UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Campus de Botucatu

Pós-graduação
Botânica
Unesp - Botucatu

FATORES ASSOCIADOS AO SUCESSO REPRODUTIVO DE DUAS ESPÉCIES COOCORRENTES DE *JACARANDA* JUSS. (BIGNONIACEAE)

CAMILA VAZ DE SOUZA

Tese apresentada ao Instituto de Biociências, Campus de Botucatu, UNESP, para obtenção do título de Doutor no Programa de Pós-Graduação em Ciências Biológicas (Botânica), Área de concentração: Morfologia e Diversidade Vegetal.

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SOUZA, C.V. **FATORES ASSOCIADOS AO SUCESSO REPRODUTIVO DE DUAS ESPÉCIES COOCORRENTES DE *JACARANDA* JUSS. (BIGNONIACEAE).** 2019. 107p. TESE (DOUTORADO) – INSTITUTO DE BIOCIÊNCIAS, UNESP – UNIVERSIDADE ESTADUAL PAULISTA, BOTUCATU.

Resumo - Nessa tese estudamos duas espécies melitófilas de Bignoniaceae, *Jacaranda caroba* e *J. decurrens*, que coocorrem em formações savânicas de cerrado e que se mostraram modelos interessantes para explorarmos aspectos ecológicos e evolutivos relacionados à sua reprodução sexual, tendo em vista que apresentam uma complexa rede de interações com mutualistas e antagonistas. A sobreposição geográfica dessas duas espécies nos permitiu avaliar padrões de coexistência relacionados à dinâmica de florescimento, bem como seu anúncio floral relacionado à atração de visitantes florais mutualistas e antagonistas. Além disso, essa interação com mutualistas e antagonistas possibilitou averiguar o impacto da atuação de antagonistas que roubam néctar sobre a polinização de ambas as espécies e como os danos realizados por abelhas roubadoras de néctar interferem na sinalização visual e química aos polinizadores. Também avaliamos a exploração de néctar por diferentes grupos funcionais de visitantes florais de *J. caroba* e *J. decurrens*. Por fim, considerando que o sucesso reprodutivo está diretamente relacionado com a eficiência da polinização investigamos o sistema reprodutivo dessas duas espécies e o efeito da qualidade do pólen transferido entre flores associado ao comportamento dos polinizadores durante o forrageamento, buscando compreender os fatores intrínsecos relacionados à seleção mecanismos de autoincompatibilidade nas populações naturais amostradas.

Palavras-chave: anúncio floral, exploração de néctar, mutualistas, sinalização química, sinalização visual, sistemas reprodutivos, sucesso reprodutivo.

Factors associated to the reproductive success of two co-occuring *Jacaranda* Juss. species (Bignoniaceae)

Abstract - In this dissertation we studied two mellithiphilous Bignoniaceae species, *Jacaranda caroba* and *J. decurrens*, that co-occur in savanic cerrado formations. This is an interesting model to explore ecological and evolutionary aspects related to their sexual reproduction taking into account that they present a complex network of interactions with mutualists and antagonists. These species' geographical overlap allowed us to evaluate patterns of coexistence related to the flowering dynamics, as well as its floral display related to the attraction of mutualists and antagonists' floral visitors. Besides, their interactions with mutualists and antagonists allowed us to evaluate the impact of nectar-robbing antagonists on both species and how damage by nectar-robber bees interferes with visual and chemical signaling to pollinators. We also evaluated the nectar exploitation by different functional groups of *J. caroba* and *J. decurrens* floral visitors. Finally, considering that these species' reproductive success and the effect of pollinator behavior during foraging on the quality of the pollen that is transferred among flowers. Considering that species' reproductive success is directly related to the pollination efficiency, we investigated the reproductive system of these two species and the effect of the quality of the pollen transferred between flowers associated to the pollinators' behavior during the foraging, trying to understand the intrinsic factors related to mechanisms of self-incompatibility in the sampled natural populations.

Key words: Chemical signaling, floral display, mutualists, nectar exploitation, reproductive success, reproductive systems, visual signaling.

INTRODUÇÃO GERAL

O baixo sucesso reprodutivo é comum em diversas espécies vegetais (Stephenson 1981, Sutherland 1987, Burd 1994, Ashman et al. 2004, Knight et al. 2005), e isso pode ser atribuído a fatores tanto ecológicos quanto intrínsecos à própria planta. Em espécies zoófilas, a atração de animais polinizadores é fundamental para garantir a transferência de pólen compatível, sendo a eficiência desse processo determinante para o sucesso reprodutivo destas espécies (Conner & Rush, 1996). Existem diversas características relacionadas à atratividade aos visitantes florais incluindo o anúncio floral, que pode apresentar diferentes intensidades ao longo do período reprodutivo do indivíduo, da população e da espécie ao longo de sua distribuição geográfica (Elzinga et al. 2007). Diversos estudos têm sugerido que o aumento de visitas é densidade-dependente e que, portanto, um maior investimento na produção de flores pode ocasionar um aumento na quantidade de pólen recebida e no número de sementes formadas devido a uma maior oferta de recursos tróficos disponíveis aos polinizadores (Mitchell 1994, Engel & Irwin 2003, Karron & Mitchell 2011). Entretanto, essa relação nem sempre é diretamente proporcional uma vez que um maior número de flores pode influenciar a amplitude de movimento dos polinizadores, levando à transferência de pólen incompatível entre flores da mesma planta ou entre plantas próximas e aparentadas, afetando negativamente a formação de progênies (Ohashi & Thomson 2009). Isso ocorre principalmente em espécies autoincompatíveis, pois quando esses animais visitam sequencialmente diversas flores do mesmo indivíduo podem transferir alta proporção de grãos pólen provenientes da mesma planta, interferindo nas chances dos grãos de pólen oriundos de polinização cruzada germinarem (Duncan et al. 2004). Experimentos em condições naturais, nos quais as variações de anúncio floral são monitoradas conjuntamente à frequência de visitas de mutualistas e antagonistas e ao sucesso reprodutivo da espécie vegetal (Capítulo 1) podem ajudar a elucidar os efeitos do anúncio floral dobre a reprodução sexual em espécies zoófilas.

Nesse contexto, ao abordarmos a reprodução sexual em espécies vegetais é fundamental considerar a ação dos antagonistas, pois a pilhagem ou o roubo de recursos pode ter consequências negativas sobre a polinização, seja pela diminuição da atratividade visual ou química da flor aos polinizadores, seja pela menor quantidade de recurso disponível aos mesmos (Zimmerman & Cook 1985, Irwin & Brody 1999, Zhang et al. 2007). Os pilhadores de néctar são caracterizados por obterem recursos florais de forma não legítima, pois geralmente esses animais apresentam dimensões corpóreas incompatíveis às dimensões das flores efetuando a coleta de recurso sem realizar a transferência dos grãos de pólen (Maloof & Inouye 2000). Já, os roubadores de néctar também coletam esse recurso de forma ilegítima, sem realizar a transferência de grãos de pólen, entretanto para acessá-lo, causam danos aos verticilos estéreis da flor, violando a integridade da corola (Inouye 1980). Estudos recentes têm enfocado as potenciais interferências diretas e indiretas da atuação destes antagonistas sobre os mutualistas e consequentemente sobre a reprodução sexual das espécies vegetais (Irwin et al. 2015, Rojas-Nossa et al. 2016, Hazlehurst & Karubian 2016, Mallinger & Prasifka 2017). Esses danos podem representar alterações visuais e químicas, as quais podem ser associadas à falta de recursos pelos polinizadores, podendo levá-los a rejeitar as flores danificadas, afetando assim a reprodução da espécie vegetal. *Oxaea flavescens* é uma espécie de abelha considerada especializada em roubar néctar de espécies de Bignoniaceae de cerrado (Gottsberger & Silberbauer-Gottsberger 2006), entretanto os impactos desse roubo sobre essas espécies vegetais é pouco conhecido (Quinalha et al. 2017). Assim, investigar o potencial efeito das marcas deixadas por essas abelhas sobre as visitas dos mutualistas e sobre a reprodução de espécies de Bignoniaceae (Capítulo 2), bem como entender o impacto da exploração de néctar por várias espécies de agentes antagonistas (Capítulo 3) pode ampliar nossa compreensão sobre os mecanismos envolvidos na manutenção de interações complexas como as que envolvem antagonistas-flores-mutualistas.

Outro importante fator a ser levado em consideração é a compreensão das respostas intrínsecas de espécies vegetais frente à qualidade do pólen depositado em seus estigmas, uma vez que os polinizadores podem depositar pólen da própria planta devido a seu comportamento de visitas sequenciais, podendo afetar negativamente o sucesso reprodutivo de espécies vegetais. Um componente intrínseco que pode ter evoluído como resposta adaptativa associada à prevenção da autogamia foi o surgimento de mecanismos de autoincompatibilidade genética, fundamentais na manutenção da variabilidade genética em populações naturais (De Nettancourt 1977, Gibbs & Bianchi 1999, Oliveira & Gibbs 2000, Gibbs 2014). Diversos estudos referentes ao sistema reprodutivo de espécies da família Bignoniaceae sugerem que o mecanismo predominante nesse grupo é o de autoincompatibilidade de ação tardia (LSI), em que os tubos polínicos provenientes de flores da mesma planta germinam e se desenvolvem no tecido transmissor do estilete, porém não chegam a formar frutos, uma vez que o pistilo sofre abscisão poucos dias após a polinização (Bittencourt et al. 2003, 2011, Bittencourt & Semir 2004, 2005, Gandolphi & Bittencourt 2010, Alves et al. 2013, Sampaio et al. 2016, Duarte et al. 2017). Assim, estudar os sistemas reprodutivos dessas espécies, pode ajudar na compreensão de como componentes intrínsecos atuam selecionando os tubos polínicos com características genéticas adequadas para o processo de fertilização e como esses mecanismos interferem no sucesso reprodutivo de espécies dessa família (Capítulo 4).

Nesse contexto, elegemos representantes da família Bignoniaceae para estudar todos os aspectos elencados aqui de maneira integrada, uma vez que as baixas taxas de frutificação natural vêm sendo referidas há décadas em diversas espécies dessa família (Vieira et al. 1992, Barros 2001, Correia et al. 2005, 2006, Guimarães et al. 2008, Quinalha et al. 2017, Souza et al. 2017). De fato, o baixo sucesso reprodutivo verificado em espécies de *Jacaranda* (Guimarães et al. 2008, Quinalha 2016) pode estar relacionado tantos aos fatores ecológicos anteriormente elencados, e.g. anúncio floral, qualidade do pólen transferido pelos polinizadores e comportamento dos mutualistas e antagonistas, quanto à fatores intrínsecos às plantas, como mecanismos de autoincompatibilidade. Assim, o objetivo geral dessa tese foi estudar os fatores que afetam o sucesso reprodutivo de *Jacaranda caroba* (Vell.) DC. e *Jacaranda decurrens* Cham. (Bignoniaceae) em populações naturais ocorrentes em fisionomias savânicas de cerrado.

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Conforme estabelecido pelo Programa de Pós-Graduação em Ciências Biológicas (Botânica) do IBB, UNESP, os resultados obtidos durante a execução da presente tese foram reunidos na forma de artigos científicos para publicação em periódicos da área, os quais serão apresentados em 4 capítulos.

CAPÍTULO 1

How do floral display and its interaction with mutualistic and antagonist bees affect reproductive success of two co-occuring savanna species?

CAPÍTULO 2

Do medium-sized bees avoid flowers with nectar robbery signals?

CAPÍTULO 3

Nectar exploitation by distinct functional groups of floral visitors in a savanna shrub species

CAPÍTULO 4

Breeding systems of two co-occurring species of the *Jacaranda* genus

CAPÍTULO 1

How do floral display and its interaction with mutualistic and antagonist bees affect reproductive success of two co-occuring savanna species?

1. ABSTRACT

In outcrossing species pollinated by animals, attractiveness is essential for pollen transfer, being the efficiency of this process decisive reproductive succes. Pollinators can adjust the number of visits in synchrony with the population flowering curve and exert a stabilizing selection on phenology. In this context, floral display besides signaling the presence of flowers, acts as an indication of the amount of resources available to them. Although there is a set of information for mutualists, little is known about the behavior of antagonists associated with floral display and if antagonist variations interfere in the attractiveness and behavior of mutualists due to the competition for resources. Thus, we aimed to investigate the occurrence of an association between floral display, mutualists and antagonist performance, and reproductive success in two co-occurring species of *Jacaranda*, which show partial overlap in flowering. For that, we characterized the flowering dynamic and used the intensity of floral display to determine the key periods in which we verified the behavior of our response variables (mutualists' visits frequency, antagonists' visits frequency, fruit / flower ratio). We verified that this flowering dynamics is associated with the interactions that *J. caroba* and *J. decurrens* establish with a network of mutualistic and antagonistic visitors and the formation of fruits. However, although the interaction with mutualists intensifies in the periods when there is a greater production of flowers, the relation with the reproductive success was opposite. This may be related to the pollinators' behavior of sequential visits that leading to transfer of incompatible pollen between flowers of the same plant or between related plants. In this context, we show that, for self-incompatible hermaphrodite species, more moderate flower display may represent a more important component for increase reproductive success than excessive flower production.

Key words: Bees, flowering dynamic, *Jacaranda*, phenology.

2. INTRODUCTION

In outcrossing species pollinated by animals, attractiveness is essential for pollen transfer, being the efficiency of this process decisive for new progenies formation and for the maintenance of plant populations in medium and long term (Conner & Rush 1996). Although flowering phenology is affected by several environmental factors imposed by bottom-up selective forces, such as temperature and photoperiod, mutualists and antagonists animals can exert top-down forces on plant species' reproductive period (Elzinga et al. 2007). Recent studies have shown that, in fact, pollinators are mediators of phenotypic selection on flowering phenology (e.g., onset) and on characters involved in floral display (Chen et al. 2017, Emel et al. 2017).

Phenology schedule can vary both in relation to the beginning of plants' flowering of a population and the duration of plants' flowering, which can lead to different patterns in the interaction with mutualists and antagonists. For example, pollinators can adjust the number of visits in synchrony with the population flowering curve and exert a stabilizing selection on phenology (Elzinga et al. 2007). In this context, floral display can be considered doubly important in the interaction with floral visitors since, besides signaling the presence of flowers, it acts as an indication of the amount of resources available to them (Ohashi & Yahara 2002, Makino & Sakai 2007), and can act not only on the attractiveness of pollinators, but also on their behavior (Parachnowitsch & Kessler 2010, Sletvold et al. 2015). In general, greater investment in flower production is related to more resources and a higher number of visits, which may lead to an increase in the amount of pollen received and the number of seeds produced (Mitchell 1994, Engel & Irwin 2003, Karron & Mitchell 2011). In the case of nectariferous species pollinated by medium-sized bees, the association between advertisement and resource is even more important, since they are able to identify scenarios with greater availability of nectar (Makino & Sakai 2007).

In addition to the effect on plant-animal interactions, variations in phenology may also affect plant-plant interaction when different plant species are visited by generalists' mutualists and antagonists (Elzinga et al. 2007). For example, plant species with flowering overlap that share pollinators may have their fitness positively or negatively affected by pollinator-mediated indirect effects such as facilitation and competition (Smith-Ramirez et al. 1998, Gross et al. 2000, Sarjent & Ackerly 2008, Fantinato et al. 2017, Ha & Ivey 2017). On the one hand, convergence in multi-species flowering phenology can act as a facilitation mediated by pollinators, since it increases the attractiveness due to the massive flower production, entailing in greater availability of resources (Aizen & Vasquéz 2006, Sargent & Ackerly 2008). On the other hand, if pollinators are a limiting component, competition may occur. Fantinato et al. (2017) verified that a remarkable amount of co-occuring species is specialized, attracting different pollinators because, despite the positive effects that the flowering synchrony can bring, it can also increase the competition for pollinators as well as the heterospecific pollen transfer, resulting in negative impacts on the plant reproductive success (Price 1984, Potts et al. 2003, Carvalheiro et al. 2014).

Although there is a set of information for mutualists, little is known about the behavior of antagonists associated with floral advertisement and blooming overlap. In addition, a fundamental aspect to be investigated is whether these potential antagonist variations interfere in the attractiveness and behavior of mutualists due to the competition for resources, which would be one more factor to consider in this complex scenario. Thus, the objective of this work was to investigate the occurrence of an association between flowering time dynamics, mutualists and antagonist performance, and reproductive success in two co-occurring species of *Jacaranda* Juss., which show partial overlap in flowering. For this, we aimed to answer specifically the following questions: (1) Is there an association between floral display and reproductive success in these plant species? (2) Do *J. caroba* and *J. decurrens* share mutualists and antagonists bees' species? (3) What is the effect of the specific floral display of each species alone and of the sum of both species' floral display during the flowering overlap period on the frequency of visits by mutualists and antagonists?

3. MATERIAL AND METHODS

3.1. Study site and plant species

We conducted the study at Estação Ecológica de Santa Bárbara (EESB), which belongs to Instituto Florestal do Estado de São Paulo. This area is located in the municipality of Águas de Santa Bárbara, between 22º46'30" to 22º50'30'S and 49º10'30" to 49º15'30'W. The vegetation covers an area of 2715 ha and is characterized by savanna physiognomies of cerrado. According to Koeppen's classification the region's climate is Cwa, with average temperatures of the hottest month (January) between 23° and 24° C and average temperatures of the coldest month (July) around 16° C (Alvares 2013). Annual rainfall is approximately 1200 mm, with a cold and dry season and hot and rainy one (Melo & Durigan 2011).

