in the population, reducing the species ability to survive and reproduce (inbreding depression), limiting the adaptability to environmental changes, leading to the extinction of populations and species (Goldberg et al., 2010).

The obtained index of self-pollination (ISA) (0.25) can be considered low, despite the absence of a self-incompatibility mechanism, such as hercogamy (anthers below the stigma) (Table 1). The absence of wind due to the physical barrier caused by bagging the balloon may have compromised the displacement of pollen grains to the stigma or caused pollen viability loss before reaching the stigma due to its recalcitrant behavior.

Study on *Sparattosperma leucanthum* presented an ISA of zero, denoting the need for pollinators even though the flower does not have mechanisms to prevent spontaneous self-pollination (Polatto & Alves-Junior, 2009), as found for ubajai plants.

The results of the reproductive efficiency (RE) analysis were high (Table 1), indicating the efficient transfer of viable pollen to the stigma by pollinators. Similar results were found for *C. adamantium*, with a high RE (1.33), indicating high efficiency of pollinators (Nucci & Alves-Junior, 2017); however, a low RE (0.19) was found for *S. leucanthum*, indicating low pollination efficiency, despite the large number of pollinator visits (Polatto & Alves-Junior, 2009).

Despite the high RE found, the presence of

pollinating insects was constant, but open pollination (control) presented only 9% success. The low fruiting rates found may be due to the amount and quality of pollen transported to the stigma. However, the ideal fruit set may also vary, depending on the species and environmental factors.

Danner et al., (2011a) evaluated different genotypes of *P. cauliflora*, *P. trunciflora*, and *P. jaboticaba* and found similar results for some and different for others when compared to ubajai. In 2008, they found fruit set percentages of 4.5% and 5.2% (Genotypes 1 and 4); 34.6%, 58.4%, and 34.8% (Genotypes 1, 3, and 4); and 32.8% (Genotype 1), respectively; and in 2009, they found 9.1% and 8.7% (Genotypes 1 and 4); 32.0%, 33.7%, and 34.2% (Genotypes 1, 3, and 4); and 34.9% (Genotype 1), respectively, denoting high variation among evaluation periods, species, and genotypes.

Conclusions

The anthesis of ubajai tree (*Eugenia myrcianthes*) is diurnal, occurring during the morning, beginning at approximately 6:00 a.m. and extending until 9:30 a.m.

Anthers are the main attractive structure for pollinating insects.

Flowers emit a fruity odor with slightly sweet notes, specifically emitted by anthers and pollen.

The main floral visitors belong to the families Apidae and Chrysomelidae, but honeybees are the effective pollinators of ubajai.

Adding 40% sucrose to the culture medium, when using fresh pollen grains from pre-anthesis flowers, is recommended to obtain higher germination rates. Ubajai pollen presents a recalcitrant behavior.

Ubajai tree is self-compatible, however, fertilization of its flowers by cross-pollination also occurs, and apomixis does not occur.

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