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First report on floral morphology, pollen release dynamics and behavioral biology of Xylocopa (Neoxylocopa) frontalis (Olivier, 1789) in Crotalaria juncea L. (Leguminosae, Papilionoideae)

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Abstract

Pollination is a clear example of an ecosystem service that provides benefits to people. Over 87.5% of the world flowering plants have been estimated to be pollinated by animals, and about one-third of global food volume produced depends on them. Our goals were to characterize for the first-time details about a) floral morphology, b) pollen release dynamics, c) behavioral biology in *Xylocopa* (*Neoxylocopa*) *frontalis*(Olivier, 1789) bees and their influence on the reproduction of green manure *Crotalaria juncea* L. We used male and female floral reproductive structures for scanning electron microscope. We collected all pollen grains of different anther morphologies. The pollen viability was obtained by acetic carmine dye test at 2%. We used four controlled tests (spontaneous self-pollination, a single, two and three visits – n = 30 floral buds to all) to evaluate pollen limitation in the crop. Keel blossom morphology in *C. juncea* allowed their opening and exposing the reproductive structures during *X. frontalis*foraging. There was statistical difference in the number of pollen grains between anther morphologies, as well as pollen viability. The rates of fruit and seed set were significantly higher as the number of visits by *X. frontalis*increased. Seed abortions occurred mainly in the basal position (n = 93), followed by the median (n = 58) and apical (n = 53). We concluded that pollinators can be useful for agricultural crops increasing fruit and seed production. Information about *X. frontalis* contribution to pollination will be useful to greenhouse and outdoor growers.

Introduction

Pollination is a fundamental ecosystem service to plant reproduction, agricultural production and maintenance of terrestrial biodiversity. Over 300,000 species (87.5%) of the world flowering plants have been estimated to be pollinated by animals, and about one-third of global food volume produced depends on animal pollination (Ollerton et al. 2011). Agricultural crop pollination is a clear example of an ecosystem service that provides benefits to people (Millennium Ecosystem Assessment 2005). Bees and other animals supply a valuable input to agricultural production that can increase both the size and quality of harvests (Allen-Wardwell et al. 1998; Klein et al. 2007; Santos et al. 2014). Recent researches suggested that agricultural crop pollination by insects reached £430 million of crop production in the UK (Smith et al. 2011), with an equivalent of US$361 billion in worldwide (Lautenbach et al. 2012). Thus, as a precursor to fruit and seed set, the pollination is a crucial ecosystem service for the reproductive processes and evolution of flowering plants (IPBES 2015).

Bees are the most frequent pollinators in angiosperms, being dependent on flowers, mainly for their food (Pirani and Cortopasso-Laurino 1993; Pinheiro et al. 2014). Bees use floral resources such as nectar for energy source and pollen for protein source, resin for nest building, floral fragrances as pheromones for copula and wax for hives building (Roubik 1989; Velthuis 1997; Agostini et al. 2014). On the other hand, the plants, when receiving visitors, may have the pollen deposited on the stigmas, thus contributing to the ovule's fertilization, fruit and seed set (Faegri and van der Pijl 1980). Fruit and seed set are reproductive processes that share resources (Lee and Bazzaz 1982; Lee 1988; Oliveira and Maruyama 2014) and a few studies have evaluated the effects of resource constraints on reproductive patterns of angiosperms

(Lee and Bazzaz 1986; Solomon 1988; Diggle 1995). In most parts of the world, bees are the most abundant and diverse pollinators, mainly in many agricultural crops, about 20,000 species were recorded around the globe (Neff and Simpson 1993; Klein et al. 2007; Michener 2007).

The pollination by bees in agricultural crops has a strong influence in the reproductive success of species. Some crops are totally dependent on biotic pollination for fruit and seed set, and without floral visitors there would be a decrease of 90% in reproductive success. These include atemoya fruit (*Annona cherimola* Mill. x *Annona squamosa* L.) (Annonaceae), coconut (*Cocos nucifera* L.) (Arecaceae), kiwifruit (*Actinidia deliciosa* (A.Chev.) C.F.Liang & A.R.Ferguson) (Actinidiaceae), yellow passion fruit (*Passiflora edulis* Sims) (Passifloraceae), macadamia nut (*Macadamia integrifolia* Maiden & Betche) (Proteaceae), hazelnut (*Corylus avellana* L.) (Betulaceae), pumpkin (*Cucurbita moschata* Duchesne) (Cucurbitaceae), watermelon (*Citrullus lanatus* L.) (Cucurbitaceae), melon (*Cucumis melo* L.) (Cucurbitaceae), acerola cherry (*Malpighia emarginata* D.C.) (Malpighiaceae), vanilla (*Vanilla planifolia* Jacks. ex Andrews) (Orchidaceae) and red banana (*Musa acuminata* L.) (Musaceae) (Klein et al. 2007). However, some agricultural crops have the ability to self-pollination, i.e., they are not dependent on pollinators to reproduce, such as tomato (*Solanum lycopersicum* L. Pizzadoro hybrid) (Solanaceae) (Hayashi-Trevizor 2014), eggplant (*Solanum melongena* L.) (Solanaceae) (Zambon 2015; Zambon and Agostini 2017), soybean (*Glycine max* (L.) Merr.) (Leguminosae) (Nogueira-Couto 1994; Ribeiro and Nogueira-Couto 2002) and coffee (*Coffea arabica* L.) (Rubiaceae) (Klein et al. 2003; Klein et al. 2007). However, if occurs pollination by bees, can have an increase of viable fruits and seeds, weight of fruits, agriculture productivity and producer profit (Kevan and Phillips 2001; Santos et al. 2014).

A study found higher production of viable seeds of soybeans resulting from flowers visited by bees (66.17%) than those not visited by bees (33.83%) for the cultivar "Conquista" (Ribeiro and Nogueira-Couto 2002). In coffee crop, the studies developed by Klein et al. (2003, 2007) showed that the species is self-compatible, but the increase in fruits set is due to the relationship with pollination by bees. Also, comparing 24 agroforestry systems in Indonesia, the coffee fruit set can be favored by increasing the species of bees that visit the flowers, from 60% of fruiting (three species) to 90% (20 species). This suggests that minimal differences in the quantity and pollinator's efficacy can lead to variations in the agricultural productivity (Freitas and Nunes-Silva 2012). Thus, the first measure to promote the pollinators conservation is to motivate studies on the relationships between pollinators and economically important plants, which includes the analysis of the ecological and economic roles of pollinators, and essential information such as the effect of pollination on fruit and seed set patterns (Allen-Wardwell et al. 1998; Maués 2014). Also, to understand these informations is especially important when the producer needs to manage the pollinators in agricultural crops.