We used as models for this study two co-occurring species of *Jacaranda* genus (Bignoniaceae), that present sequential flowering phenophasis, with a period of overlapping (E. Guimarães, personal observation).

Jacaranda caroba (Vell.) DC. is an species that varies from shrubs to small trees (0.5 - 2.5m in height), presents glabrous branches and long internodes, bipinnate composed leaves with leaflets that vary from elliptic to rhomboid and opposite phyllotaxis; the inflorescences are tirsoid, terminal or axillary, composed by flowers with cupular vinous cup, irregularly 2-5 lobed; corolla tubular-campanulate above a strict basal tube, which correspond to the nectar chamber, puberula, 5-lobed, with coloration varying from magenta to purple; stamens didinamous with dithecae anthers; densely glandular staminodium and ovate-flattened ovary with cylindrical-pulvinate disc at the base; the fruit is an elliptical capsule 2.5-7.0 cm long and 2.2-4.5 cm wide, brown and sublime when ripe; their valves curl in the dehiscence, exposing the winged membranaceous seeds (Gentry & Morawetz 1992, Lohmann & Pirani 1996).

Jacaranda decurrens Cham. is a subshrub with a thickened subterranean organ from which aerial branches are born of 20 to 100 cm of height; is found in cerrado’s open physiognomies and in cerrado *sensu stricto*; the leaves are bipinnate sparse or densely pilose, arranged on the winged rachis; the inflorescences are small terminal panicles; the flowers have a deeply 5-parted calyx, with variable presence of glandular trichomes on the margins of the lobes, corolla violet or bluish, tubular-campanulate, flattened dorsiventrally, above a strict basal tube, which correspond to the nectar chamber, puberula externally, with capitate glandular trichomes more densely distributed at the base of the tube and sparse in the rest, 5-lobed; didynamous stamens, with monothecate anthers, the second theca is reduced, staminodium included or subexserted, densely glandular villous; ovary flattened-ovate, glabrous with pateliform-pulvinate disc at the base; elliptic-obovate fruit, strongly woody, containing seeds with hyaline membranaceous wings (Gentry 1992).

Vouchers of both species were collected and deposited in the ‘Irina Delanova Gemtchujnicov’ Herbarium (BOTU) from the São Paulo State University (Unesp), Institute of Biosciences, Botucatu, SP, Brazil, under the numbers 33198, 33319 (*J. caroba*) and 33199, 33320 (*J. decurrens*)

3. 2. Characterization of flowering dynamic

Flowering phenology can be evaluated at various levels (e.g. populations, individual plants in a population and flowers in a plant) (Elzinga et al. 2007). In this work, we characterized it in terms of population. We considered that the period of flowering of a plant corresponds to the number of days between the opening of the first flower and the senescence of the last one. The flowering season was considered number of days between the first and the last flower considering all the individuals of the population following Elzinga et al. (2007). For that, we randomly took 20 plants for each species evaluated the phonological events every fifteen days from September 2016 to November 2018. From these data we obtained the activity index and the flowering intensity, which were used as an indicative of the floral display. The activity index was used to estimate synchrony, indicating the proportion of flowering individuals every 15 days (*sensu* Bencke &Morellato 2002). The intensity was calculated from the sum of the number of flowers counted per plant every 15 days (*sensu* d'Eça-Neves & Morellato 2004).

We used the intensity of floral display to determine the key periods in which we verified the behavior of our response variables (mutualists' visits frequency, antagonists' visits frequency, fruit / flower ratio).

3.3. Determination of female reproductive success

We evaluated the female component of reproductive success in the same plants previously sampled for phenological analyzes of each *Jacaranda* species. For this, we initially left the flowers of these plants exposed to natural visitation. Later, we recorded the number of fruits produced, and followed them throughout their development, keeping them bagged individually until the opening, when seed dispersal started. We estimated the reproductive success from the total number of fruits formed and by the ratio fruits/ flowers produced during the five key periods of floral display. In order to calculate this ratio for each period made a correspondence between fruits and the flowers that originated them.

3.4. Floral visitors

3.4.1. Species richness

We evaluated the species richness of floral visitors in sampled plants by hour class (hourly) in each of the two *Jacaranda* species. Throughout the observations, we registered the time, duration and frequency of visits, as well as the behavior and type of resource collected by each floral visitor species. We recorded the visits from filming and direct visual observations according to Potts (2005), so that by the end of each flowering period we had 240 hours of observation for each species of *Jacaranda* (totaling 720 hours of observation throughout the whole period of this study).

3.4.2. Visiting behavior

We made detailed observations of the behavior of each visitor species and classified them as mutualists when they touched flower reproductive structures while searching for trophic resources, presenting behavior that included them as potential pollen vectors (pollinators) or as antagonists when they searched for resources (nectar and pollen) illegitimately, cheating them, without carrying out pollen transfer.

3.4.3. Frequency of visits

In order to verify variation in the frequency of flower visitors during *J. caroba* and *J. decurrens*' flowering period, we performed focal observations, according to the method described by Potts (2005), during the whole period of the day, beginning at 5:00 h and ending at 18:00 h, totaling three replicas of each class of horary in each sampled period determined in sub-item *3.3.1*. We evaluated the total number of visits in each period as well as the number of visits per flower.

3.5. Statistical analysis

To determine the phenological patterns, we applied the circular statistic (Morellato et al. 2010) using total number of flowers produced by each species of *Jacaranda* from September 2016 to November 2018. We used the Rayleigh test to evaluate the existence of seasonality in the production of flowers of *J. caroba* and *J. decurrens* (Zar 2010). In this test, each month corresponds to 30 °, e.g., January corresponds to 0 °, February corresponds to 30 °, and so on. The results of this test are significant when $p <0.01$ and $r > 0.5$ (Morelatto et al. 2010), indicating the occurrence of a peak for the analyzed variable. To test whether there is a difference between the flowering peaks of each species, we used the Watson-Wheeler test (Zar 2010). The analysis were performed in R v. 3.4.3 (R Development Core Team 2017) using standard and additional packages: plotrix (Lemon 2006) and circular (Agostinelli & Lund 2013).

In order to verify the effect of floral advertisement on our response variables (fruit/flower ratio, mutualist frequency, antagonist frequency), we used Local Regression (LOESS) with a 95% confidence interval in the R v 3.4.3 environment (R Development Core Team 2017). We used as a sampling unit the plants evaluated fortnightly ($N_{plant} = 21$ for *J. caroba* and $N_{plant} = 20$ for *J. decurrens* per period) to verify the number of flowers produced and the number of fruits produced per flower. For the analysis of the floral visitors frequency we used days of observation (1 day = 12 hours of observation, equivalent to the period of light) as the sampling unit (N_{dias} = 3 per period).

4. RESULTS

4.1. Flowering temporal dynamics of *J. caroba* and *J. decurrens*

Jacaranda caroba and *J. decurrens*, are annual flowering species (according to the classification of Newstrom et al. 1994), showing seasonality in this phenophases **(Table 1)**. Flowering of both species began in the coldest and driest period of the year, with *J. caroba* beginning to flower in July and ending in October and *J. decurrens* beginning in September and ending in November **(Fig. 1, Fig. 2)**. The flowering peak of *J. caroba* differed among the three sampling periods ($W = 322.18$, $p < 2.2\ 10^{-16}$, Watson-Wheeler test) occurring in September 2016 and 2017, but in 2018 occurring in August. During the peak, in 2016 33% of the individuals produced a total of 293 flowers, in 2017 88% of the individuals produced a total of 3268 flowers and in 2018 75% of individuals produced a total of 2123 flowers **(Fig. 1, Fig. 2A, B)**. In *J. decurrens* the flowering peak also differed over the sampling period ($W = 1004.7$, $p < 2.2\ 10^{-16}$, Watson-Wheeler test) occurring in September in 2016 and in October 2017 and 2018. During the peak, in 2016 90% of individuals produced a total of 943 flowers, in 2017 100% of individuals produced a total of 1186 flowers and in 2018 90% of the individuals produced a total of 321 flowers **(Fig. 1, Fig. 2A, C)**. Comparing both species year by year, we observed that in 2016 there is no difference between the flowering peaks ($W = 1.35$, $p = 0.5$, Watson-Wheeler test), but in 2017 and 2018 the peaks differ between *J. caroba* and *J. decurrens* (2017: $W = 1852.6$, $p < 2.2\ 10^{-16}$; 2018: $W = 475.35$, $p < 2.2\ 10^{-16}$, Watson-Wheeler test).

According to the mean angle of each species, we observed that *J. caroba* reached its peak approximately 31 days before the flowering peak of *J. decurrens* in the three years sampled **(Table 1, Fig. 1)**.

The flowering period of *J. caroba* was longer when compared to *J. decurrens*, with partial overlapping in September and October **(Fig. 1, Fig. 2A)**. When we considered the individuals sampled from each species, it was possible to observe that all of them have seasonal flowering and that the individuals of *J. decurrens* are more synchronous with each other when compared to the individuals of *J. caroba* **(Fig. 2B-C)**.

Table 1. Circular statistical analysis corresponding to the number of individuals with flowers from 2016 to 2018 in natural populations of *J. caroba* and *J. decurrens* (Bignoniaceae) present in savanna physiognomies of cerrado, municipality of Águas de Santa Bárbara, São Paulo, Brazil.

	J. caroba	*J. decurrens*
Mean vector	Aug-Sep	Sep-Oct
Length of mean vector (*r*)	0,93	0,96
Mean angle (α)	224,9°	255,0°
Rayleigh test (p)	0,93 (< 0,0001)	0,96 (< 0,0001)
Watson-Wheeler test (p)	1764,5(< 2,2 . 10^{16})	

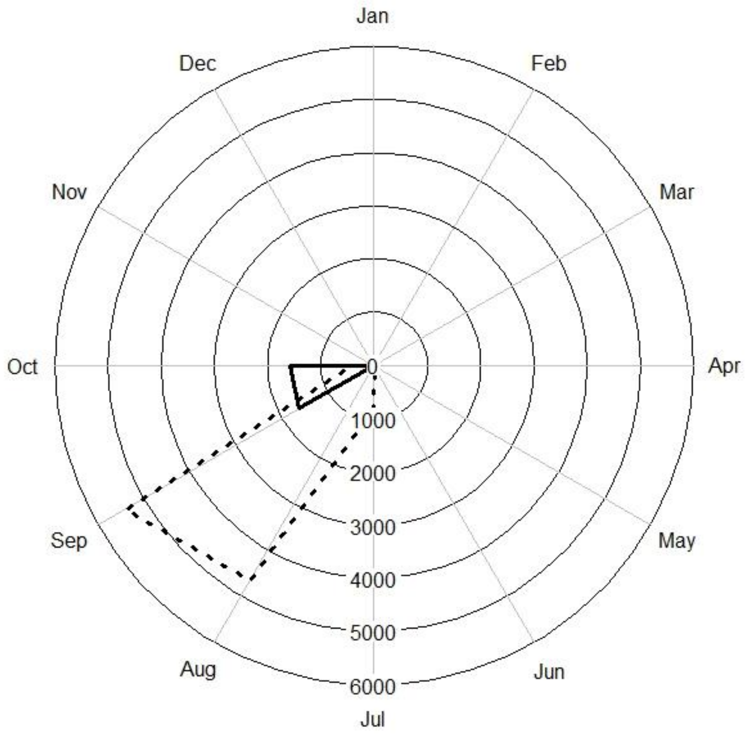


Fig. 1. Flowering phenology of *Jacaranda caroba* and *J. decurrens* (Bignoniaceae), co-occurring species in cerrado areas, municipality of Águas de Santa Barbara, São Paulo, Brazil. Dashed line indicates the number of flowers produced by *J. caroba* and full line corresponds to the number of flowers produced by *J. decurrens* from 2016 to 2018.

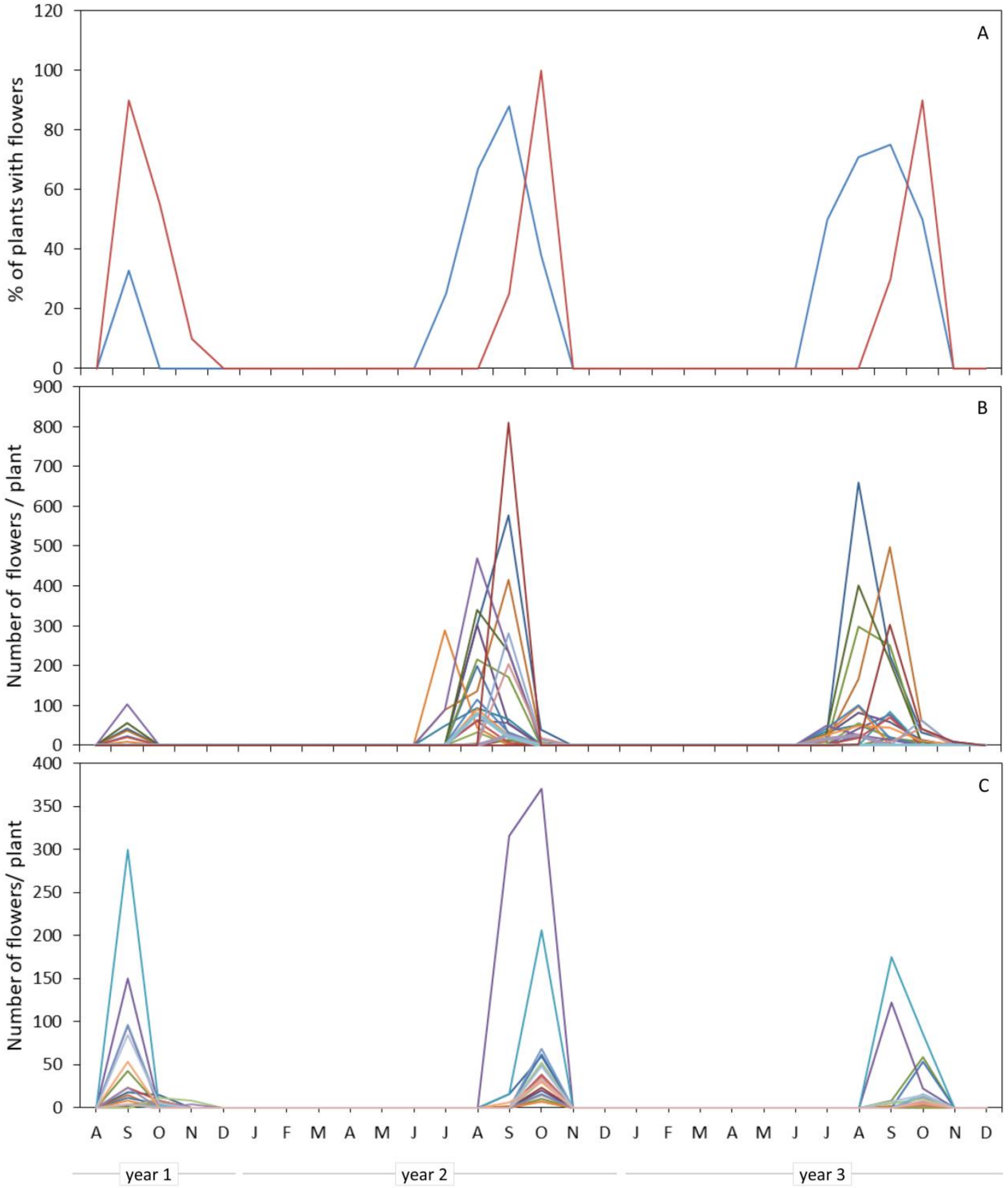


Fig. 2. Flowering phenology of two species of *Jacaranda* genus (Bignoniaceae). (A) Index of activity of *J. caroba* (—) and *J. decurrens* (—). (B) Number of flowers produced by each individual sampled throughout the flowering period of *J. caroba*. (C) Number of flowers produced by each individual sampled throughout the flowering period of *J. decurrens*.

The intensity of flower production varied throughout the reproductive period and between years. Thus, based on the phenology data, we defined the months as periods of floral advertisement for each year.

In 2016 we determined the August second fortnight (period 4) as the beginning of flowering of *J. caroba* and *J. decurrens* since the intensity of flowers production is still low; September first fortnight (period 5) as the peak of *J. caroba* and September's second fortnight (period 6) as the peak of *J. decurrens* flowering, since it is the period with the greatest number of open flowers; October first fortnight (period 7) as the end of the flowering period of *J. caroba* and October second fortnight (period 8) as the end of the flowering period of *J. decurrens* **(Fig. 3A)**.

In 2017 we determined the two fortnights evaluated in July (periods 1 and 2) as the beginning of the flowering of *J. caroba* since the intensity in the flower production is still low; the two fortnights evaluated in August (periods 3 and 4) correspond to half of *J. caroba* flowering period, with an intermediate number of flowers; the first half of September (period 5) as the peak of flowering of *J. caroba*, since it is the period with the greatest number of open flowers; the second fortnight of September (period 6) as the overlapping peak of *J. caroba* and *J. decurrens* flowering, corresponding to the period in which the number of flowers produced by *J. caroba* decreases considerably and *J. decurrens* is starting to emit flowers; the first fortnight of October (period 7) as the flowering peak of *J. decurrens*, because it is the moment with the greatest number of open flowers in this species; the second fortnight of October (period 8) as half of *J. decurrens* flowering period, since the number of open flowers begins to decrease considerably; and the first fortnight of November (period 9) as the end of the *J. decurrens* flowering period, since it is no longer possible to find opened flowers from that moment **(Fig. 4A)**.

In 2018 we found a flowering pattern similar to 2017, however, the peak of flowering of both species anticipated, corresponding to August second fortnight (period 4) in *J. caroba* and to September second fortnight (period 6) in *J. decurrens*. Period 6 also corresponding to flowering overlapping peak in these species **(Fig. 5A)**.

Fig. 3. Variation in flower production, frequency of mutualists, frequency of antagonists, and fruiting over periods with different intensity of floral display in the year of 2016. Trend lines describe the relationship between two variables based on lowest smoother using a Locally Weighted Regression (LOESS). Dashed lines correspond to the upper limit of the 95% confidence interval. The lower limit is zero and, therefore, the line was omitted. (A) Total number of flowers produced per plant in *J. caroba* (N_{plants} = 21 / period) and *J. decurrens* (N_{plants} = 20 / period). (B) Number of fruits / flower in *J. caroba* (N_{plants} = 21 / period) and *J. decurrens* (N_{plants} = 20 / period). (C) Total number of mutualists visits on *J. caroba* and *J. decurrens* flowers (N_{days} = 3 / period for each species). (D) Number of visits per flower of mutualists (N_{days} = 3 / period for each species). (E) Total number of antagonists visits on *J. caroba* and *J. decurrens* flowers (N_{days} = 3 / period for each species). (F) Number visits per flower of antagonists (N_{days} = 3 / period for each species). Hollow circles correspond to individuals of *J. caroba* and full circles correspond to individuals of *J. decurrens*.

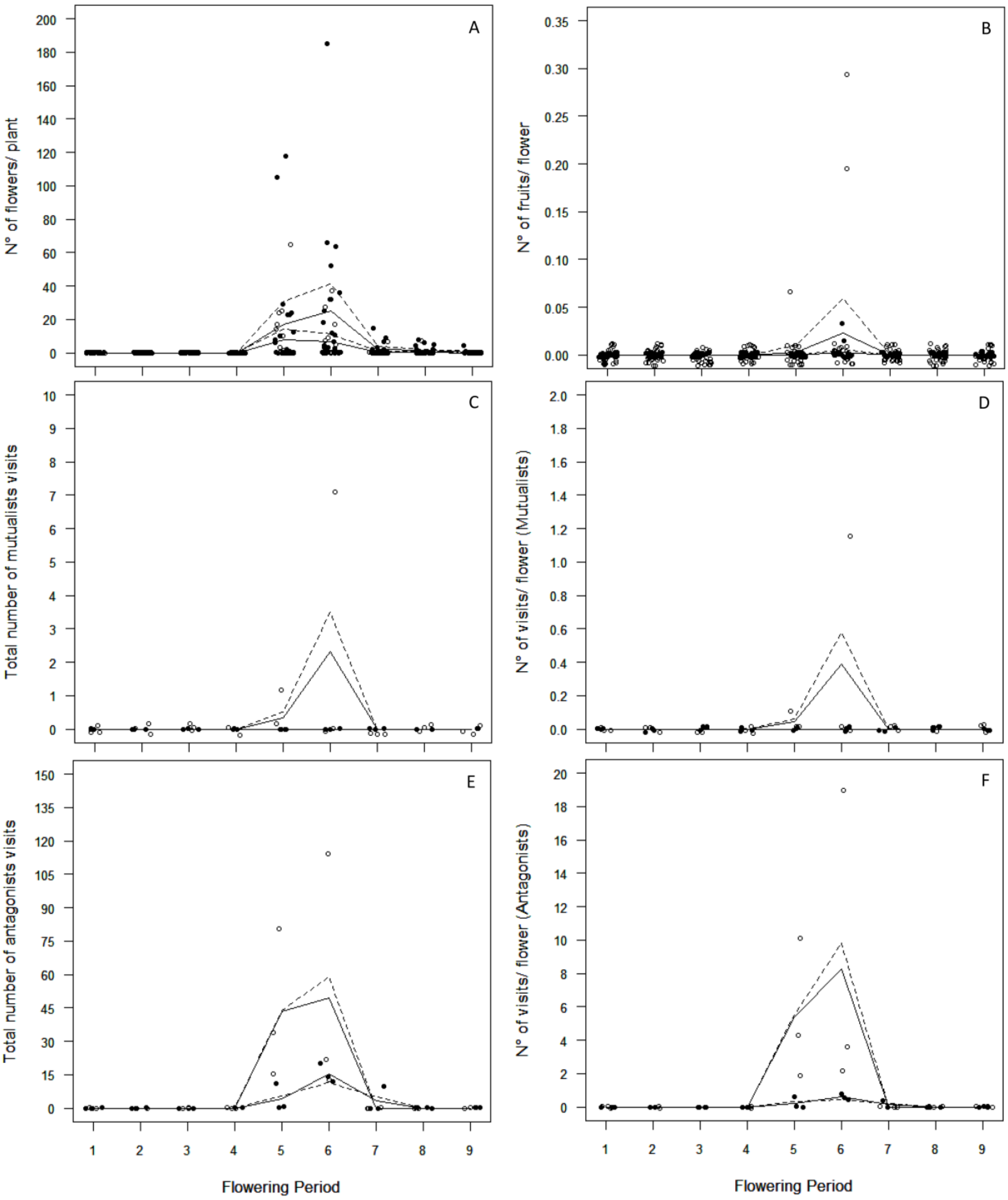

A
N° of flowers/ plant
0
20
40
60
80
100
120
140
160
180
200
B
N° of fruits/ flower
0.00
0.05
0.10
0.15
0.20
0.25
0.30
0.35
C
Total number of mutualists visits
0
1
2
3
4
5
6
7
8
9
10
D
N° of visits/ flower (Mutualists)
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
2.0
E
Total number of antagonists visits
0
15
30
45
60
75
90
105
120
135
150
F
N° of visits/ flower (Antagonists)
0
2
4
6
8
10
12
14
16
18
20
1
2
3
4
5
6
7
8
9
Flowering Period
1
2
3
4
5
6
7
8
9
Flowering Period

Fig. 4. Variation in flower production, frequency of mutualists, frequency of antagonists, and fruiting over periods with different intensity of floral display in the year of 2017. Trend lines describe the relationship between two variables based on lowest smoother using a Locally Weighted Regression (LOESS). Dashed lines correspond to the upper limit of the 95% confidence interval. The lower limit is zero and, therefore, the line was omitted. (A) Total number of flowers produced per plant in *J. caroba* (N_{plants} = 21 / period) and *J. decurrens* (N_{plants} = 20 / period). (B) Number of fruits / flower in *J. caroba* (N_{plants} = 21 / period) and *J. decurrens* (N_{plants} = 20 / period). (C) Total number of mutualists visits on *J. caroba* and *J. decurrens* flowers (N_{days} = 3 / period for each species). (D) Number of visits per flower of mutualists (N_{days} = 3 / period for each species). (E) Total number of antagonists visits on *J. caroba* and *J. decurrens* flowers (N_{days} = 3 / period for each species). (F) Number visits per flower of antagonists (N_{days} = 3 / period for each species). Hollow circles correspond to individuals of *J. caroba* and full circles correspond to individuals of *J. decurrens*.

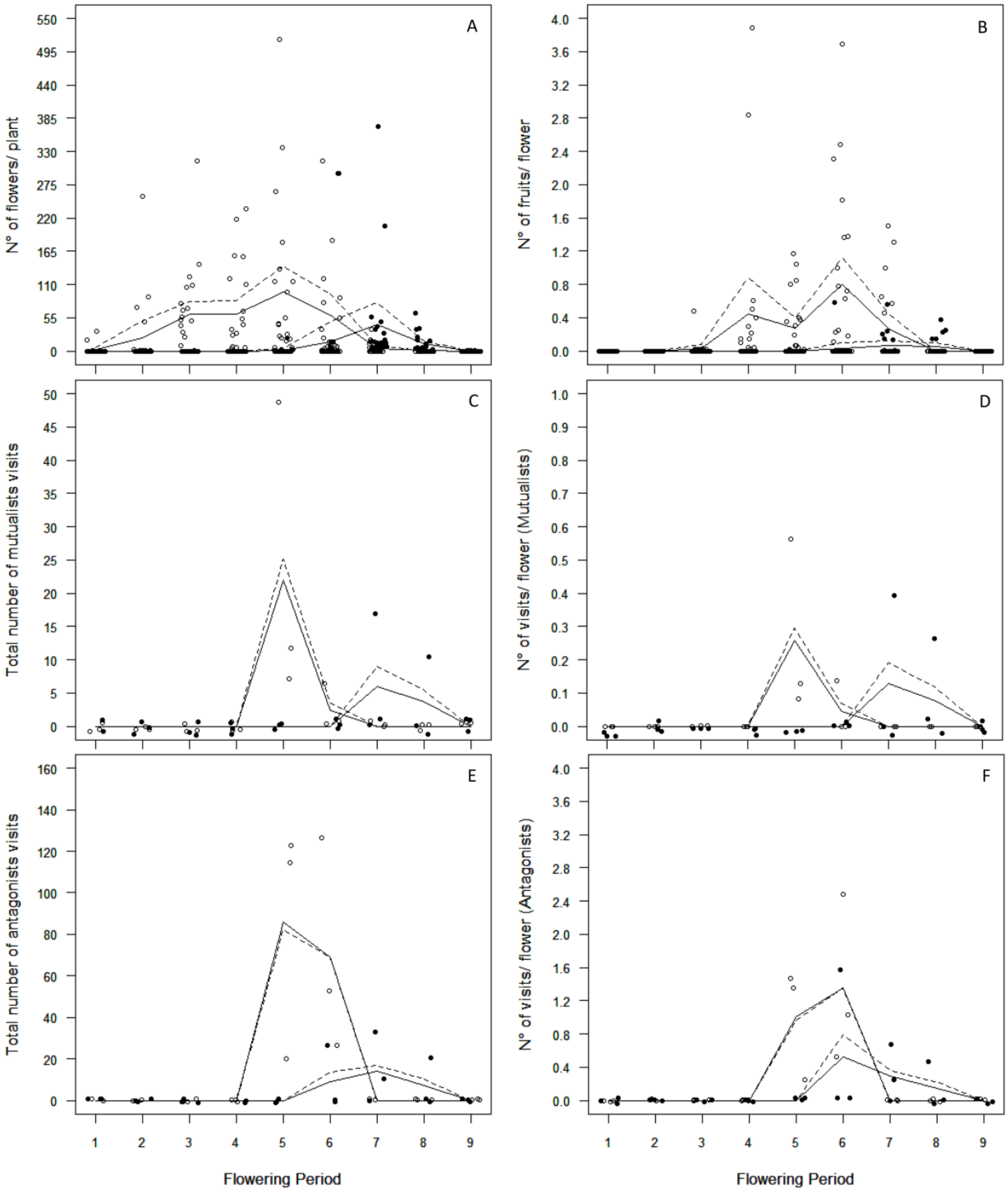
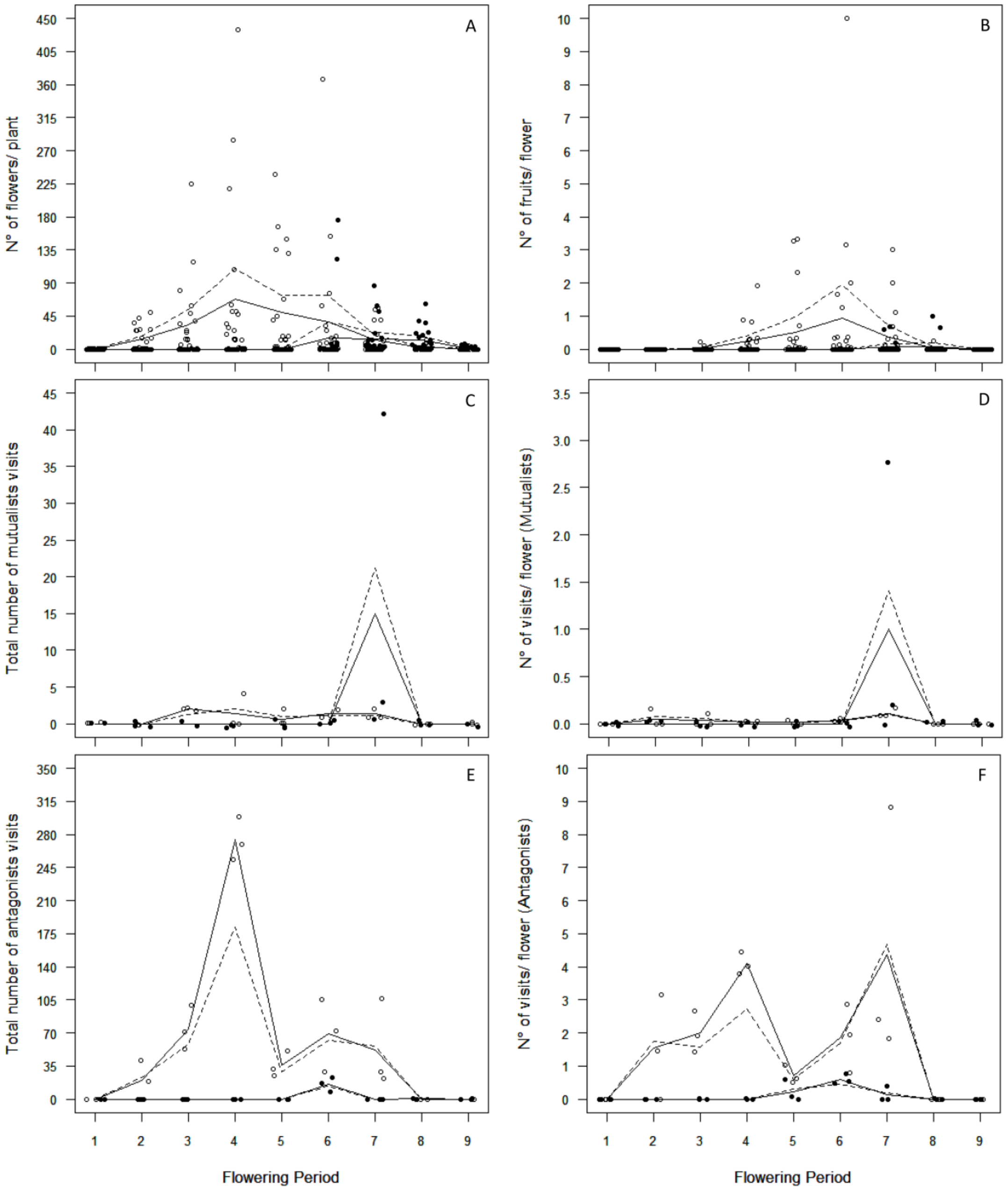

A
B
C
D
E
F
N° of flowers/ plant
N° of fruits/ flower
Total number of mutualists visits
N° of visits/ flower (Mutualists)
Total number of antagonists visits
N° of visits/ flower (Antagonists)
0
55
110
165
220
275
330
385
440
495
550
0.0
0.4
0.8
1.2
1.6
2.0
2.4
2.8
3.2
3.6
4.0
0
5
10
15
20
25
30
35
40
45
50
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
0
20
40
60
80
100
120
140
160
1
2
3
4
5
6
7
8
9
Flowering Period
Flowering Period

Fig. 5. Variation in flower production, frequency of mutualists, frequency of antagonists, and fruiting over periods with different intensity of floral display in the year of 2017. Trend lines describe the relationship between two variables based on lowest smoother using a Locally Weighted Regression (LOESS). Dashed lines correspond to the upper limit of the 95% confidence interval. The lower limit is zero and, therefore, the line was omitted. (A) Total number of flowers produced per plant in *J. caroba* (N_{plants} = 21 / period) and *J. decurrens* (N_{plants} = 20 / period). (B) Number of fruits / flower in *J. caroba* (N_{plants} = 21 / period) and *J. decurrens* (N_{plants} = 20 / period). (C) Total number of mutualists visits on *J. caroba* and *J. decurrens* flowers (N_{days} = 3 / period for each species). (D) Number of visits per flower of mutualists (N_{days} = 3 / period for each species). (E) Total number of antagonists visits on *J. caroba* and *J. decurrens* flowers (N_{days} = 3 / period for each species). (F) Number visits per flower of antagonists (N_{days} = 3 / period for each species). Hollow circles correspond to individuals of *J. caroba* and full circles correspond to individuals of *J. decurrens*.


A
N° of flowers/ plant
0
45
90
135
180
225
270
315
360
405
450
B
N° of fruits/ flower
0
1
2
3
4
5
6
7
8
9
10
C
Total number of mutualists visits
0
5
10
15
20
25
30
35
40
45
D
N° of visits/ flower (Mutualists)
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
E
Total number of antagonists visits
0
35
70
105
140
175
210
245
280
315
350
F
N° of visits/ flower (Antagonists)
0
1
2
3
4
5
6
7
8
9
10
1
2
3
4
5
6
7
8
9
Flowering Period
Flowering Period

4.2. Flowering dynamics and reproductive success

We verified that fruit production varied according to the sampled period **(Fig. 3B, 4B, 5B)**. In 2016 the fruit production curve was directly proportional to the flower increasing, concentrated in the month of September (periods 5 and 6) as well as the flowering of *J. caroba* and *J. decurrens* **(Fig. 3A-B)**. However, over the years 2017 and 2018, this relation changed.