Studies that emphasize the influence of floral morphology and pollinator's behavior on reproductive success, in agricultural crops, are still not very representative. Floral attributes were demonstrated by van der Pijl (1960), as a set of convergent adaptations of flowers to the sensory mechanism of biotic or abiotic vectors responsible for pollination (Vogel 1954; Faegri and van der Pijl 1960, 1961, 1980; Culley et al. 2002; Rech et al. 2014). These attributes may restrict or guide the accessibility of visitors to resources

and determine the degree of dependence on plant-pollinator relationships (Sakai et al. 1998). Therefore, the richness of pollinators is closely related to their floral features, which are able of attracting, repelling and even mobilizing the learning process in the manipulation of reproductive structures that have restricted access to most species, a process resulting from probable competition between plants and floral visitors (De Luca and Vallejo-Marín 2013). Comparative studies are being developed to evaluate the pollinator's efficacy in many agricultural crops (Imperatriz-Fonseca et al. 2007; Hayashi-Trevizor 2014; Zambon 2015; Martarello 2016; Zambon and Agostini 2017; Martarello et al. 2021). These studies were conduced through the controlled access of the pollinators to the flowers, varying their number of visits, in order to compare the pollinator's efficacy on the reproductive success, thus providing relevant data for key-pollinators in several agricultural crops (Klein et al. 2007; Maués 2014; Santos et al. 2014).

Leguminosae has a cosmopolitan distribution and is the second botanical family in economic importance, being surpassed by Poaceae (Judd et al. 1999; LPWG 2013). Some species of Leguminosae (e.g., *Canavalia ensiformis* (L.) D.C., *Crotalaria juncea* L. and *Mucuna pruriens* (L.) D.C.) are used in green manure practice, offering many advantages for both soil and plants. Among these benefits are the erosion control, potential for mulching avoiding warming, ecological balance, soil moisture conservation, biological fixation of nitrogen and nutrient cycling from deeper soil layers to regions where cultivated plants can remove them (Fávero et al. 2000; Faria et al. 2004; Formentini 2008). To understand the pollination and reproductive biology of these species used as green manure is important, because the propagation occurs through seeds, requiring the intervention of the pollinators. Thus, obtaining seeds is essential for the soil management through green manuring (Mazzuco 2008). Other point to be mentioned is about the producer who adopts crop rotation or intercropping to perform the planting of green manure around the main agricultural crops and not cutting before flowering. The green manure species in the surroundings are important for fruit and seed set, in addition to maintaining pollinators in the area, because the pollination of cultivated plants depends on crop management and also the quality of adjacent habitats (Klein et al. 2003; Hayashi-Trevizor 2014; Santos et al. 2014).

From India, *C. juncea* is a legume, herbaceous, reaching a maximum height from two to three meters (Graner et al. 1973; pers. obs., NA Polizelli-Ricci 2016). The flowers are yellow, pentamers and bisexual, distributed in inflorescences containing from four to 20 flowers (pers. obs., NA Polizelli-Ricci 2016). The species offers nectar and pollen, having as main pollinators large bees (bumblebees) (Silva et al. 2009). Also, has self-incompatibility system (Maeda et al. 1986; Thimmaiah et al. 2018), and flowering period occurs from 60 to 120 days after planting (Salgado et al. 1987). In field, our individuals bloomed 61 days after planting (pers. obs., NA Polizelli-Ricci 2016).

Flowers of *C. juncea* have an anthesis of five days, and sequential flowering, starting in the base and finishing in the apex of the inflorescence (acropetal opening) (pers. obs., NA Polizelli-Ricci 2016). Calix is dialysepal, with four sepals. Corolla is composed of five yellow petals, divided into a flag, two wings and two fused petals forming the keel blossom, whose function is to shelter and protect the floral reproductive structures. It has zygomorph symmetry (bilateral) and free petals among them (dialypetal). Flowers have 10 free stamens, erects, greenishes in color and present a dimorphism of the male floral structures

(heterantry and heterostemony), being five fillets with rounded anthers and five with elongated anthers, both with longitudinal dehiscence. Pollen grains are viscous and yellowishes in color. Stigma is greenish and the surface is composed of several trichomes, mainly at their apex. Fruits are legume type, cylindrical, greenishes in color and the surface is covered by trichomes (velvety appearance). Ovary has ovules arranged linearly and in two columns, varying between six and 16 in number (pers. obs., NA Polizelli-Ricci 2016).

Our manuscript brings a new descriptive study about pollination and reproductive biology, highlighting the data about specialized behavior of pollinators to access floral reproductive structures and their influence in the fruit and seed set. In addition, we reported the dimorphism of floral male structures in *C. juncea* (heterantry and heterostemony) through floral morphological studies, and also, we performed a pollinic study (quantitative and viability tests) comparing the different anther morphologies. For that, we compared the *Xylocopa* (*Neoxylocopa*) *frontalis* (Olivier, 1789) efficacy according to number of visits in flowers of *C. juncea*. Our questions were: (1) Was it necessary any specific behavior of *X. frontalis* to approach the keel blossom and the access to the floral reproductive structures? (2) Did the amount and viability of pollen grains produced vary between different anther morphologies? (3) Did the increase in the number of visits of *X. frontalis* result in greater fruiting and viable seeds? (4) Was there an increase in the average weight of fruit set, according the number of visits of *X. frontalis* increased? (5) Did the aborted seeds occur more frequently in the basal position of the fruit (related to competition for pollen tubes) or in the apical position (related to maternal resources)?

We expected the pollination system by bees in *C. juncea*, because flowers visited by bees usually present blue, yellow or purple colors, landing platform, diurnal anthesis and different floral resources (nectar and pollen) (Pinheiro et al. 2014). We also hypothesized that the highest number of *X. frontalis* in *C. juncea* resulted in more pollen grains transported to the stigma, a greater number of pollen tubes growth, resulting in fertilization and providing an increase in the average weight and number of viable fruits and seeds. About components of fecundity, we suggested that the basal positions of the inflorescences presented greater fruit set, since it is possible to increase the use of mother-plant resources for the progeny's development (Lloyd 1980; Teixeira et al. 2006), thus a higher probability of fruit set in positions close to access to resources. As for seeds abortion, it should occur in greater numbers at the ovary positions further away from the stigma, as this is the most common for Leguminosae (Bawa and Buckley 1989; Teixeira et al. 2014). In this case, basal ovules would be fertilized by the slower growing or lower pollen tubes competitive, and therefore more likely to be aborted (Gibbs and Sassaki 1998; Otárola and Rocca 2014).

Material and methods

I. Components of fecundity experiments

We used n = 120 individuals of *C. juncea* in an urban area of Santa Bárbara d'Oeste, São Paulo, Brazil (22° 44' 21.9" S; 47° 21' 22.6" W) grown from seed to adult plant. Flowering period occurred from

February to April 2016, and fruits and seeds set from March to June 2016. We performed four treatments to determine if the number of visits of *X. frontalis* influences the fruits and seeds set: 1) spontaneous self-pollination, 2) one visit, 3) two visits, and 4) three visits by *X. frontalis*. We used n = 30 randomly selected individuals and bagged four floral buds of each one (n = 120 floral buds).

After floral anthesis, we exposed individually the flowers to the pollinators. We re-bagged the flowers after pollinators visited them. When the fruits have ripened, we collected them (about 40 days after tests) and verified the number of viable and aborted seeds and the fruit fresh weight. Also, we evaluated if there was a difference among three treatments. We registered the number of seeds per fruit and the position inside the fruit where the seeds abortion occurred in (n = 684) fruits. We verified the position of aborted and viable seeds in immature fruits (n = 10 inflorescences per specimen in a sampling of n = 30 specimens). From the aborted seeds, we noted their position in the fruit, with the first position being the one with the seed closest to the pedicel (basal position) and the last position being the one with the seed closest to the floral stigma (apical position) (Teixeira et al. 2006; Agostini 2008). We considered as aborted seeds when presented irregular shape and/or very small size. In addition, we planted 60 seeds of *C. juncea*, distributed in 20 plastic bags, with three seeds in each bag, and we stored them in a greenhouse, located in Santa Bárbara d'Oeste, São Paulo, Brazil (22° 43' 49.6' 'S; 47° 24' 18.5" W) to spontaneous self-pollination test. The seeds were acquired from the same lot, at "Piraí sementes", Piracicaba, São Paulo, Brazil, accredited in the National System of Seeds and Seedlings of the Ministério da Agricultura, Pecuária e Abastecimento of Brazil through the National Register of Seeds and Seedlings - RENASEM SP0811/2006.

We used parametric (ANOVA) and non-parametric (Kruskal-Wallis) statistical tests, followed by the Tukey's HSD and Dunn's tests, to investigate pairwise comparisons in the presence of significant differences for the ANOVA and Kruskal-Wallis. We also applied the G-test and chi-square-test (χ^2) to evaluate if the number of developed fruits varied according to the number of visits of *X. frontalis*, using software R version 3.1.1® (R Core Team 2017).

II. Characterization of the reproductive structures and keel blossom

We fixed male and female floral reproductive structures and keel blossoms in FAA (ethyl alcohol 70%, formaldehyde 37% and glacial acetic acid – 90:5:5 v/v) and dehydrated in an ethylic series (10%, 30%, 50% and 70%) and acetone (90% and 100%). We submitted the samples to the critical point for the removal of water, using the Balzers Critical Point Dryer 030®. After, we covered with gold by the method of "Sputtering", through Balzers Sputter Coater 050®. Micrographs were obtained by scanning electron microscope Hitachi TableTop Microscope TM3000®, operating at 15 kV. Analyze were performed at the Microscopy Lab of the State University of São Paulo "Júlio de Mesquita Filho" (UNESP), *Campus* of Rio Claro, São Paulo, Brazil.

III. Pollen grains counting in different anther morphologies (heterantry) and details of heterostemony

We collected all pollen grains of elongated and rounded anthers (one to each morphology), in 10 flowers. For each flower, the pollen grains were placed in Eppendorf with 10 drops of ethyl alcohol 70%. After shaking the Eppendorf, a drop of the solution was removed, placed on a glass slide and observed by the scanning method in a light microscope under 400x. We counted all the pollen grains through manual counter. We estimated the pollen grains o fone anther by multiplying the total found in a drop taken from the solution by the total drops. To estimate the number of pollen grains in each flower, we multiplied the total resulting from Eppendorf (referring to one anther) by the number of anthers of each morphology. Flowers of *C. juncea* also present heterantry and heterostemony, detailed later in this article. To verify the quantity of pollen grains in different morphologies (n = 10), we applied the *t*-test paired at 5% using software R version 3.1.1®. We collected floral samples in our planting and we measure the pollen grains in the Biology Lab of the Federal University of São Carlos (UFSCar), *Campus* of Araras, São Paulo, Brazil.

IV. Pollen viability

We used acetic carmine dye 2% (Radford et al. 1974). We sampled three floral opening stages - stages two (24 h after opening), three (48 h) and four (72 h), with 10 replicates each one. In each flower, we removed a pollen grains sample from the anthers and distributed on a glass slide. After, we added acetic carmine dye and covered with a cover slip. To avoid the drying of samples, the observations and recording of pollen grains occurred after slides preparation. We used the slide scanning method under a light microscope, and counted 100 pollen grains. We estimated the pollen viability by the ratio between stained (viable) and non-stained pollen grains and/or with retracted cytoplasm (non-viable). We used this method for both morphologies (rounded and elongated), because we detected heterantry in flowers of *C. juncea*. We submitted the data to ANOVA and Tukey's statistical analysis at the significance level of 5%, using software R version 3.1.1®. This step was performed at the Biology Lab of the Federal University of São Carlos (UFSCar), *Campus* of Araras, São Paulo, Brazil.