In 2017 *J. caroba* the efficiency in fruit production was higher in periods with low availability of flowers. The fruit / flower ratio was inversely proportional to the floral display intensity, with the highest values at middle and end of flowering (periods 4 and 6, respectively) and the lowest values at the peak of flowering (period 5) **(Fig. 4B)**. However, one of the periods in which the fruit/ flower ratio was higher corresponds to the overlapped period of *J. caroba* and *J. decurrens* flowering (period 6) **(Fig. 4A-B)**. In *J. decurrens* the fruit production started at the beginning of the flowering period, which corresponds to the moment of overlap with *J. caroba* (period 6) **(Fig. 4A-B)**. However, in general, the relationship between fruiting and floral display intensity was different from that found in *J. caroba*, considering that the highest fruit / flower ratio values were found at peak flowering (period 7) and maintained until the time when flower production presented the lowest values in this species (period 8) **(Fig. 4A-B)**.

In 2018, the fructification of *J. caroba* was similar to 2017 pattern, with a peak production in a period in which the production of flowers decreases (periods 5 and 6) **(Fig. 5A-B)**. One of the periods in which the fruit/ flower ratio was higher also corresponds to the overlapped period of *J. caroba* and *J. decurrens* flowering (period 6). *Jacaranda decurrens* also presented higher fruit production in periods with low number of flowers (periods 7 and 8) **(Fig. 5A-B)**.

4.3. Flowering dynamics and interactions with floral visitors

4.3.1. Species richness and visiting behavior

Both plant species were visited by small and medium-sized bees belonging to different functional groups **(Fig. 6A-D)**, as well as interacting with hummingbirds. We classify floral visitors according to their behavior and the type of resource collected as mutualists (pollinators) or antagonists (nectar robbers and nectar thieves) **(Table 2)**.

Table 2. Floral visitors of *Jacaranda caroba* and *J. decurrens* (Bignoniaceae), co-occuring species in savanna physiognomies of cerrado, municipality of Águas de Santa Bárbara, São Paulo, Brazil

Species	*J. caroba*	*J. decurrens*	Visiting behavior	Collected resource
ANDRENIDAE				
Oxaea flavescens	present	present	Non legitimate	Nectar
APIDAE				
Bombus morio	present	present	Legitimate	Nectar
Ceratina sp.	present	present	Non legitimate	Nectar
Epicharis sp.	absent	present	Legitimate	Nectar
TROCHILIDAE				
Chlorostilbon lucidus	present	absent	Non legitimate	Nectar

4.3.1.1. Mutualists

The only bee species classified as a legitimate visitor of *J. caroba* was *Bombus morio*, since this bee contacted both stigma and anthers during the visits **(Fig. 6A)**. This bee visited the flowers searching for nectar that is stored in the nectariferous chamber present in the bottom of the corolla tube. For this, it approached the flower and used the lower lobe of the corolla as landing platform, grabbing it and dragging towards the basal portion of the tube. During this process, the upper part of its head and the dorsal portion of its thorax touched the reproductive structures, first contacting the fertile portion of the stigma (inner face of the lobes) and then the anthers. After collecting nectar, the bee left the flower, usually with a head and torax covered with pollen. Upon leaving the flower, these bees contacted only the sterile portion of the stigma (outer face of the lobes). The dimensions of *B. morio*, especially the diameter of the thoracic region (dorsiventral thickness = 8.37 ± 0.48 and laterolateral thickness = 8.49 ± 0.28), allow a fit in relation to the dimensions of the flowers of *J. caroba* (largest diameter of corolla entry = 17.34 ± 2.12, smaller diameter at corolla entry = 6.98 ± 1.74). *Bombus morio* visited on average 3.52 ± 2.04 flowers per turn, remaining approximately 3 seconds in each flower, being its most frequent visits in the morning, with peak between 9:00 and 11:00 h.

In *J. decurrens,* we observed two species of bee classified as mutualists: *B. morio* and *Epicharis* sp. **(Fig. 6A-B)**. *Bombus morio* behavior and period of visits are the same described to *J. caroba*, however the body size of these bees are larger than the diameter of the corolla tube of some flowers, so, when entering the flower they can tear the corolla. On the other hand, *Epicharis* sp. has smaller body sizes when compared to *B. morio* (dorsiventral thickness = 4.94 ± 0.16 and laterolateral thickness = 5.23 ± 0.29), presenting a complete fit to the flowers of *J. decurrens* (the largest diameter at the corolla entrance = 11.70 ± 1.73, smaller diameter at the corolla entrance = 5.08 ± 1.08). *Epicharis* sp. presented a behavior similar to that described to *B. morio* visiting the flowers of *J. decurrens,* apparently searching for nectar as food resource. These bees approached the flower landing on corolla’s lower lobe and crawled into the tube collecting nectar stored in the nectariferous chamber. During this process the whole body of the bee came in contact with the reproductive structures of the flower. When leaving the flower, the dorsal portion of its body was covered with pollen grains. The foraging schedule of this species was concentrated in the afternoon, with peak activity between 14:00 and 16:00 h.

4.3.1.2. Antagonists

Ceratina sp. and *Oxaea flavescens* bees established antagonistic interactions with both *J. caroba* and *J. decurrens,* and we classified them as nectar thieves and nectar robbers respectively **(Fig. 6C-D)**. Bees of the genus *Ceratina* sp. **(Fig. 6C)**, approaching the flowers of both species apparently searching for nectar, they entered on the corolla and walked laterally to the staminodium until reaching the nectariferous chamber at the bottom of the floral tube where the nectar is stored. However, because of their small body dimensions they did not contact the flower reproductive structures. These bees remained in the flower for approximately 20 seconds and visited on average 1.48 ± 0.9 flowers per turn. These bees collected nectar throughout the day, concentrating the visits between 10:00 and 14:00 h.

Oxaea flavescens was classified as a nectar robber in both *Jacaranda* species because they collected this resource by piercing the corolla base externally, causing damage to the flower without transfering pollen grains to the stigma **(Fig. 6D)**. Besides, the action of these bees may have a negative indirect effect on the visit of pollinators, because they can associate the damage left by antagonists to the absence of resource (Chapter 2), these bees also presented agonistic behavior chasing away any approaching visitors of the flowers they were patrolling. They contacted on average 3.81 ± 2.70 flowers per turn and remained approximately 2 seconds in each flower. Their visits were concentrated in the morning, with peak between 9:00 and 12:00 h.

Finally, another species that we classified as nectar robber was *Chlorostilbon lucidus* (Trochilidae). These hummingbirds had similar visiting behavior to *O. flavescens.* They interacted only with *J. caroba* flowers, approaching the flowers and collecting nectar externally piercing the corolla basis. These hummingbirds were sighted in the early morning between 7:00 and 9:00 am.

4.3.2. Floral display and frequency of visits

The visitation of *J. caroba* and *J. decurrens* flowers varied according to the floral advertisement. In general, we observed a higher frequency of mutualists visits on the flowering peaks of both *Jacaranda* species, with the visit curve showing a directly proportional relation to the intensity of flowers produced by each plant species in terms of total visits **(Fig. 3C, 4C, 5C)**, and in terms of number of visits per flower **(Fig. 3D, 4D, 5D)**. However, in 2017 the frequency of mutualist visits decreased during the period of flowering overlap of both species (period 6), presenting values similar to the beginning and the end of each flowering, when the floral advertisement intensity was lower **(Fig. 4C-D)**.

In relation to the antagonists, the total number of visits was proportional to the intensity of floral advertisement during the nine periods sampled, since we observed a higher number of visits of these animals in the peak of flowering of *J. caroba* and in the period of flowering overlap between *J caroba* and *J. decurrens* **(Fig. 3E, 4E, 5E)**. However, in terms of visit number per flower, the highest values occurred in the flowering overlapping period, presenting an inverse relation to that found for mutualists **(Fig. 3F, 4F, 5F)**. In 2018, we observed that the higher frequency of antagonistic visitors of *J. caroba* corresponding to the flowering peak of this plant species (period 4) and overlapping peak of *J.caroba* and *J. decurrens* (period 6).

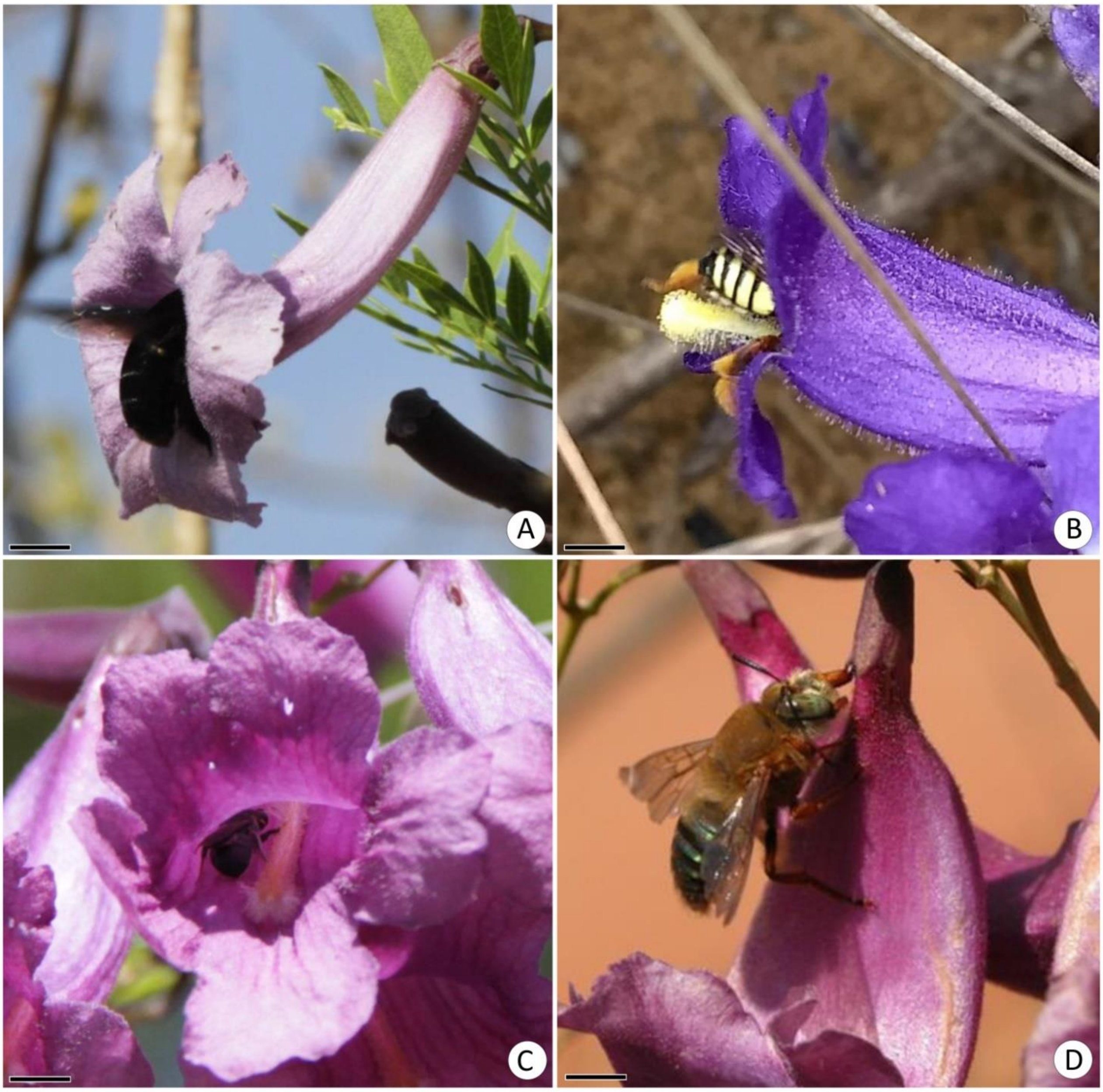


Fig. 6. Floral visitors of *J*. *caroba* and *J*. *decurrens*. (A) *Bombus morio* legitimately visiting a *J*. *caroba* flower. (B) *Epicharis* sp. legitimately visiting a *J*. *decurrens* flower. (C) Nectar thief *Ceratina* sp. entering the corolla of *J*. *caroba* by the lateral portion, walking next to the staminodium. (D) *Oxaea flavescens* robbing nectar through a slit produced externally to the corolla of *J*. *caroba* flower.

5. DISCUSSION

In our study, we evaluated the influence of floral display on reproductive success and interaction with mutualists and antagonists of two co-occurring species of the genus *Jacaranda*. We evaluated the effect of flowering dynamics of these species individually as well as in the period in which they overlap over three consecutive years. We verified that this dynamic is associated with the interactions that both establish with a network of mutualistic and antagonistic visitors and the formation of fruits.

Jacaranda caroba and *J. decurrens* showed seasonal flowering, concentrated in a few months of the year, besides sequential. Some studies have shown that plants pollinated by animals, such as the species of the present study, show greater variation in the flowering period than species pollinated by the wind (Proctor et al. 1996, Bolmgren et al. 2003). This suggests that the displacement in the flowering period observed in species pollinated by biotic agents may represent an evolutionary strategy in response to pollinator competition (Sargent & Ackerly 2008). However, *J. caroba* and *J. decurrens* present a period of flowering overlap in the three years sampled. In general, convergence in flowering phenology may have a function of pollinator-mediated facilitation, since flowering of distinct species may increase floral display and attract more pollinators together (Aizen & Vásquez 2006, Sargent & Ackerly 2008). In fact, we observed that the *Jacaranda* species sampled in this study share pollinators. Both are pollinated by *Bombus morio,* as described previously (Yanagizawa & Maimoni Rodella 2007, Quinalha et al. 2017). In this context, sequential flowering dynamics with a period of overlap presented by these two species can favor coexistence, increasing attractiveness and reducing competition.

Jacaranda caroba presented a more intense and long floral display when compared to *J. decurrens*. The second, on the other hand, presented more synchronous individuals, with many plants reaching the peak of flowering together, while the plants of *J. caroba* had peaks distributed throughout flowering months. In fact, the pattern presented by *J. caroba* seems to be more common, since plants in natural populations rarely have a perfectly synchronous flowering, since there are, besides environmental variations, intrinsic differences of each individual (Hendry & Day 2005). This variation in the flowering phenology influences the population genetic structure of the species, since it can determine how the pollen flow occurs according to the compatible pairs available in the population, leading to different selective responses (Kitamoto et al. 2006). This is because, in species with lower synchrony between the beginning and end of flower emission, the pollen flow tends to be higher among more distant individuals, since there would be few individuals close flowering at the same time (Kitamoto et al. 2006). In contrast, species synchronous with massive flower production in the same period could have the proportion of pollen flow increased among close individuals (Kitamoto et al. 2006). This variation is directly related to the behavior of floral visitors, since, according to MacArthur & Pianka's (1966) theory of optimal foraging, pollinators would tend to forage closer individuals, obtaining greater amount of trophic resources with lower energy.

In this sense, the variation in intraspecific flowering phenology is especially important due to the influence on plant-animal interactions since the intensity of the floral display may be related to the attractiveness of floral visitors (Elzinga et al. 2007, Zhao et al. 2016). Several studies have reported directional selection of more intense floral advertisements, since they are more attractive to anthophilous animals (Sandring & Ågren 2009, Parachnowitsch & Kessler 2010, Sletvold et al. 2010). In fact, we observed that the intensity of biotic interactions changed throughout the flowering period of *J. caroba* and *J. decurrens*. We observed an increase in the frequency of both mutualists and antagonists proportional to the increase of the flowering curve of both species over the three years sampled.

At the beginning of the flowering period, we did not observe visits of pollinating bees. This may be due to the fact that bees need to learn that the resource is available, which may take some time to link the visual stimulus to the resource supply (Strang & Sherry 2014). In the case of nectariferous plant species pollinated by medium-sized bees, the association between advertisement and resource is even more important as they are able to distinguish greater availability of nectar (Makino & Sakai 2007).

At the peak of flowering of each species of *Jacaranda* we observed the highest values of frequency of visits in both total number and number of visits per flower. This was even more evident in the year of 2017 when *J. caroba* showed a higher frequency of mutualist visits compared to *J. decurrens*, evidencing the importance of floral advertisement intensity in the attractiveness of anthophilous animals (Sandring & Ågren 2009, Sletvold et al. 2010). However, in both species there was a decrease in these frequencies in the period after the flowering peak. Our observations corroborate several studies that affirm that the frequency of visits of pollinators is dependent on the intensity of floral display (see Elzinga et al. 2007), and that after an intense peak in flower production there may be a reduction of pollinators leading to limitation of pollen flow (Gomez 1993).

In the years 2017 and 2018 the period after the flowering peak in *J. caroba* corresponds to the period of overlapping of this phenophasis with *J. decurrens*. In this period, the frequency of interactions with mutualists decreased in 2017 and remained low in 2018. This scenario can be attributed to two factors: (1) the density of floral advertisement: this is the time when *J. caroba* has considerably reduced the number of flowers and *J. decurrens* have few plants in activity; (2) the effect of the antagonists, since in this period the visits of these animals reached the high values. This increase of the antagonists in the period of overlap is associated with the fact that they visit the flowers of *J. caroba* and *J. decurrens* indiscriminately which indicates that the floral display of the two species together has an effect on the attraction of these animals.