V. Frequency and behavior of floral visitors

We observed 10 flowers for each one of the three floral stages (stages two, three and four), because the flowers are in anthesis and attracting visitors. We observed the behavior of foraging over three consecutive weeks in February 2016 (five days - from 6:00 a.m. to 6:00 p.m., and ten days - from 10:00 a.m. to 6:00 p.m.), totaling 140 hours in field. We performed these observations, *in situ*, to relate the visitor's behavior to floral morphology. Also, we verified if the visitor reached the floral resources and touched on the reproductive structures. We calculated the average and standard-deviation of visits over three floral stages, using Shapiro-Wilk normality and ANOVA's tests using software R version 3.1.1®.

Results

I. Characterization of the reproductive structures and keel blossom

Although the anthers of *C. juncea* were large enough to be viewed under a light microscope, the scanning electron microscopy allowed a more detailed analysis of all structure. Scanning electron microscopy allowed to observe that flowers of *C. juncea* are heterantries, that is, they have different morphologies of the anthers (Fig. 1A-B), both with longitudinal dehiscence (LD). Style (SY) has superior length to the fillets and the trichomes (TR) on its surface occurs in a spiral distribution, from the base to the apex (stigma, ST) (Fig. 1C). Stigma has a hook shape, which may be connected to the pumping motion of the pollen grains at the keel blossom (KB) apex, at the time of *X. frontalis* bumblebee foraging (Fig. 1D). Keel blossom in *C. juncea* is covered by a large quantity of trichomes, and is composed of two molten petals (Fig. 1E), so that their trichomes join at the ends, allowing their opening and exposing the reproductive structures during *X. frontalis* foraging (Fig. 1F).

II. Pollen grains counting in different anther morphologies (heterantry) and details of heterostemony

It was obtained difference between the number of pollen grains in different anther morphologies, based in the paired *t*-test (t = 1.8^{-9}, p < 0.001). In 10 flowers sampled, we verified the estimated values of pollen grains varying between 201,150 and 248,500 for elongated anthers, 223,135 = and σ = 14,660. For rounded anthers, we verified the estimated values of pollen grains varying between 50,150 and 93,950, = 64,705 and σ = 11,990.

In *C. juncea*, we verified a phenomenon known as heterostemony, i.e., flowers containing a distinct growth along floral anthesis (five fillets with greater length and five fillets with smaller length, both presenting longitudinal dehiscence - LD). This event occurs as follows: In the floral stage one (Fig. 2A), the shorter fillets have anthers with rounded teaks (RT), while the longer fillets have anthers with elongated teaks (ET). In the stages two and three (Fig. 2B-C), when the flowers are open, and the anthers are releasing the pollen grains, we verified that the fillets that had less length, in stage one, had a growth in length and equalized to the fillets with greater length, but the size of the anther teaks did not change. In the stage four (Fig. 2D), the length of the fillets with rounded teaks exceeded the fillets with elongated teaks (Fig. 2A), evidencing the heterosemony.

III. Pollen viability

We not found a statistical difference in the viability of pollen grains in anthers with rounded teaks ($F_{[2,27]}$ = 2.884, p = 0.073), but we found a statistical difference in the anthers with elongated teaks ($F_{[2,27]}$ = 9.258, p < 0.001). Using the Tukey's test, we verified the discrepance between two and four stages (p = 0.010) and between three and four stages (p < 0.001).

IV. Frequency and behavior of floral visitors

During 140 hours of field experiments, we found only visits of *X. frontalis* in flowers of *C. juncea*. In the floral stages two, three and four, we obtained an average of visits of 30.75, 32.88 and 26.10, respectively.

ANOVA's test not showed statistical difference when comparing the values obtained in floral stages two, three and four ($F_{[2,21]}$ = 0.615873, p = 0.549642). The day period with greater foraging by floral visitors occurred from 3:00 p.m. to 3:59 p.m., at floral stages two, three and four (n = 55, n = 68 and n = 50), respectively (Fig. 3).

Observations in field showed to us that, during foraging, the carpenter bees *X. frontalis* grasps the flowers with the anterior and median legs and, thus, two movements occur, concomitantly, in the wings: backwards and laterally. This lateral movement makes it possible the passage to the nectary, initially closed by the wings, and the buccal parts are inserted in the central region of the nectary. With the keel blossom-wings complex moving, the distal part opens, and the reproductive structures, which are immobile, are finally exposed. During pollen grains release, the stigma, which contains small trichomes, rubs on the anthers and agglutinates a large amount of viscous pollen grains on its surface. The stigma, containing the pollen grains, is released by the keel blossom apex and adheres to the ventral thoracic and abdominal regions and anterior and medial legs of *X. frontalis*. When the visitor leaves the flower, all reproductive structures return to their initial position.

V. Components of fecundity (fruit and seed set)

We verified that the fruits development had an increase, according to the number of visits of pollinators on the flowers. Treatment with only one visit was different statistically from treatment with three visits (G = 12.2364, df [(*)] = 2, p < 0.001), but there was no difference between one visit treatment with the two visits treatment (G = 3.277, df = 2; p = 0.0694) and between two and three visits (G = 2.1584, df = 2, p = 0.1418) (Table 1). [(*)] df means degrees of freedom.

Fruit weight was greater according to the number of visits of pollinators (H = 16.13, df = 2, p < 0.001). Treatment with only one visit was different statistically from treatment with three visits (H = 14.78, df = 2, p < 0.001), and there was no difference between one and two visits (H = 4.353, df = 2, p = 0.1127083) and two and three visits (H = 5.05, df = 2, p = 0.1340360) (Table 2).

Seed development was greater according to the number of visits of pollinators (H = 14.86, df = 2, p < 0.001). Treatment with only one visit was different statistically from two visits (H = 4.254, df = 2, p = 0.02712) and three visits (H = 13.99, df = 2, p < 0.001). There was no difference between two and three visits (H = 2.954, df = 2, p = 0.05261) (Table 3).

We observed that seed abortions occurred mainly in the basal (peduncular) position of the fruit (n = 93), followed by the median (n = 58) and apical (n = 53) (χ^2 = 0.000925, df = 2, p = 0.05) and there was no difference between position of seed abortions and the three treatments related to the number of pollinator visits (F = 2.558, df = 2, p = 0.157) (Table 4).

Table 1

Number and percentage of fruiting, in all treatments with *Xylocopa* (*Neoxylocopa*) *frontalis* (Olivier, 1789) in *Crotalaria juncea* L. (Leguminosae, Papilionoideae) flowers, as well as G-test and p-values. * Statistical difference at 5%.

Treatments	Number and percentage of fruiting	Comparative treatments	G-test	p-value
One visit	11/30 (36.66%)	Between one and two visits	3.297	0.0694
Two visits	19/30 (63.33%)	Between one and three visits	12.2364	0.0005*
Three visits	25/30 (83.33%)	Between two and three visits	2.1584	0.1418

Table 2

Number of fruits and weight average of fruiting, in all treatments with *Xylocopa* (*Neoxylocopa*) *frontalis* (Olivier, 1789) in *Crotalaria juncea* L. (Leguminosae, Papilionoideae) flowers, as well as p-values, through Kruskal-Wallis's index (H) and Dunn's-test. * Statistical difference at 5%.

Treatments	Number of fruits	Weight average (g) of fruiting ± standard-deviation	Comparative treatments	p-value
One visit	11	0.58 ± 0.37	Between one and two visits	0.1127083
Two visits	19	0.63 ± 0.36	Between one and three visits	0.0000687*
Three visits	25	0.74 ± 0.34	Between two and three visits	0.1340360

Table 3

Total number of fruits and seeds developed, in all treatments with *Xylocopa* (*Neoxylocopa*) *frontalis* (Olivier, 1789) in *Crotalaria juncea* L. (Leguminosae, Papilionoideae) flowers, as well as p-values, through Kruskal-Wallis's index (H) and Dunn's-test. * Statistical difference at 5%.

Treatments	Number of fruits	Number of seeds developed (total)	Comparative treatments	*P* value
One visit	11	87	Between one and two visits	0.02712*
Two visits	19	167	Between one and three visits	0.0000775*
Three visits	25	229	Between two and three visits	0.05261

Table 4
Total number of seed abortions, in *Crotalaria juncea* L. (Leguminosae, Papilionoideae), according to the fruit positions (basal, median and apical), through efficacy tests of *Xylocopa* (*Neoxylocopa*) *frontalis* (Olivier, 1789). [a] Means with equal letters do not differ statistically from each other at $p < 0.05$.

Visits	Basal	Median	Apical	Total
1	26	13	12	51a
2	29	14	25	68a
3	38	31	16	85a
Total	93	58	53	

Discussion

I. Characterization of the reproductive structures and keel blossom

The tribe Crotalarieae (Benth.) Hutch. (Leguminosae, Papilionoideae), in which is circumscribed our studied species (*C. juncea*), is part of the Genistoide core (Crisp et al. 2000). Currently, comprises around 1,204 species distributed especially in tropical regions (van Wyk 2005; LPWG 2013), with tribe boundaries that may be the result of morphological and chemotaxonomic changes and molecular systematics (Boatwright et al. 2008a, 2009, 2011). After, we discussed about some aspects analyzed in our research: male and female reproductive structures and keel blossom within tribe Crotalarieae.

a) Androecium: In *C. juncea*, the flowers are heterantries and heterostemonies, that is, they present at least two different sizes of stamens, which may vary in size or shape of the fillet, insertion, size or colors of the anthers (Tucker 2006; Vallejo-Marín et al. 2009; Paulino et al. 2013). The presence of heteromorphic stamens may be associated with enantiomorphism, buzz-pollination and/or bee pollination (Buchmann 1983; Luo et al. 2008; Vallejo-Marín et al.2009; Almeida et al. 2013, 2015). Heterostemony probably evolved via pollinator-mediated selection to reduce the conflict caused by different pollinic functions (Vallejo-Marín et al. 2009). According to Vallejo-Marín et al. (2009), dimorphism in stamens is found about 16 families distributed in 12 orders, and Leguminosae has the highest richness of species (about 24,000 species). A study developed by Barrett (2010), showed that the Caesalpinioideae is a group that present the most distinct types of floral stamens. However, studies have found that the majority of genera with heterostemonies flowers were found in the subfamily Papilionoideae (Barroso 1991; Endress 1994; Mansano and Souza 2004; Prenner 2004a, b; Filardi et al.2007).

Within tribe Crotalarieae can have different arrangements in the anthers (Polhill 1976) that are informative at the generic level (Boatwright et al. 2008b, 2009). Keel blossom species tend to have

monomorphic anthers (genera with straight or curved style) or dimorphic anthers (rounded style) with an arrangement of (1) four basifix long anthers and six shorter dorsifix anthers; (2) four basifix anthers, carinal anther and five shorter dorsifix anthers; (3) markedly differentiated anthers into five long basifix anthers and five short dorsifix anthers (Polhill 1976). Also, the dimorphism appears to be directly linked to the keel blossom's twisted degree, and is strongly developed in the genus *Crotalaria* (Le Roux and van Wyk 2012).

b) Gynoecium: Polhill (1976) noted different types of pollen release in genera within Crotalarieae. Four basic types of pollen release in Papilionoideae were described by Delpino (1876): valve, explosive, brush and pumping. Furthermore, Lavin and Delgado (1990) distinguished four variations of brush type: (1) the ciliated style, with trichomes present from the base to the apex; (2) the ciliated stigma; (3) penicillary stigma, with trichomes present in the distal region of the style, above the anthers, preventing self-pollination and (4) erect trichomes (uniform in shape and orientation), located at the apex of the style conducting pollen grains outside the keel blossom promoting cross-pollination and decreasing self-pollination (Etcheverry 2001a, b, c; Etcheverry et al. 2003). Within genus *Crotalaria*, pollen grains are released through the pumping mechanism, with the keel blossom serving as a cylinder and the style and anthers acting as a piston; pollen grains are pushed and pumped out by brush action (Arroyo 1981) at the time of foraging. In *Crotalaria*, the style brush is always present with trichomes arranged upwards at the distal portion (Polhill 1976, 1982). Three types of trichomes are evident in *Crotalaria*: (1) a single line along the upper edge, (2) two lines along the sides or (3) distributed around the style (spiral) (Le Roux and van Wyk 2012), being the last type found in *C. juncea*.

c) Keel blossom: In flowers where the keel blossom is twisted, as *C. juncea*, the trichomes are arranged in a spiral following the style and keel blossom's shapes (Le Roux and van Wyk 2012). The close relationship between genera *Bolusia* Benth. and *Crotalaria* was mentioned earlier (Polhill 1976, 1982): The presence of the coiled keel blossom in *Bolusia* and a strongly curved keel blossom apex (becoming slightly coiled) in *Crotalaria cornu-ammonis* R. Viguier appear to provide a link between the two genera. The keel blossom's trichomes, then, serve to bind the keel blossom along their upper edges, and to ensure that the keel blossom protects the androecium and gynoecium, being a single structure during pollination process (NA Polizelli-Ricci, pers. obs. 2016).