In our study, two bee species (*Oxaea flavescens* and *Ceratina* sp.) and one species of hummingbird (*Chlorostilbon lucidus*) were classified as antagonists due to their behavior of collecting nectar without transfer of pollen grains. Most plant species exhibit simultaneous interactions with mutualists (e.g., pollinator) and antagonists (e.g., thieves and robbers of floral rewards) (Strauss & Irwin 2004). Cheating behavior is commonly observed in animals that interact with Bignoniaceae species, including the mutualist-plant-antagonist system of interactions involving *Jacaranda* species, were subject of previous studies conducted by our research group (Guimarães et al. 2008, Quinalha et al. 2017). The interaction of these antagonists may interfere directly in flower signaling to the pollinators (Chapter 2), and may lead to changes in nectar volume and concentration (Castro et al. 2009, Chapter 3). In this context, the direct and indirect effects caused by these agents should be considered simultaneously for a more complete and integrative understanding of the dynamics of interactions and the consequences for the reproductive success of *Jacaranda* species.

In general, the studies show the importance of the attractiveness of pollinators on the plant species' reproductive success, and that floral display intensity is an important factor in this process efficiency (Mitchell 1994, Conner & Rush 1996, Engel & Irwin 2003, Karron & Mitchell 2011). However, this relationship is not always directly proportional. Evaluating the fruiting during the floral display periods determined in this study, we observed that the peak of flowering of *J. caroba* was the period in which this species presented the lowest values of fruits produced by flower, indicating that in this species the intensity of floral advertisement has an inversely proportional relation to reproductive success. This can be attributed to the fact that in hermaphrodite and self-incompatible species (eg, *J. caroba* and *J. decurrens* - Chapter 4), the behavior of the pollinator is crucial in the formation of new progenies. We observed that both *B. morio* and *Epicharis* sp. visited sequentially several flowers of the same individual which may have been responsible for the decrease in the reproductive success of these species, since these pollinators tend to transfer an increasing proportion of pollen grains from the same plant in subsequent visits, interfering in the chances of pollen grains from cross-pollination germinate (Duncan et al. 2004). However, in *J. decurrens* the relationship between floral display intensity and reproductive success was different from that found in *J. caroba*, considering that the highest fruiting values were found at the peak of flowering and were maintained until the moment when the production of flowers presented the lowest values in this species. In this sense, floral display less expressive and a high flowering synchrony of plants in *J. decurrens*, seems to be an important factor to the reproductive success of this species, since, when finding fewer flowers per plant, the pollinator will need to include more plants in its foraging route to supply its energy demands, increasing the chances of crossing between plants in the population.

6. CONCLUSIONS

In this study we verified that the floral display has several effects on the attractiveness of antagonists and mutualists, as well as on the reproductive success of two co-occurring plant species. The intensity of floral advertising influences the interaction with a network of mutualistic and antagonistic visitors, who, although increasing the foraging to the flowers of these species as the floral display becomes more expressive, present different behaviors during the flowering overlapping period.

However, although the interaction with mutualists intensifies in the periods when there is a greater production of flowers, the relation with the reproductive success was opposite. In this context, we show that, for self-incompatible hermaphrodite species, more moderate flower display may represent a more important component for increase reproductive success than excessive flower production, which in addition to affect the reproductive success' female component negatively requires high energy demand from plants. Thus, for *Jacaranda* species studied here, this would be an "excellent display" considering the effectiveness of fruit production. In this case, the importance of flowering peak would be more directly related to the performance of visitors, ensuring a resources supply compatible with their energy needs. Thus, we could consider that, in *Jacaranda*, the flowering peak would be conditioning pollinators to visit and the end of the flowering period would be more directly related to the plants fitness, because with fewer flowers per plant and with the visitation of pollinators already established in the area, the chances of compatible crosses would increase leading to greater reproductive success of plant species.

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CAPÍTULO 2

Manuscrito submetido ao periódico científico Plant Biology

Do medium-sized bees avoid flowers with nectar robbery signals?

1. ABSTRACT

Some floral visitors collect nectar by piercing the flower's external whorls, acting as robbers. In addition to reducing the amount of nectar available to pollinators, they leave vestiges of the robbery, which can cause changes in floral characteristics. These changes include physical and chemical signals that may influence flower recognition by pollinators. Thus, if pollinating bees associate these changes with absence or reduction in nectar volume, they can avoid these flowers, negatively impacting pollination. Therefore, we aimed to investigate if there is an association between the vestiges left by nectar robbers and the pollination of two bee-pollinated species of the *Jacaranda* genus. Both plant species have their floral nectar stolen by *Oxaea flavescens,* a bee referred as specialized in nectar robbing of cerrado species of Bignoniaceae, which makes slits at the corolla base during the nectar gathering. In order to evaluate the effects of the vestiges of slits left by *O. flavescens* on the pollination of *J. caroba* and *J. decurrens* we performed field experiments on flowers with and without damage and counted the pollen grains deposited on the stigmas in flowers of both treatments. We also performed chemical analysis of volatile organic compounds (VOCs) on flowers with and without damage. We observed that there was no difference in pollen deposition between treatments with and without damage in *J. caroba* flowers. Besides, we also didn't found difference in VOCs emitted in both treatments. Thus, we showed that nectar-robbery by bees, although very frequent, apparently do not affect the pollinator visitation *J. caroba* and *J. decurrens*, suggesting that this system may be in a complex balance encompassing mutualists and antagonists.

Key words: Chemical signaling, floral damages, nectar-robbers, *Oxaea flavescens*, visual signaling.

2. INTRODUCTION

Some groups of floral visitors feed on floral nectar without pollinating the flowers, being considered robbers for violating the integrity of flower by collecting this resource, causing holes or slits on external whorls (sensu Inouye 1980). These robbers, in addition to reducing the amount of nectar available in flowers, may negatively impact plant performance by altering other floral characteristics (Irwin et al. 2000, Rojas-Nossa et al. 2016). These changes include signs, which may be physical, and chemical, which may indicate to pollinators the reduction in the amount of nectar (Mallinger & Prasifka 2017), causing them to avoid flowers with vestiges of robbery and thus, reducing the chances pollination (Hazlehurst & Karubian 2016).

Young bees, at the beginning of their foraging activities, usually learn which flowers provide the largest amounts of resource (Waddington et al. 1998) and, by influence of floral traits that attract them, such as shape, size, color, flower texture; decision-making for visitation is made (Chittka & Thomson 2004). An important factor in bees' foraging strategies is their innate preference for certain flower colours. For example, bumblebees and honeybees prefer blue and blue-green flowers (Giurfa et al. 1995, Hempel de Ibarra et al. 2014), and are able to learn to associate the coloration of the flower with the resources present therein (Gumbert 2000, Ings et al., 2009; Strang & Sherry 2014). Besides the perception of the coloring pattern and the shape of the flowers, the bees are able to associate the chemical characteristics of the flower, i.e. odor, with its reward (Chittka 2017).

Thus, due to the association and bees’ learning ability, it is possible that the damages promoted by the nectar robbers negatively affect the plant pollination by visual signaling, since it may change the colour patterns and the original appearance of the corolla, as well as by chemical signaling, by altering the volatile compounds emission from damaged flowers (Mallinger & Prasifka 2017). Both means of perception could lead bees to restrict their visits to undamaged flowers, where there would be a better cost-benefit ratio (MacArthur & Pianka 1966).

In this scenario, we chose as a model a mutualist-plant-antagonist system involving Bignoniaceae species, whose interaction network includes both pollinating bees and bees that steal nectar from their tubular flowers (Genini et al. 2010). Thus, our objective was to investigate if there is an association between the vestiges left by nectar-robbers-bees and the pollination of two species of the *Jacaranda* genus.

3. MATERIAL AND METHODS

3.1. Study area and plant species

The study was carried out at the Estação Ecológica de Santa Bárbara (EESB), belonging to the Instituto Florestal de São Paulo (IF-SP), located at municipality of Águas de Santa Bárbara, São Paulo, Brazil (22º46 '- 22º50' S and 49º10 '- 49º15' W). The reserve has 2712 hectares of cerrado *sensu lato*. The humid-dry tropical climate has average temperatures between 16ºC and 23ºC in the colder and hotter months, respectively. Annual rainfall varies is on average 1200 mm (Melo & Durigan, 2011).

In order to answer our questions, we choose as models the species *Jacaranda caroba* (Vell.) DC. and *Jacaranda decurrens* Cham. These plant species co-occur in cerrado's open physiognomies and in cerrado *sensu stricto* (a savannah-like vegetation). Previous studies have shown that *Bombus morio* Swederus (1787) acts as a pollinator of both species (Quinalha et al., 2017, Chapter 1) and *Epicharis* sp. Klug. (1807) act as pollinator of *J. decurrens* (Yanagizawa & Maimoni Rodella 2005, Souza et al. in prep.). Both plant species have their floral nectar stolen by *Oxaea flavescens* Klug. (1807) (Quinalha et al., 2017, Souza et al. in prep) a bee referred as specialized in nectar robbing of cerrado species of Bignoniaceae family, which makes slits at the corolla base, due to adaptations in its proboscis that allows a fast and efficient perforation of the floral tube (Gottsberger & Silberbauer-Gottsberger 2006).

3.2. Nectar robbing experiment

We made focal observations in the field in order to better characterize the behavior of pollinating bees and the nectar-robber bees.

In order to evaluate the effects of the slits left by *O. flavescens* on the pollination of *J. caroba* and *J. decurrens* we performed field experiments in two consecutive years (2017 and 2018). In 2017, we randomly took 13 flowering individuals of *J. caroba* ($N_{flowers}$ = 16) and 19 individuals of *J. decurrens* ($N_{flowers}$ = 33), and divided them into two treatments based on the presence / absence of slits caused by *O. flavescens*: with damage - nine flowers in *J. caroba* and 17 in *J. decurrens* and; without damage - seven flowers in *J. caroba* and 16 in *J. decurrens*. In 2018, we took 66 flowering individuals of *J. caroba* ($N_{flowers}$ = 101) and 69 of *J. decurrens* ($N_{flowers}$ = 120) and also were divided them into two treatments: with damage - 51 flowers in *J.* caroba and 59 in *J. decurrens*; without damage - 50 flowers in *J. caroba* and 61 in *J. decurrens*.

In each individual plant, we used one to two freshly opened flowers to perform each the experiment. The flowers remained exposed to floral visitors for 10 hours. To ensure that the undamaged flowers remained intact from the beginning of their opening until the end of the day, we wrapped the corolla basis with adhesive tape. In order to standardize and avoid any interference of the adhesive tape in the visitation of mutualistic and antagonistic agents, we also coated with tape the flowers with slits. By the end of the exposure period, we collected the stigmas of all flowers from both treatments and fixed them on slides containing acetic carmine solution for counting the pollen grains deposited onto the surface under a light microscope.

3.3. Chemical analysis

In order to determine if the damage promoted by nectar robbers on the corolla tubes of *J. caroba* and *J. decurrens* affect the compounds emitted by the flowers, we compared the floral scent of flowers with presence and absence of slits from both plant species. For that, we followed the protocol by Dötterl et al. (2005). We wrapped, individually, in polyethylene bags three flowers of each treatment ($n_{flowers}$ = 1/ plant) from *J. caroba* and three flowers of each treatment from *J. decurrens* ($n_{flowers}$ = 1/ plant), totaling 12 flowers. We collected the VOCs accumulated inside the bag with adsorbent traps connected to a membrane pump with an airflow of 200 ml/min. We used adsorbent tubes made from micro vials, filled with a mixture of 1.5 mg Tenax-TA (60–80 mesh) and 1.5 mg of Carbotrap B (20–40 mesh). We collected each sample during one hour. The samples were stored in a freezer at approximately -20 °C prior to the analysis. We analyzed the VOCs samples on a Thermo Scientific GC-MS, model FOCUS with an automatic sampler (Thermo – triplus DUO) and coupled to a Thermo – ISQ 230ST mass detector. We used a TG-5MS column and maintained a constant 1 mL/min flow of helium as the carrier gas. Before the injection, we put the traps in vials for 20 min, at 200°C, in a heating oven. We collected from inside the vial two microliters of sample with an automated gas tight syringe at 140°C and injected immediately into the GC injector in splitless mode, with the injector temperature being 200°C. We co-injected the samples with a 500-µL mixture of n-alkanes (C7–C30) at 0.1% of concentration. The column temperature was initially 50°C and was increased to 250°C increasing 5°C per minute and kept constant for 5 min. The

MS interface worked at 250ºC. The detector was operated in electron impact ionization mode (70 eV), with a scanning range of 34–350 m/z. In order to try identifying the volatile compounds we used NIST 08, and Adams (2007) mass spectral libraries. Besides, we compared the mass spectra and Kovats Retention Indices (RI) of target compounds with that of authentic standard compounds. For quantitative analysis of VOCs we injected 100 ng each of ca. 150 components into the GC-MS system. Among these compounds there were monoterpenes, aliphatic, and aromatic compounds. To estimate the total amount of scent available in the scent samples, we used the mean of the peak areas (total ion current) of these compounds (Etl et al. 2016).

3.4. Statistical analysis

To evaluate the effect of the damage left by *O. flavescens* on the deposition of pollen on the stigma of the two *Jacaranda* species, we used the Wilcox-Mann-Whitney test *U*.

To verify the relationships between the different groups of compounds emitted by flowers of each treatment performed in *J. caroba* and *J. decurrens* we visualized this compounds on a two dimensional ordination of the scent space, aiming to see if distinct scent clusters or groups appear. This ordination was obtained through the method of non-linear multidimensional scaling (NMDS) using a package vegan in R v. 3.4.3 (R Development Core Team 2017). We used Bray-Curtis dissimilarity index to calculate distance matrix from the scent data.

4. RESULTS

4.1. Floral visitors

Oxaea flavescens behaves as an nectar robber in both plant species, because it lands on the floral tube with the head facing the calix and pierces the corolla tube base, accessing externally the nectar accumulated in the nectariferous chamber **(Fig.1A-B)** and leaving one to two slits at each visit **(Fig. 1C-D)**. In *J. caroba*, *O. flavescens* visited approximately three flowers and two inflorescences per individual, remaining around two seconds in each flower. His visits began at about 8:30, with a peak of visitation from 10:00 to noon. In J. *decurrens*, its visits were less frequent, but followed the same pattern.

Regarding pollinating bees, in the two years of field observations, we registered *Bombus morio* legitimately visiting *J. caroba* flowers by entering the floral tube apparently searching for nectar **(Fig. 2A)**. We noticed that its visits occurred from 8:00 to 9:00. In *J. decurrens*, we observed legitimate visits of *Epicharis* sp in 2017 **(Fig. 2B)**, but in the flowering of 2018, we did not register any visit of this bee. In 2018, we just observed medium-sized bees, apparently *Bombus morio,* also legitimately visiting *J. decurrens* flowers **(Fig. 2C)**.

Fig. 1. *Oxaea flavescens* Klug. (1807) visiting *Jacaranda* Juss. flowers (Bignoniaceae) in savannah physiognomies of cerrado vegetation, at Águas de Santa Bárbara municipality, São Paulo state, Brazil. (A) *O. flavescens* visiting *J. caroba* flowers. (B) *O. flavescens* visiting *J. decurrens* flowers. (C) Damage caused by *O. flavescens* in flower of *J. caroba*. (D) Damage caused by *O. flavescens* in flower of *J. decurrens*. (Bar = 0.5 cm).

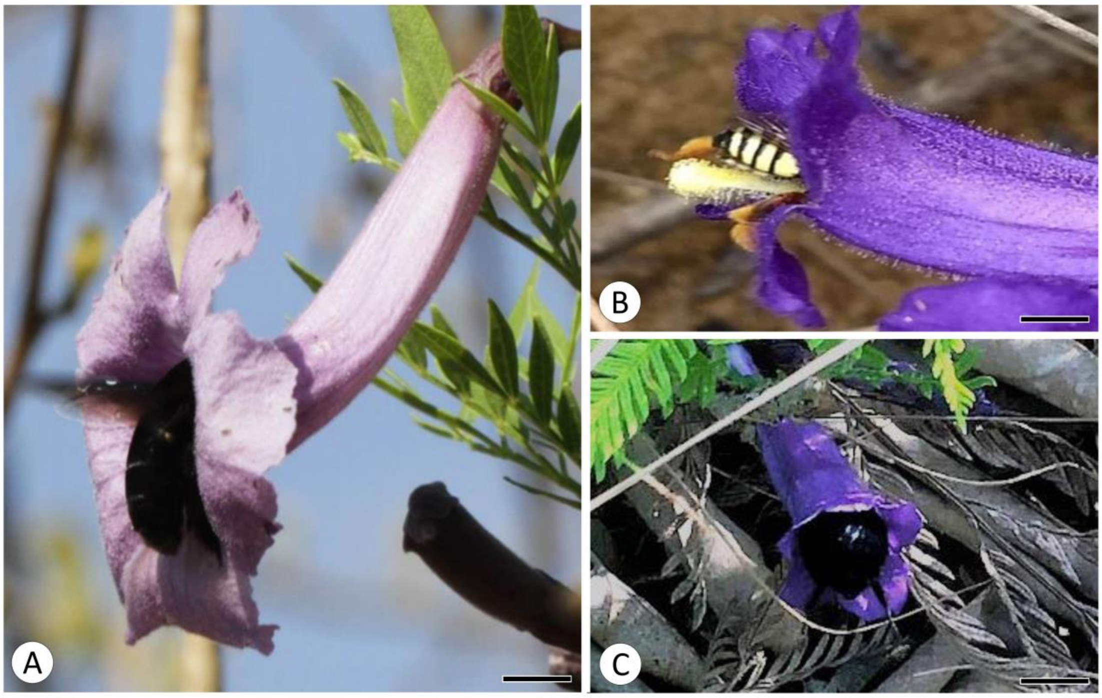

Fig. 2. Legitimate floral visitors of *Jacaranda caroba* and *Jacaranda decurrens* (Bignoniaceae) in physiognomies of cerrado, at the municipality of Águas de Santa Bárbara, São Paulo, Brazil. (A) *Bombus morio* visiting *J. caroba* flower. (B) *Epicharis* sp visiting a *J. decurrens* flower. (C) *Bombus morio* visiting *J. decurrens* flower. (Bar = 0.5 cm)

4.2. Nectar robbing experiment

There was no difference in pollen deposition between treatments with and without damage in *J. caroba* flowers (W = 2189, p = 0.1593) **(Fig. 3A)**. We also did not detect difference in pollen deposition on stigmas surface between treatments performed on flowers of *J. decurrens* (W = 2776, p = 0.5842) **(Fig. 3B)**.