II. Pollen grains counting in different anther morphologies (heterantry) and details of heterostemony

Some floral features can be interpreted as adaptations against excessive pollen grains removal by bees. In some species, such as the genus *Senna* Mill. (Leguminosae), there is a division of labor between anthers of different sizes - heterantry (Vogel 1978), while anthers deposit non-viable pollen grains on the ventral region or on the legs of the bees, later collected for food (protein source), others deposit viable pollen grains on the visitor's back (case of inverted keel blossom), which will not be removed by the cleaning behavior of these bees, and this is the pollen which will be transferred to the stigma of another flower (Gottsberger and Silberbauer-Gottsberger 1988; Marazzi et al. 2007). Androecium's dimorphism, as observed in *C. juncea*, occurs in some species with longitudinal anthers - e.g. genus *Swartzia* or poricides

- e.g. *Senna macranthera* (D.C. ex Collad.) H.S. Irwin & Barneby, *Senna multijuga* (Rich.) H.S. Irwin & Barneby and *Tibouchina langsdorffiana* (Bonpl.) Baill. (Melastomataceae). From an evolutionary point of view, the heterantry has been considered by some authors as a response from plants to ensure reproductive success, but with economy of floral resources (Vogel 1974; Buchmann 1983; Vallejo-Marín et al. 2009).

We verified a difference in the quantity of pollen grains produced when compared the different morphologies in anthers of *C. juncea*, refuting the same data analyzed in the study by Leite (2016), with the species *Tibouchina granulosa* (Desr.) Cogn. (Melastomataceae), which also presents the floral heterantry pattern. However, it corroborates data from the same study, which also analyzed the different morphologies in anthers of *Tibouchina pulchra* (Cham.) Cogn., and points out that elongated anthers contain more pollen than rounded ones, probably due to the size of the anthers being discrepant (high degree of heterantry). Regarding pollen dynamics, the results with species of the genus *Tibouchina* show that pollen is not fully available to bees at the starting of anthesis, which differs the result of this study with *C. juncea*, where anthers present pollen grains release from the first day of anthesis, coinciding with the foraging onset of *X. frontalis*.

Vallejo-Marín et al. (2014) described that the spatial arrangement of reproductive structures in a flower influences self- and cross-pollination rates, since the position of the style and stamens can interfere with anther pollen grains deposition to the stigma of the same flower, especially if the style it is longer than the length of the stamens, such as the *C. juncea* flowers, and it is mandatory to transfer pollen grains from the anthers of an emitting flower to the stigma of the receiving flower.

III. Pollen viability

In a study by Coelho et al. (2012) about pollinic viability in *C. juncea*, it was observed that the average percentages found ranged from 96% to 100%, therefore, close to the averages found in our analysis. The same study did not evaluate the viability percentage of pollen grains in different anther's morphologies, a differential in relation to the data presented in our manuscript. In *C. juncea* it was found that pollen grains are highly viable when tested with acetic carmine dye, which was not expected according to the division of labor hypothesis suggested in other studies (Müller 1882; Luo et al. 2008; Vallejo-Marín et al. 2009). This may be indicative of the high probability of reproductive success and higher crop productivity.

Thus, there was no difference in pollinic viability in the rounded anthers indicates that the floral stage does not affect the production of viable pollen grains in this morphology. However, in the elongated anthers there was a difference, indicating the influence of the stage in the pollinic viability. In this scenario, Souza et al. (2002) indicated that during floral opening, the pollen grains must be fully viable, but over the anthesis, pollen grains viability tends to decrease and reduces its fertilization rate. In addition, Giordano et al. (2003) reported that during the plant maturation process, pollen grains viability may also be affected by endogenous or exogenous factors such as floral development, high or low external temperatures, sun light, plant nutrition and use of agricultural inputs.

IV. Frequency and behavior of floral visitors

Around *C. juncea* plantation area showed other angiosperms species, such as *Passiflora edulis* Sims. (Passifloraceae), *Abelmoschus esculentus* (L.) Moench. (Malvaceae), *Bixa orellana* L. (Bixaceae), *Delonix regia* (Hook.) Raf. (Leguminosae, Caesalpinioideae), *Tecoma stans* (L.) Juss. ex Kunth. (Bignoniaceae), *Mangifera indica* L. (Anacardiaceae) and *Lagerstroemia indica* L. (Lythraceae). Some of these species bloomed in the same period as *C. juncea*, so the high availability of floral resources may explain the foraging of some pollinator species, including the *Apis mellifera* L. (1758, Apidae), *Tetragonisca angustula* (Latreille, 1811) and *X. frontalis*, the latter being the species observed in *C. juncea* flowers. *X. frontalis* is one of the main pollinators of *P. edulis* f. *flavicarpa* (yellow passion fruit) and *Passiflora alata* (sweet passion fruit) (Passifloraceae) for their large size and abundance in agricultural crops. These bees usually nest and remain in agricultural crops. However, studies showed that the species can guarantee natural pollination in more anthropized areas of São Paulo and other Brazilian States (Camillo 2003; Oliveira-Filho and Freitas 2003). Moreover, Marchi and Santos (2013), in a floristic survey of species visited by *Xylocopa* carpenter bees in São Paulo State, reported that *X. frontalis* visits a wide variety of angiosperms in urban and agricultural areas, including *T. stans*, *D. regia*, *B. orellana* and *C. juncea*, these species being present in our study site.