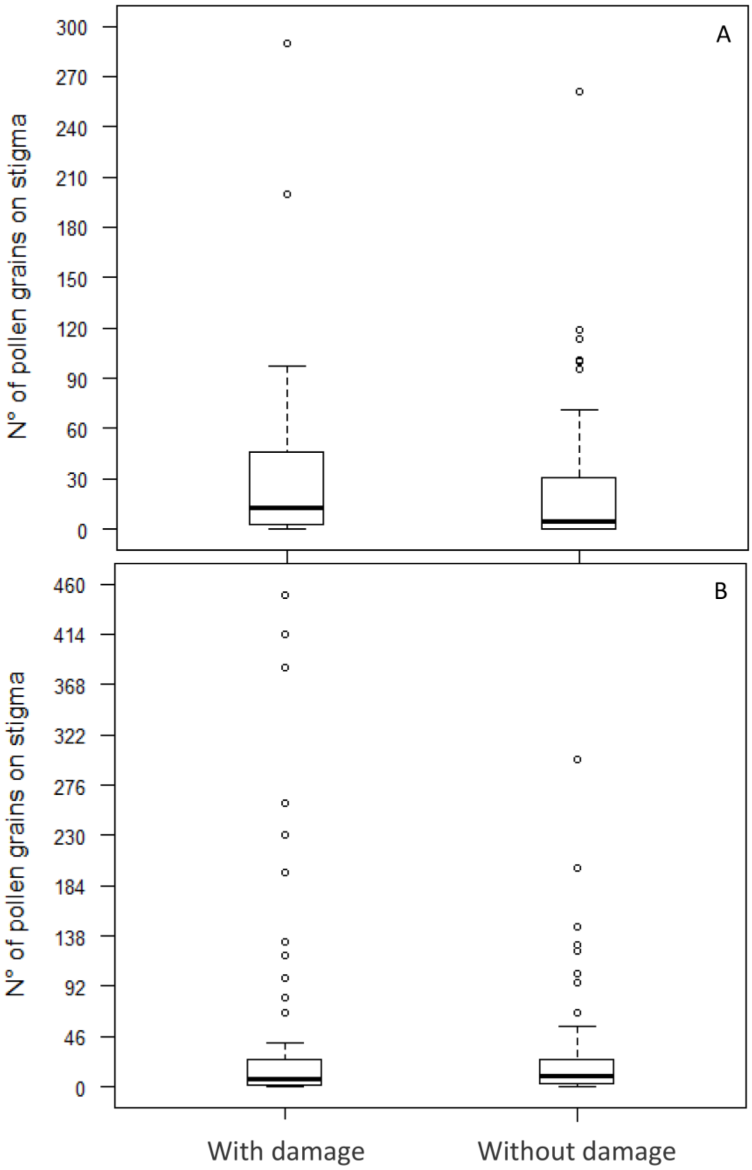

Fig. 3. Pollen grains deposited on the stigmas of flowers with and without damages in physiognomies of cerrado. (A) Number of pollen grains deposited on the stigmas of *Jacaranda caroba* flowers. (B) Number of pollen grains deposited on the stigmas of *Jacaranda decurrens* flowers.

4.3. Chemical analysis

The chemical analysis of flowers with and without damage revealed no difference in almost all the compound emitted, except for one aliphatic compound (2-tridecanone), which was detected only in one intact flower of *J. caroba* **(Fig. 4, Table 1)**.

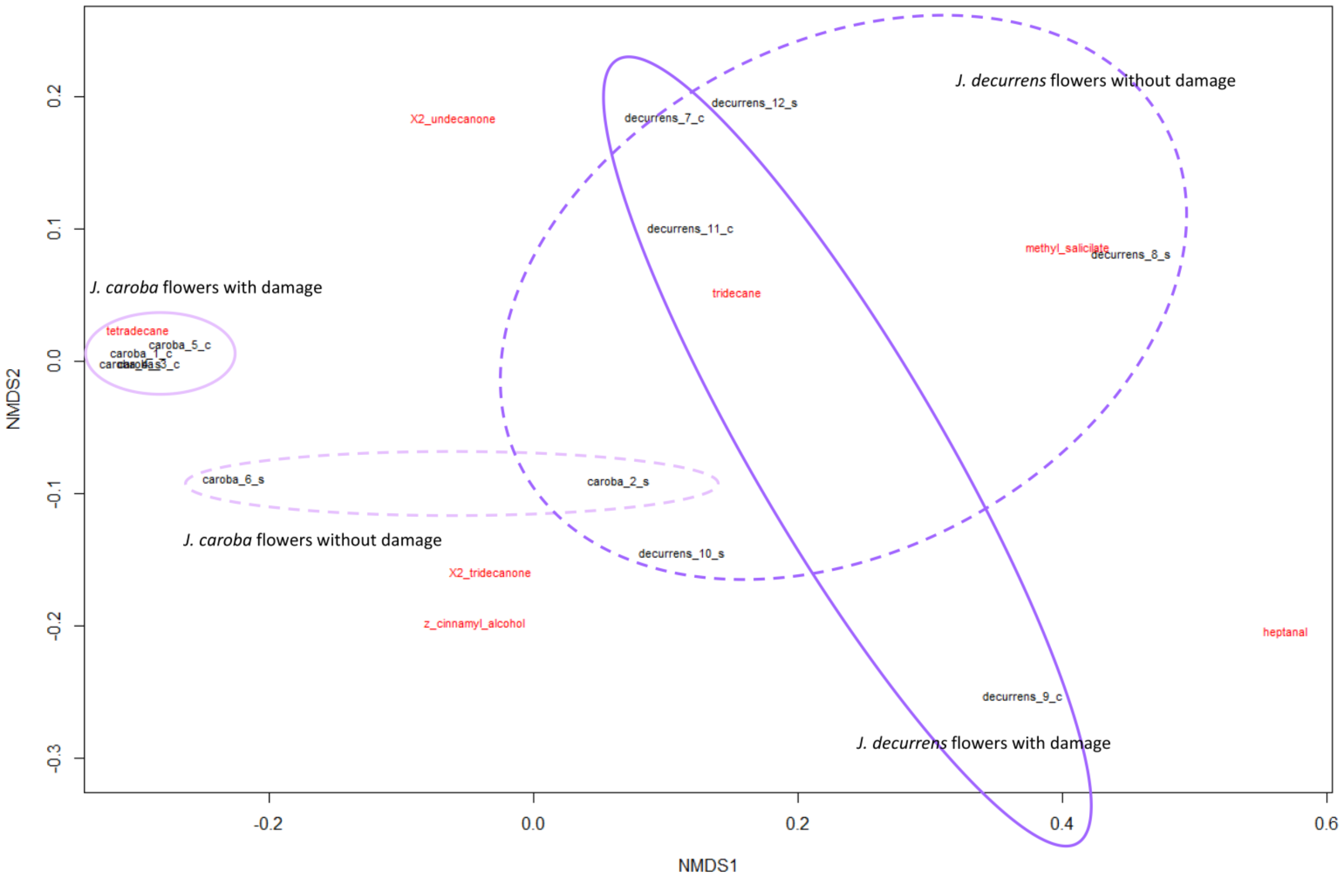

Fig. 4 The scent space representation of two *Jacaranda* species (Bignoniaceae) visualized in two dimensions by non-metric multidimensional scaling (NMDS) based on Bray-Curtis similarities of the floral scent composition.

Table 1. Absolute amounts (mean ± sd) of scent compounds found in flowers of the genus *Jacaranda* (Bignoniaceae).

	J. caroba		*J. decurrens*	
	Flowers with damage	Flowers without damage	Flowers with damage	Flowers without damage
Tridecane			7.95 ± 1.46	6.90 ± 1.49
Tetradecane	10.59 ± 0.89	9.81 ± 1.85	10.15 ± 1.48	9.01 ± 2.88
2-tridecanone	1.68 ± 1.68		13.39 ± 4.46	3.11 ± 0
Heptanal			140.89 ± 0	88.79 ± 0
Methyl-salicilate			37.88 ± 18.77	22.09 ± 11.55
(Z)-Cinnamyl alcohol			13.56 ± 0	4.93 ± 0
2-Undecanone			6.66 ± 3.40	7.28 ± 0

5. DISCUSSION

In this study we performed experiments under natural conditions to evaluate the effect of nectar robbing on pollination of two co-occuring Bignoniaceae cerrado' species. We found that pollinators of *J. caroba* and *J. decurrens* visited flowers indiscriminately, despite the intense foraging of *O. flavescens* on this system and the signs of damage left by these bees on the corolla basis. Besides visual signaling, we registered no difference on chemical signaling in flowers that suffered damages by antagonists.

Nectar robbery may cause various effects on plants' reproductive success (Maloof and Inouye, 2000; Irwin et al., 2010) since it can influence the pollinator behavior because, besides the nectar depletion, the robbers may create visual signs (damages) that could be recognized by pollinators (Irwin et al 2010, Quinalha et al. 2017). Thus, pollinators could recognize nectarless flowers and avoid them. A possible negative consequence of the decrease in pollinator visits to stolen flowers would be that, in an extreme scenario, it could lead the pollinator to abandon this species and start to explore another plant species, affecting negatively the robbed plants' reproductive success. However, according to Laverty (1994) and Chittka & Thomson (1997), the process of learning new rewarding flowers requires time investment and a high energy cost, which could explain the our results, which showed that *Bombus morio* and *Epicharis* sp. visit legitimately flowers with and without damage of both plant species.

The localization of flowers by anthophilous animals depends on communication mechanisms that are enhanced when floral characters are more suited to their sensory abilities (Schaefer & Ruxton, 2011). Attractive floral chemicals play an important role in directing animals to flowers (Raguso 2001, Dobson et al. 2005, Schaefer & Ruxton 2011). The ability to associate floral scent with the presence of nectar has an even greater significance in the case of generalist pollinators, such as bees, which present high olfactory acuity (Wright et al. 2009). Thus, the fact of *J. caroba* and *J. decurrens* did not present differences in the floral scent composition may represent a key factor for *Bombus morio* and *Epicharis* sp. to visit both robbed and intact flowers.

In fact, the sensory perceptions of pollinators could be important in driving this complex mutualist-plant-antagonist system. Evidences suggest that hummingbird pollinators may be able to avoid nectar-robbed plants and flowers (Gass & Montgomerie 1981, Irwin 2000, Roubik 1982), while bumblebees pollinators may have less ability to avoid stolen flowers not ceasing to visit flowers with low amounts of nectar (Rust 1979, Stout et al. 2000, Richardson 2004). In this case, pollinators that keep to foraging on stolen flowers may need to increase the number of flowers they visit to compensate their daily metabolic requirements. This could have a cost to pollinators' fitness reducing their growth and decreasing energy allocation to reproduction (Irwin et al 2010).

However, this behavior could have a positive consequence to the plant reproductive success since pollinators increase the number of visited plants, covering greater distances among them them, in order to meet their daily metabolic needs favoring, thus, the pollen flow among plants(Irwin, 2010). This is especially important in self-incompatible plants as is the case of *Jacaranda* species used as a model for this study (Chapter 2).

Thus, there are many examples in the literature showing that nectar robbing may influence multiple mechanisms at the same time, being able to affect positively and negatively the behavior of pollinators and, consequently, the reproductive success of plant species (Zimmerman & Cook 1985, Irwin et al. 2010, Mayer et al. 2014, Irwin et al.2015, Quinalha et al. 2017, Bergamo & Sazima 2018). However, we showed that nectar-robbery by bees, although very frequent; apparently do not affect the pollinator visitation of these two Bignoniaceae species, *J. caroba* and *J. decurrens,* suggesting that this system may be in a complex balance encompassing mutualists and antagonists.

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CAPÍTULO 3

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Nectar exploitation by distinct functional groups of floral visitors in a savanna shrub species

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1. ABSTRACT

Nectar is the main energy source exploited by floral visitors, being essential to the pollination of many outcrossing plant species. Bignoniaceae species interact with a wide variety of floral visitors, establishing most of their interactions with antagonists. *Jacaranda caroba* is a Bignoniaceae species that has an extensive interactions network, including pollinators, nectar robbers and nectar thieves. Thus, in this study we characterized the exploitation of floral nectar by different functional groups that visit *Jacaranda caroba*'s flowers, aiming to better understand the potential role of the antagonists on mutualistic systems. For this, we performed a set of experiments in order to describe the nectar exploitation by each different functional group of floral visitors. We observed that the accumulated amount of nectar produced by *J. caroba* flowers was higher than the amounts found in flowers exposed to pollinators, nectar thieves and nectar robbers during the same period. This finding demonstrates that the three functional groups of bees exploited nectar as food resource. Additionally, we verified that all floral visitors, mutualists and antagonists, exploited nectar equally in terms of volume. It is remarkable that antagonists, nectar robbers together with nectar thieves, can exploit the resource almost to its entire depletion, which may interfere directly in the flower relationship with pollinators, by altering nectar availability.

Keywords: Bees**,** nectar larceny, mutualists, antagonists.

2. INTRODUCTION

Nectar is the main trophic resource exploited by pollinators, being essential to the establishment of interactions between flowers and their visitors, enabling the pollen transfer and, therefore, reproductive success in outcrossing species (Wilmer 2011). Bignoniaceae is composed mainly of species that have tubular corolla and accumulate floral nectar at the base of the tube (Gottsberger & Silberbauer-Gottsberger 2006, Galetto 2009). The species of this plant family interact with a wide variety of floral visitors, including several types of antagonists that exploit floral resources without performing pollination (Guimarães et al 2008, Genini et al. 2010, Quinalha et al. 2016, Souza et al. 2017). These antagonists can be classified according to their behavior as *thieves*, those visitors that access the floral resources by entering in the floral tube without producing damage to the flower, or *robbers* those that always collect floral resource causing damage to floral whorls (Inouye 1980).

All the visitors, mutualists or antagonists, benefit with nectar collection, since that is a caloric, sugar rich resource that can supply their different energy and nutritional requirements (Nicolson 2007). However, nectar exploitation by antagonists may have effects on plants' reproductive success, since it could affect pollinator behavior, compromising the pollen transfer (Maloof & Inouye 2000, Bronstein et al. 2003, Irwin et al. 2010).

Jacaranda caroba (Vell.) DC. (Bignoniaceae) is a savanna species which has an extensive interactions network, including nectar thieves and robbers, with 75% of interactions established with antagonists (Genini et al. 2010). Only one medium-sized bee is recognized as pollinator in this system (Quinalha et al. 2016) which led us to think about the impact of the nectar exploitation by several antagonistic species on the pollination and reproductive success of this vegetal species. Thus, the aim of this study was to evaluate the exploitation of nectar by different functional groups of *Jacaranda caroba*'s floral visitors, focusing on the potential role of the antagonists' impact on mutualistic systems.

3. MATERIAL AND METHODS

3.1. Study site and plant species

We conducted the study at Estação Ecológica de Santa Bárbara (EESB), belonging to Instituto Florestal do Estado de São Paulo. This area is located in the municipality of Águas de Santa Bárbara, between 22º46'30" to 22º50'30'S and 49º10'30" to 49º15'30'W. The vegetation covers an area of 2715 ha and is characterized by savanna physiognomies (locally named as cerrado). According to Koeppen's classification the region's climate is Cwa, with average temperatures of the hottest month (January) between 23° and 24° C and average temperatures of the coldest month (July) around 16° C (Setzer 1966). Annual rainfall varies from 1000 to 1300 mm, with dry winters and rainy summers (Melo & Durigan 2011).

Jacaranda caroba (Vell.) DC. is an species that varies from shrubs to small trees (0.5 - 2.5m in height), presents glabrous branches and long internodes, bipinnate composed leaves with leaflets that vary from elliptic to rhomboid and opposite phyllotaxis; the inflorescences are tirsoid, terminal or axillary, composed by flowers with cupular vinous cup, irregularly 2-5 lobed; corolla tubular-campanulate (with 50.84 ± 6.79 in size and 15.85 ± 2.15 in diameter) above a strict basal tube, which correspond to the nectar chamber, puberula, 5-lobed, with coloration varying from magenta to purple; stamens didinamous with dithecae anthers; densely glandular staminodium and ovate-flattened ovary with cylindrical-pulvinate disc at base; the fruit is an elliptical capsule 2.5-7.0 cm long and 2.2-4.5 cm wide, brown and sublime when ripe; their valves curl in the dehiscence, exposing the winged membranaceous seeds (Gentry & Morawetz 1992, Lohmann & Pirani 1996).

Jacaranda caroba is a savanna species, which interacts with different guild of visitors: Medium-sized bees *Bombus morio* behaved as pollinators visiting the flowers in a legitimate way; small-sized bees *Ceratina* sp. and *Epicharis* sp. behaved as nectar thieves collecting nectar by entering the corolla tube without touching reproductive structures due to their reduced body size, while medium-sized bee *Oxaea flavescens* behaved as nectar robber, collecting nectar from the outside of the corolla tube, causing damage to the external flower whorls (Quinalha et al. 2016).

3.2. Nectar exploitation characterization

In order to evaluate the nectar exploitation by each different functional groups of floral visitors we performed the following experiment, using 15 different plants for each treatment (n = 60 plants) and taking, in a non-systematic way, one flower per plant: <u>Treatment 1</u>: we closed the corolla tube entrance with cotton to quantify the effect of nectar removal by nectar robbers (medium-sized bees that access the resource by piercing the corolla externally) **(Fig. 1A)**; <u>Treatment 2</u>: we isolated the base of the corolla tube with transparent tape and placed a wire box with a mesh of 1,0 x 1,0 cm which avoided medium sized-bees to enter in the floral tube, allowing the access only for nectar thieves (small bees which access the nectar when entering the tube, however, do not act as pollinators as they do not touch the reproductive structures in their way to the nectar chamber) **(Fig. 1B)**; <u>Treatment 3</u>: we isolated the corolla base with transparent tape, precluding nectar robbers to access the floral resource, allowing only pollinators and nectar thieves to achieve the nectar **(Fig. 1C)**; <u>Treatment 4</u>: we protected the inflorescences with bridal veil bags in order to obtain data on the potential nectar production per flower without nectar depletion by any floral visitors **(Fig. 1D)**.

All the treatments started at the beginning of the first day of anthesis (recently opened flowers) and we collected the flowers to perform the nectar measurements by the end of the first day of anthesis, totaling approximately 12 hours of exposure. We measured the remaining nectar volume (µL) in each flower by using calibrated syringes.

3.3. Statistical analysis

In order to characterize nectar exploitation by different floral visitors of *J. caroba,* we performed a one-way analysis of variance (ANOVA) and used Tukey post-hoc test for multiple comparisons among pairs of means (Zar 2010). For this analysis, we tested the differences in nectar remaining among flowers visited by pollinators, nectar thieves, nectar robbers and the potential nectar production by flower without depletion. The analyses were performed in R v. 3.4.3 (R Development Core Team 2017).

Fig. 1. Flowers of *Jacaranda caroba* submitted to different treatments to evaluate nectar exploitation by each functional group of visitors. (A) Corolla tube entrance closed with cotton to quantify the effect of nectar removal by nectar robbers. Scale bar: 15 mm. (B) Corolla basis isolated with transparent tape and inflorescence wrapped with a wire box allowing access only by nectar thieves. Scale bar: 13 mm. (C) Corolla basis isolated with transparent tape precluding nectar robbers to access the nectar. Scale bar: 8 mm. (D) Inflorescences protected with bridal veil bags from all floral visitors. Scale bar: 50 mm.

4. RESULTS

Flowers of *J. caroba* opened predominantly at around 8:00 h and anthesis lasted about two days. Nectar production in *J. caroba* started at pre-anthesis stage so, as soon as the flowers opened, the resource was available to floral visitors.

The measurements of the remaining nectar in the flowers submitted to the distinct treatments differed ($F_{3,60}$= 8.44, $p < 0.0001$). Flowers exposed to pollinators, nectar thieves and nectar robbers had an average of remaining nectar volume of 1.71 ± 1.72 µL, 1.77 ± 1.74 µL and 1.55 ± 1.87 µL, respectively meanwhile flowers protected from the foraging of all floral visitors presented a mean nectar production of 5.12 ± 3.41 µL of nectar production. The potential nectar volume produced by flowers of *J. caroba* differed from that found in flowers visited by pollinators, nectar thieves and nectar robbers and these visitors exploited floral nectar equally **(Fig. 2)**.

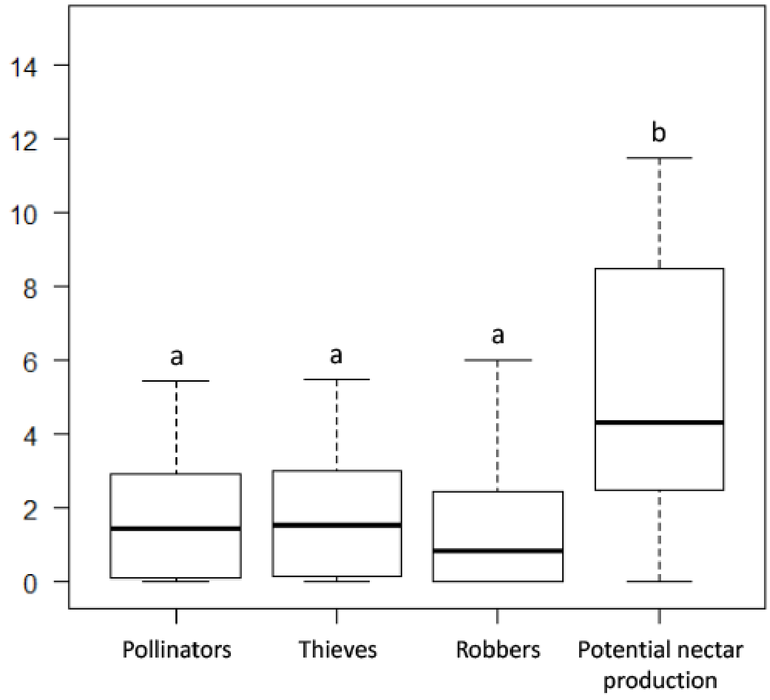

Figure 2. Nectar volume remaining after 12 hours of exposure to floral visitors of *J.* caroba. For each functional group of visitors, boxes accompanied by the same letters did not differ in the post-hoc Tukey test (confidence interval = 95%).

5. DISCUSSION

In this study we evaluated the nectar exploitation by different floral visitors of *J. caroba* measuring the remaining nectar in flowers exposed to each group of animals. We found that there was no difference in the exploitation of nectar among floral visitors suggesting that both mutualists and antagonists exploit the nectar equally in terms of volume.

Tactics of foraging are largely known to influence plant fitness (Schmid-Hempel and Schmid-Hempel 1998, Pelletier and McNeil 2003, Baker et al 2018), so the way the bee visits the flower to collect nectar will have costs and benefits to the plant fitness, since nectar foraging is associated with pollination (Irwin et al 2010, Baker et al 2018).

Animals can adopt different forage behaviors and usually choose one of them (Irwin et al. 2010, Baker et al. 2018). Commonly, nectar foragers visit the flowers through the opening of corolla tube, which enables to them touch reproductive structures and perform pollination. However, many animals can steal the nectar by producing a hole in the corolla tube or by collecting it through the corolla entrance, but without contacting flower anthers and stigma, thus impairing plant reproduction (Inouye 1980, Irwin et al 2010). The morphological fit between animals and flowers could help explain variations in nectar exploitation (Newman and Thompson 2005). Species with short tongues are referred to adopt more frequently nectar robbing behavior (Newman and Thompson 2005). In fact, *Oxaea flavescens,* referred as a bee specialized in nectar robbing on Bignoniaceae spcecies (Gottsberger and Silberbauer-Gottsberger 2006), shows shorter tongues (4.0 ± 0.56 mm) when compared with the bee pollinator *Bombus morio* (10.11 ± 2.04) (unpublished data).

Jacaranda caroba interacts with different bee species that have distinct foraging strategies, being the cheaters much more abundant and frequent when compared with pollinators (Quinalha et al. 2016). These cheaters often reduce the amount of floral nectar available and could take a negative indirect effect on the pollinator behavior since they could avoid robbed and thieved flowers (Hazlehurst and Karubian 2016). Our results showed that the amount of nectar removed by each functional group of visitors of *J. caroba* is the same but, nectar robbers and thieves, together, can exploit the resource almost to its entire depletion. The action of these antagonists may interfere directly in the flower relationship with pollinators, since it may lead to changes in the volume and concentration of floral nectar (Castro et al. 2009).

We observed that legitimate visitors of *J. caroba* search for nectar despite the high frequency of visits of antagonists. There are several costs for pollinators to continue to forage robbed flowers including the need of increasing the number of flowers visited to supply their energetic demands (Irwin et al 2010). These metabolic costs may impact pollinators' fitness by reducing caloric intake, compromising the pollinator growth and decreasing energy allocation to their reproduction (Irwin et al 2010).

Thus, the direct and indirect effects caused by cheaters in plant-pollinator systems should be considered simultaneously for a more complete and integrative understanding of the dynamics of interactions and their consequences for the reproductive success of plant species.

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CAPÍTULO 4

Breeding systems of two co-occurring species of the *Jacaranda* genus

1. ABSTRACT

Most of the angiosperms have hermaphrodite flowers, with functional male and female whorls. This arrangement can favors self-pollination due to the proximity of reproductive structures. Thus, the emergence of self-incompatibility mechanisms was a crucial adaptation for the prevention of self-fertilization, being fundamental in the generation of genetic variability in natural populations. Thus, we aimed to investigate the reproductive system of two species of *Jacaranda,* seeking to elucidate if *Jacaranda caroba* and *J. decurrens* present a mechanism of self-compatibility similar to the other species of Bignoniaceae studied. For this, we performed manual pollinations submitting flowers to self-pollination and cross-pollination. We verified in each treatment if there were fruit formation and the germination of the pollen grains, growth of pollen tubes and quantified the ovules penetrated under a fluorescence microscope. Our results regarding the manual pollination experiments indicate that both species of *Jacaranda* show self-incompatibility mechanisms we did not register the formation of fruits in any of the pistils submitted to self-pollination. We observed that in both species there is a higher incidence of ovules penetrated in the cross-pollination treatments. However, several pollen tubes from self-pollination penetrate the ovules. Thus, the fact that self-pollen penetrates a large number of ovules suggests that the current mechanism of self-incompatibility in the species of this study is the late-acting SI (LSI). This mechanism can be essential to maintain the genetic variability in the progeniesis of *J. caroba* and *J. decurrens* since these species have an exuberant and massive flowering. This type of floral display, associated to the behavior of pollinator bees, which visit sequentially several flowers in the same plant, can negatively impact the reproductive success of these species due to the deposition of pollen of low quality since when a high deposition of self-pollen occurs on the stigmas, this can be able to decrease the chances cross-pollen germinate.

Key words: Bignoniaceae, breeding systems, late-acting self-incompatibility

2. INTRODUCTION

Most of the angiosperms have hermaphrodite flowers, with functional male and female whorls (Seavey & Bawa 1986, Gibbs & Bianchi 1999, Gibbs 2014). This organization seems to have evolved in a way to favor the pollination process since in a single pollinator visit occurs pollen deposition on the stigma concomitant with the pollen removal from the anthers (Gibbs 2014). However, this arrangement also favors self-pollination due to the proximity of reproductive structures. In this context, the emergence of self-incompatibility mechanisms was a crucial adaptation for the prevention of self-fertilization, being fundamental in the generation of genetic variability in natural populations (Gibbs & Gibbs 2000, Gibbs 2014).

These mechanisms are genetically controlled and classified according to the location where the male gametophyte recognition and rejection reactions occur, and three fundamental types of self-incompatibility systems are recognized: homomorphic gametophytic system (GSI), homomorphic sporophytic system (SSI) and the heteromorphic system (HetSI) (Nettancourt 1977). These systems prevent the germination of pollen from the same sporophyte in the stigma, or the growth of the pollen tubes until reaching the ovary and, therefore, are mechanisms that act in the pre-zygotic stage, thus preventing self-fertilization.

However, after Seavey & Bawa (1986) conducted a literature review study on reproductive systems of 29 species, they observed that these species did not produce fruit after being pollinated with pollen from the same plant, although the pollen tubes germinated and grew vigorously until reaching the ovary. This mechanism was termed late-acting self-incompatibility (LSI), in which self-incompatibility barriers operate on the ovary.

In the Bignoniaceae family, among 62 species had their reproductive systems as the target of study, 80% presented self-incompatibility generally characterized as a result of the late-acting self-incompatibility mechanism (LSI) (Bittencourt et al. 2003, Bittencourt & Semir 2004, 2005, Gandolphi & Bittencourt 2010, Alves et al. 2013, Sampaio et al. 2016, Duarte et al. 2017). In this family, pistils receiving pollen from the plant itself show normal growth of pollen tubes with the majority of ovules penetrating the ovary. However, the pistils undergo abscission within a few days after pollination. In addition, the initial development of the endosperm is lower when compared to pistils pollinated with pollen of other plants (Bittencourt et al. 2004, Bittencourt & Semir 2004, Bittencourt et al. 2011, Sampaio et al. 2016). In this sense, although the fertilization mechanism of this family seems to be consensus, only 7% of its 827 species had their reproductive systems studied so far (Bittencourt 2011).

Jacaranda Juss. is a Neotropical genus belonging to the Bignoniaceae family that has 48 species distributed in South America from Guatemala to northern Argentina (Gentry, 1992; Lohmann, 2015). Although some studies on the reproductive biology of species of this genus have been developed in the last decades (eg, Yanagizawa 1983, Vieira et al. 1992, Bittencourt & Semir, 2006, Gottsberger & Silberbauer-Gottsberger 2008), only two species had their reproductive systems as the target of study (Vieira et al. 1992, Bittencourt & Semir, 2006). Thus, the objective of this work was to investigate the reproductive system of two species of *Jacaranda*, seeking to elucidate if *Jacaranda caroba* and *J. decurrens* present a mechanism of self-compatibility similar to the other species of Bignoniaceae studied.

3. MATERIAL AND METHODS

3.1. Study area

The study was conducted at the Estação Ecológica de Santa Bárbara (EESB), belonging to the Instituto Florestal de São Paulo (IF-SP). This area is located in the municipality of Águas de Santa Bárbara between the coordinates 22º46'30 " at 22º50'30 "S and 49º10'30 "' at 49º15'30"O. The vegetation occupies 2715 ha and is characterized by savannah physiognomies of cerrado.

3.2. Plant species

For this study we used two species of the genus *Jacaranda* Juss., belonging to the Bignoniaceae plant family.

Jacaranda caroba (Vell.) DC. is an species that varies from shrubs to small trees with a ovate-flattened ovary with cylindrical-pulvinate disc at base; the fruit is an elliptical capsule 2.5-7.0 cm long and 2.2-4.5 cm wide, brown and sublime when ripe; their valves curl in the dehiscence, exposing the winged membranaceous seeds (Gentry & Morawetz, 1992; Lohmann & Pirani, 1996).

Jacaranda decurrens Cham. is a subshrub with a thickened subterranean organ from which aerial branches are born of 20 to 100 cm of height with a ovary flattened-ovate, glabrous with pateliform-pulvinate disc at the base; elliptic-obovate fruit, strongly woody, containing seeds with hyaline membranaceous wings (Gentry 1992).

3.3. Floral functionality

Initially, we verified the period of functionality of the flowers by investigating (1) stigmatic receptivity using the peroxidase test paper solution (Dafni & Maués 1998); and (2) the viability of pollen grains throughout the floral lifespan using acetic carmine as a vital dye (Radford et al. 1974), as well as their size and morphology under optical microscopy. For this, we randomly took three flowers per individual in 15 individuals of each species (n = 45 flowers / species).

3.4. Breeding systems

To determine the breeding system of both plant species we performed manual pollinations in 15 individuals of *J. caroba* and in 12 individuals of *J. decurrens*. For this, we marked pre-anthesis buds with distinct colour wires identifying each treatment (12 flowers / individual / treatment) and isolating them with voile bags preventing the flowers from being contacted by any visitors. In addition, we left a set of flowers bagged to identify if the species form fruits by spontaneous self-pollination. Subsequently, we submitted flowers on the first day of anthesis to self-pollination (pollen from the same flower) and cross-pollination (a pollen mixture from 30 donor plants, distant at least 100 meters from the receivers plants) and re-bagged them. We did manual pollinations covering all internal portion of the stigmatic lobes, which corresponds to the receptive portion of the stigma, ensuring that the absence of ovule penetration and fruit formation was not due to pollen limitation. After pollinations two flowers of each treatment were left in the individuals to observe fruit formation after the experiments (control). We monitored the individuals biweekly for three months after the experimental pollinations in order to follow the development of the fruits until their maturation. We kept flowers, pistils and fruit bagged during the whole period of the experiment aiming to avoid a possible action of predators.

For the *in situ* analysis, we collected the pistils at the intervals of 6, 12, 24, 48 and 72 hours after manual pollinations (2 pistils / plant at each interval), fixed them with FAA50 and performed pollen tube growth analysis registering the number of ovules penetrated in both species. We dissected the ovaries by removing their walls and exposing the ovules. We clarified and softened the pistils with 9N NaOH at 60 °C for two hours and stained them with aniline blue to verify the germination of the pollen grains, growth of pollen tubes and quantify the ovules penetrated under a fluorescence microscope (Gibbs & Bianchi 1993).