The results obtained in the field corroborated the hypothesis of the relationship between keel blossom and *X. frontalis*. Regarding the keel blossom, many plants in different families have a keel blossom, a feature that probably arose independently in each one (Westerkamp 1997a, b), but is even more evident in the Leguminosae (Simpson 2006). Several authors have suggested over time different adaptive aspects that may have led to the emergence of this trait: (1) adaptation to protect pollen grains from rain (Sprengel 1793), (2) ovary protection from bird beaks, in the case of flowers originally pollinated by birds (Grant 1950) or (3) adaptations that emerged in flowers that were originally pollinated by bees (Faegri and van der Pijl 1980). However, more recent works suggested that keel blossoms have emerged as an adaptation against excessive pollen collection by bees (Westerkamp 1997a, b; Westerkamp and Claβen-Bockhoff 2007; Amaral-Neto 2011).

Other point to be mentioned is that Westerkamp (1997a, b) described the standard as immovable, since the folds at the base of the petal make the structure rigid and make it one of the flower's strongest points. The rigidity of the petal means that during this movement of raising the flag, the body of *X. frontalis* is forced down, increasing the efficacy of the style exposure mechanism (Amaral-Neto 2011) and, consequently, the deposition of pollen grains on the pollinator's body. As shown, the interaction between the petals, especially the flag and wings create barriers that cannot be overcome by bird beaks, as suggested by Grant (1950), requiring a specific approach to access floral resources and, in the process, to transport pollen grains to other flowers, contributing to the reproductive success of *C. juncea*.

According pollinators' visits along day, in different floral anthesis stages, there was an increase in the average of visits from stage two to three, but a decrease of this average in the stage four. These data may mean the increased availability of floral resources at stage three, especially nectar, and the reduction

of floral resources from stage four, as the flower approaches the onset of floral senescence, thus lower floral appeal to the visitor and, consequently, decreased number of visits. The effect of robbers in reducing the number of visits from one flower stage to another is discarded, as some studies report the negative effects of these behaviors by decreasing the number of visits by effective pollinators due to the reduction of available resources (Irwin and Brody 1999; Dedej and Delaplane 2004). However, we did not observe robbers in *C. juncea* during field observations.

The afternoon period from 3:00 p.m. to 3:59 p.m. was the most representative in the number of visits to the three floral stages analyzed, which corroborates a study by Amaral-Neto (2011), in which the author verified the visiting hours of four bee species: *Xylocopa grisescens* (Lepeletier, 1841), *Xylocopa cearenses* (Ducke, 1910), *X. frontalis* and *Centris fuscata* (Lepeletier, 1841), and reported a higher number of visits from 7:00 a.m. to 10:00 a.m. (early hours of the morning) and from 3:00 p.m. to 6:00 p.m. (last hours of the afternoon), with the latter period coinciding as the most frequent for *X. frontalis*, in *C. juncea* flowers (pers. obs., NA Polizelli-Ricci 2016). According Amaral-Neto (2011), the efficient performance at foraging and the large body size of *X. frontalis* indicate that it can be considered an essential species for the development of fruits and seeds. Yet, the hairiness in the ventral region and the scopes of this species help in the adherence of pollen grains and, when approaching the flower and contact the reproductive structures, can be considered effective pollinators, with greater chance of pollen transfer between flowers, in a single visit.

Garibaldi et al. (2013), verifying the efficacy of native and exotic pollinator species, detected an increase in fruit development of many agricultural crops when flowers were pollinated by native species, even if exotic ones deposited large amounts of pollen grains. This is because native species provides effective distribution of pollen grains in the stigma and increase the cross-pollination by visiting flowers from individuals that are distant from each other.

V. Components of fecundity (fruit and seed set)

In *C. juncea* there was no fruit set in the spontaneous self-pollination test in the field and in the greenhouse. The study conducted by Thimmaiah et al. (2018) indicated that *C. juncea* is a self-incompatible species that generally reproduces through cross-pollination, as there was a significant difference in the developed pods between self- and cross-pollination, coinciding with our results, where there were no fruits set in the spontaneous self-pollination test. According to Bianchi and Gibbs (2000) and Nayak and Davidar (2010), in bisexual flowers is used a Self-incompatibility Index (ISI) to determine the reproductive success of angiosperms. In this index, ISI values lower than 0.25 indicate the presence of self-incompatibility, corroborating the ISI results of Thimmaiah et al. (2018) for the study conducted with *C. juncea*. Therefore, we consider the need for cross-pollen transfer to ensure the reproductive success of the species.

Our study did not perform experiments of floral anatomy to verify the pollen tubes growth in flowers submitted to cross- and self-pollination. However, the literature brings the study conducted by Thimmaiah et al. (2018), in which was obtained a highest number of pollen tubes reaching ovules, in pistils submitted

to cross-pollination, compared to the pistils submitted to self-pollination. The reduced pollen tubes growth in self-pollinated flowers can be attributed to self-recognition during pollen-pistil interaction, which triggers the post-pollination / pre-zygotic barrier (Oliveira and Maruyama 2014).

Our floral biology experiments revealed that the number of fruits developed was different between treatments with only one pollinator visit and three visits, corroborating the discussion of Lowenstein and Minor (2015), where a greater number of bees in the flowers can result in a higher fruits development in the eggplants crop. These authors explained that there is a longer period of time between one visit and another, allowing greater production of pollen grains in the anthers and, thus, greater pollinic transfer by the pollinators. About this, the flowers of *C. juncea* have a system of pumping the pollen grains, requiring six visits of *X. frontalis* to release all the pollen grains produced by the anthers. In addition, *C. juncea* pollen grains are oily, allowing for easy attachment to the first and second pairs of legs and abdomen of *X. frontalis* (pers. obs., NA Polizelli-Ricci 2016).

Furthermore, the average weight of fruits set increased by the number of visits of pollinators, suggesting that *C. juncea* requires at least two visits of *X. frontalis* for optimal fruits and seeds set (pers. obs., NA Polizelli-Ricci 2016). We verified that with a single visit of *X. frontalis* in the flower of *C. juncea* is possible to develop a fruit, but it is not always successful. Weight of fruits developed can be greater because the number of the seeds is also greater according to the number of pollinators visiting the flowers. If a fruit has more seeds, it is necessary more resources for embryo nutrition and seeds development, indeed fruits will be heavier because they are allocating more maternal resources for embryo development. Both factors can provide an increase in the production of some agricultural crops (Gingras et al. 1999; Artz et al. 2011).