3.5. Statistical analysis

In order to compare the effect of the treatments (self-pollination and cross-pollination) on the incidence of penetrated and non-penetrated ovules in each interval after manual pollinations (6, 12, 24, 48 and 72 hours after pollination), we used the Cochran-Mantel-Haenszel to estimate the odds ratios and the 95% confidence interval of incidence of ovules penetrated according to the treatment applied. The analysis was performed in R v. 3.4.3 (R Development Core Team 2017). Altogether, we observed 4096 ovules of *J. caroba* (2120 derived from self-pollinated pistils and 1976 from cross-pollinated pistils) from a total of 46 ovaries. For *J. decurrens* we observed a total of 1045 ovules (613 derived from self-pollinated pistils and 792 from cross-pollinated pistils) from a total of 24 ovaries.

4. RESULTS

4.1. Floral funcionality

The flowers of both species began to open early in the morning and although the anthesis is concentrated in the day’s early hours we observed some flowers that open throughout the day. In *J. caroba* the anthesis begins at around 7:00 am, and until 9:00 am most of the flowers are already open. In *J. decurrens*, the anthesis begins around 5:00 am, and until 8:00 am most of the flowers are already open. The stigma of *J.*

caroba and *J. decurrens* flowers were receptive and with the lobes open at the moment the flowers opening and remaining receptive until at least the second day of anthesis. The stigmas of both species are touch sensitive, closing when stimulated. However, if there is no pollen deposition, it reopens gradually after a while.

The anthers of both species have apicefix attachment in the fillets and longitudinal dehiscence. The pollen grains are whitish and are already viable at the time of opening the flowers. The viability of *J. caroba* pollen grains is 95.7 ± 2.96% and *J. decurrens* is 91.14 ± 6.19%.

4.2. Breeding systems

In both species we did not observe fruit formation in flowers that remained isolated from the visitors, indicating the absence of spontaneous self-pollination or any other mechanism independent of pollination, such as some types of apomixis. In addition, we also did not observe the formation of fruits from pistils that were self-pollinated manually. In relation to cross-pollinations, in *J. caroba*, 53% of the pollinated pistils manually developed into fruits, whereas in *J. decurrens* none of the pistils that received pollen from other plants produced fruits.

With respect to the tests to determine the self-incompatibility reaction site using fluorescence microscopy, we observed the growth of hundreds of pollen tubes in the transmitting tract, as well as penetrated ovules from both self-pollinated pistils and pistils submitted to cross-pollination (Fig 1A- D). In both species and in both treatments the pollen tubes already begin to reach the ovary in the first 6 hours after pollination. The results of the numbers of eggs penetrated and non-penetrated at each interval are shown in Table 1.

In *J. caroba* we observed that in the first hours after pollination (6 hour interval) the odds ratio of ovules of self-pollinated pistil to be penetrated is greater than the odds ratio of pistils subjected to cross-pollination (odds ratio 1.9 times higher for self-pollinated pistils). However, this proportion is reversed in subsequent intervals (odds ratio: 12h = 1.0, 24h = 2.30, 48h = 1.14 and 72 = 1.50), being that in intervals of 24 and 72 hours after pollination the incidence of penetrated ovules was significantly higher in the pistils submitted to cross-pollination than in self-pollinated pistils (24h: $z = -5.56$, $p < 0.0001$, 72h: $z = -2.15$, $p < 0.05$) (Fig. 2A).

In *J. decurrens* we observed that in the first hours after pollination (6 hour interval) the odds ratio of ovules of self-pollinated pistil to be penetrated is equal to the odds ratio of pistils subjected to cross-pollination to be penetrated (odds ratio = 1.0). However, in the subsequent intervals, the odds ratio of pistil ovules submitted to cross-pollination to be penetrated is greater than the odds ratio of self-pollinated pistils (odds ratio: 12h = 2.32, 24h = 1.23, 48h = 2 , 44 and 72 = 1.72). In the intervals of 12, 48 and 72 hours after pollination, the incidence of penetrated ovules was significantly higher in pistils submitted to cross-pollination than in self-pollinated pistils (12h: $z = -3 , 15$, $p < 0.001$, 48h: $z = -2.68$, $p < 0.01$, 72h: $z = -1.98$, $p < 0.05$) (Fig 2B).

In general, in both species of *Jacaranda*, the number of penetrated ovules depended on the type of pollination (*J. caroba*: Mantel-Haenzel Chi-square = 15.07, df = 1, $p < 0.001$, *J. decurrens*: Mantel -Haenzel Chi-square = 14.07, df = 1, $p < 0.001$), and the odds ratio of pistil ovules subjected to cross-pollination to be penetrated was higher than the odds ratio of self-pollinated pistil ovules to be penetrated (*J. caroba*: 1.38 and *J. decurrens*: 1.59).

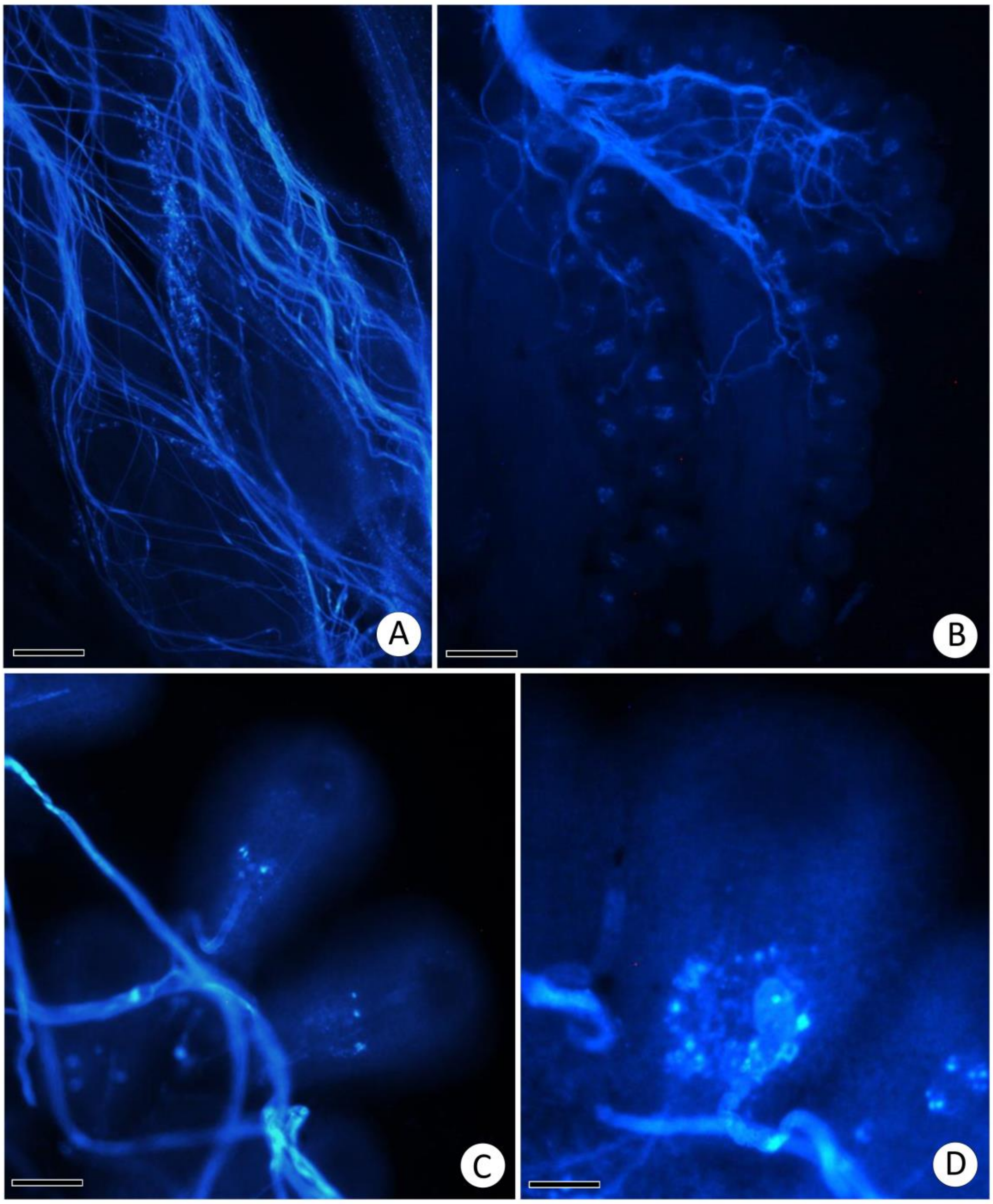

Fig. 2. Epi-fluorescence photomicrographs of self-pollinated ovaries of *Jacaranda caroba* (Bignoniaceae) within 24 hours after pollination. (A) Pollen tubes advancing the transmitting tract towards the ovary. Scale bar = 140 µm (B) Pollen tubes in ovary with several ovules already penetrated. Scale bar = 140 µm (C-D) Penetrated ovules. Scale bar = 50, 30 µm.

Table 1. Incidence of penetrated and non-penetrated ovules 6, 24, 48 e 72 hours after manual self-pollination and cross-pollination in *Jacaranda caroba* e *J. decurrens* (Bignoniaceae)

		Intervals after polination									
		6h		**12h**		**24h**		**48h**		**72h**	
Species	**Ovules**	**Self**	**Cross**	**Self**	**Cross**	**Self**	**Cross**	**Self**	**Cross**	**Self**	**Cross**
J. caroba	*Penetrated*	21 (6,07%)	11 (3,28%)	135 (35,16%)	124 (35,03%)	157 (43,25%)	244 (64,21%)	315 (83,55%)	319 (85,29%)	314 (78,70%)	311 (84,74%)
	Non-penetrated	325 (93,93%)	324 (96,72%)	249 (64,84%)	230 (64,97%)	206 (56,75%)	139 (35,79%)	62 (16,45%)	55 (14,71%)	85 (21,30%)	56 (15,6%)
	Total	346	335	384	354	363	380	377	374	399	367
J. decurrens	*Penetrated*	41 (28,47%)	38 (28,36%)	37 (32,17%)	68 (52,31%)	57 (46,72%)	69 (51,88%)	16 (13,56%)	38 (27,54%)	35 (30,70%)	54 (43,2%)
	Non-penetrated	103 (71,53%)	96 (71,64%)	78 (67,83%)	62 (47,69%)	65 (53,28%)	64 (48,12%)	102 (86,44%)	100 (72,46%)	79 (69,3%)	71 (56,8%)
	Total	144	134	115	130	122	133	118	138	114	125

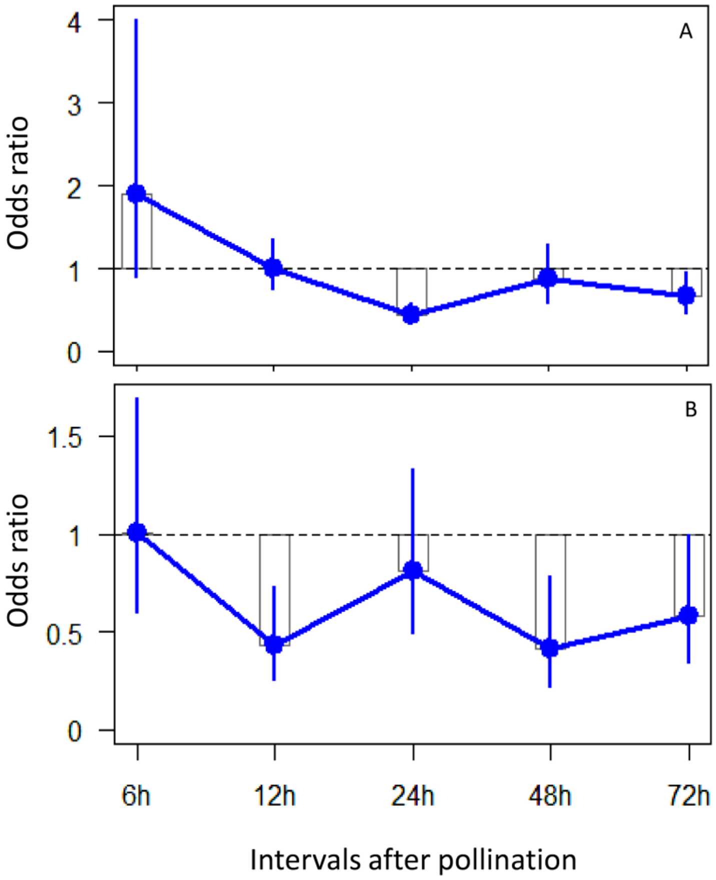

Fig. 2. Odds ratio of incidence of penetrated ovules in (A) *Jacaranda caroba* (Vell.) DC. and (B) *Jacaranda decurrens* Cham. at a 95% confidence interval. The numerator of the ratio corresponds to self-pollinated pistils (values above 1) and the denominator of the ratio corresponds to pistils submitted to cross-pollination (values below 1). Values that don't cross the line corresponding to 1 (dashed line) are statistically significant

5. DISCUSSION

Our results regarding the manual pollination experiments indicate that both species of *Jacaranda* show self-incompatibility mechanisms since we did not register the formation of fruits in any of the pistils submitted to self-pollination. We observed that in both species there is a higher incidence of ovules penetrated in the cross-pollination treatments, suggesting the occurrence of a mechanism for the recognition of pollen tubes and their rejection in self-pollinated pistils, a phenomenon previously reported in other species of Bignoniaceae (Gibbs & Bianchi 1999, Bittencourt & Semir, 2004).

Although this mechanism of recognition favors a model of self-incompatibility, several pollen tubes from self-pollination penetrated the ovules. In *J. caroba*, pollen tubes from self-pollination reach the ovules first (in the first 6 hours). However, after this period there is a predominance of pollen tubes from cross-pollination reaching the ovules. In *J. decurrens* in the first hours after pollination, the chances of reaching the ovary were the same for both treatments, with predominance of ovules penetrated in cross-pollination treatments. Thus, the fact that self-pollen penetrates a large number of ovules suggests that the current mechanism of self-incompatibility in these *Jacaranda* species is the late-acting SI (LSI), as well as described for other several species of Bignoniaceae (Bittencourt et al. 2003, Bittencourt & Semir 2004, 2005, Gandolphi & Bittencourt 2010, Alves et al. 2013, Sampaio et al. 2016, Duarte et al. 2017).

This intrinsic component may have evolved as an adaptive response associated with the prevention of autogamy being essential in maintaining genetic variability in natural populations (Gibbs & Gibbs 2000, Gibbs & Bianchi 1999, Gibbs & Bianchi 1999). This mechanism can be essential to maintain the genetic variability in the progenies of Bignoniaceae species, since this family is known by their exuberant and massive flowering (Gentry 1974). This type of floral display, associated to the behavior of pollinator bees, which visit sequentially several flowers in the same plant (Quinalha 2016, Souza et al., Cap 1), could lead to an intensive geitonogamy and to the loss of genetic variability throughout successive generations. Thus, this pollinators behavior can negatively impact the reproductive success of these species due to the deposition of pollen of low quality since when a high deposition of self-pollen occurs on the stigmas, this can be able to decrease the chances cross-pollen germinate (Duncan et al. 2004).

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CONSIDERAÇÕES FINAIS

Nessa tese nós utilizamos uma abordagem ampla e integradora para discutir os potenciais fatores relacionados ao baixo sucesso reprodutivo de espécies vegetais, utilizando duas espécies coocorrentes em formações savânicas de cerrado como modelo.

A partir dos dados obtidos nesse estudo, nós concluímos que o deslocamento temporal na fenologia de *J. caroba* e *J. decurrens* é importante já que elas compartilham os mesmos polinizadores e isso pode contribuir para sua coexistência, reduzindo a competição. Além disso, evidenciamos a importância de anúncios florais mais moderados, uma vez que estão relacionados a um maior sucesso reprodutivo nessas espécies vegetais. Assim, apesar de uma maior produção de flores conferir maior atratividade aos polinizadores, os mesmos visitam flores sequencialmente, depositando pólen da mesma planta nos estigmas e comprometendo a germinação de grãos de pólen provenientes de outras plantas. Nesse contexto, esse comportamento dos polinizadores é especialmente importante porque, apesar dessas espécies possuírem estratégias de prevenção da autogamia, o mecanismo vigente nesse processo é o de autoincompatibilidade de ação tardia (LSI). Esse mecanismo permite que os tubos polínicos se desenvolvam e penetrem os óvulos, mesmo que os pistilos sofram abscisão posteriormente, competindo com os grãos de pólen provenientes de outras plantas e comprometendo o sucesso reprodutivo nessas espécies.

Também buscamos elucidar a importância dos antagonistas nesse complexo sistema mutualista-planta-antagonista. Observamos que apesar desses agentes antagônicos serem muito frequentes, eles aparentemente não interferem na polinização de *J. caroba* e *J. decurrens*. Ao avaliarmos o impacto do roubo de néctar nessas espécies, observamos que os danos causados por *Oxaea flavescens* não interferem na sinalização visual e na sinalização química aos polinizadores e os mesmos continuam visitando indiscriminadamente flores com dano e intactas. Além disso, nós verificamos que todos os visitantes florais de *Jacaranda*, sejam eles mutualistas ou antagonistas, exploram o néctar igualmente em termos de volume. Isso sugere, que o sistema mutualista-planta-antagonista nessas espécies vegetais parece estar em equilíbrio.