In our study, seeds abortion frequently occurred in basal (peduncular) position, and it is related to the number of pollinator visits. Some studies reported that seeds located in basal position (peduncular position) are the strongest competitors by maternal resources. However, there are some species of Leguminosae in which an increase in aborted embryos is observed as they distance themselves from the stigma (Bawa and Buckley 1989; Susko and Lovett-Doust 1999). More pollinators foraging reflects in a greater pollen load, and if the closest ovules to the stigma are fertilized first by the sperm cells, these developing embryos will be successful in obtaining resources in than those embryos that developed later and are more distant from the stigma. Therefore, those embryos distant from the stigma and that developed later will be aborted due to the nutritional reduction required for seeds set (Teixeira et al. 2006; Teixeira et al. 2014).

We also verified that *C. juncea* increased fruit production from 36.66% (one visit from the pollinator) to 83.33% (three visits), increased the weight average of fruits developed from 0.58 g (one visit from the pollinator) to 0.74 g (three visits) and increased the seeds developed from 87 (one visit from the pollinator) to 229 (three visits). The difference between one to three visits suggests that the number of visits of *X. frontalis* could increase the production of *C. juncea*, as seen in other studies with agricultural

crops that verified the increase in productivity due to the visits of *X. frontalis* (Hayashi-Trevizor 2014; Zambon 2015; Martarello 2016; Zambon and Agostini 2017; Martarello et al. 2021).

About reproductive success of plants, it is determined by pre-emergent and the post-emergent stages. The first refers to the number of eggs that will develop into viable seeds, while the second is related to seed germination and plant development, including its reproductive stage (Wiens et al. 1987; Otárola and Rocca 2014). In several angiosperms, the viable seeds are formed from a small proportion of eggs; the other ovules do not develop by the absence of fertilization or, if they are fertilized, the embryos are aborted during the development (Oliveira and Maruyama 2014). In order to explain the occurrence of abortions in plants, there are several hypotheses, such as sexual selection, assigning abortions to the optimization of male and female reproductive success (Lloyd 1980), involving zygote paternity (Janzen 1977), the combination of lethal genes (Martin and Lee 1993), the amount of pollen grains deposited in the stigma (Mogensen 1975) and egg competition by resources required for seed development (Lloyd 1980).

Finally, in angiosperms, the viable fruits are generally less than the number of flowers produced by the individual. Possible explanations for the observed may be related to the process of fertilization and abortion during embryogenesis process. These studies of the components of fecundity make it possible to verify the existence of reproductive problems, including those related to embryo nutrition through maternal resources and to identify irregularities in the pre-emergent stage that may influence the events that will take to the reproductive success of plants (Otárola and Rocca 2014). Once this green manure is planted just by seeding, it is important to develop techniques to increase fruits and seeds production. Pollinator management can improve fruits and seeds production without the use of chemical inputs. We further suggest that producers, who adopt crop rotation, allow some individuals of *C. juncea* to reach the reproductive stage, because these individuals can produce floral resources to feed some pollinators and keep them in the area. Therefore, these pollinators can be useful for other agricultural crops that depend on pollinators for fruits and seeds production. Information about *X. frontalis* contribution to pollination will be useful to greenhouse and outdoor growers.

Declarations

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Author contribution statement

NAPR and KA planned, designed, and executed field work; NAPR and KA analysed the data; NAPR and KA wrote the manuscript; KA supervision.

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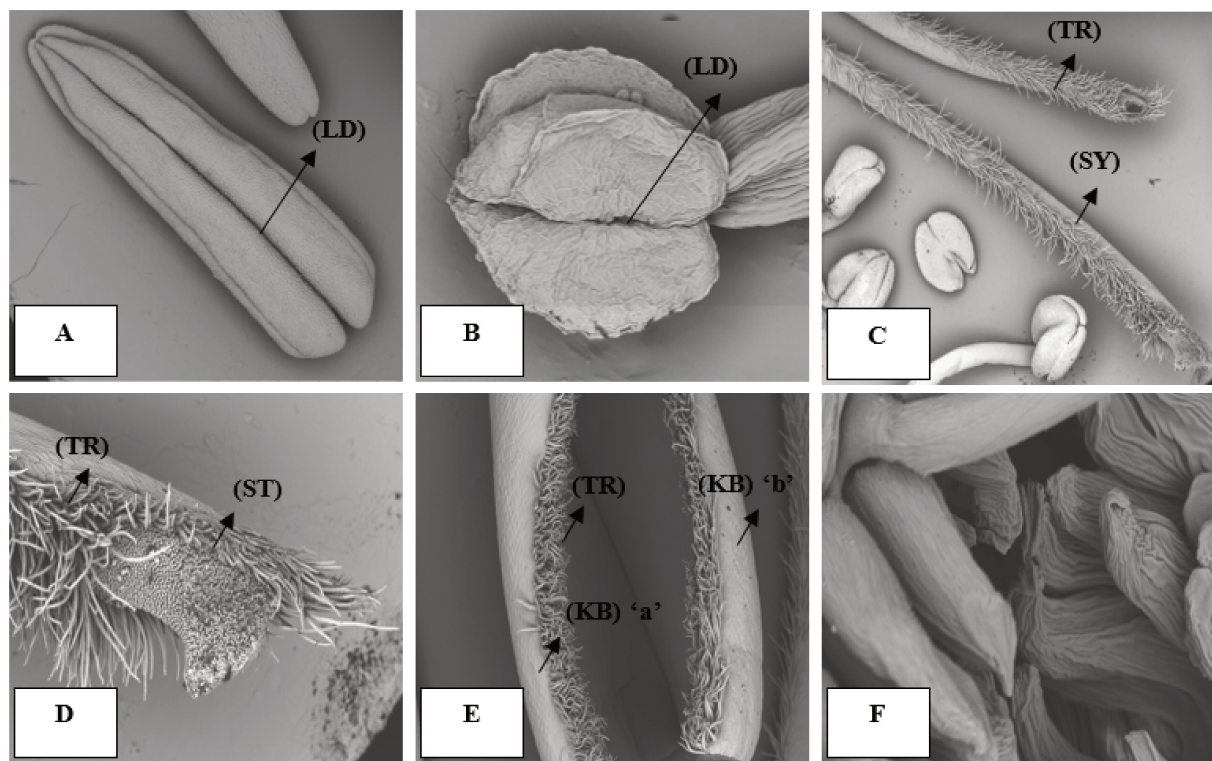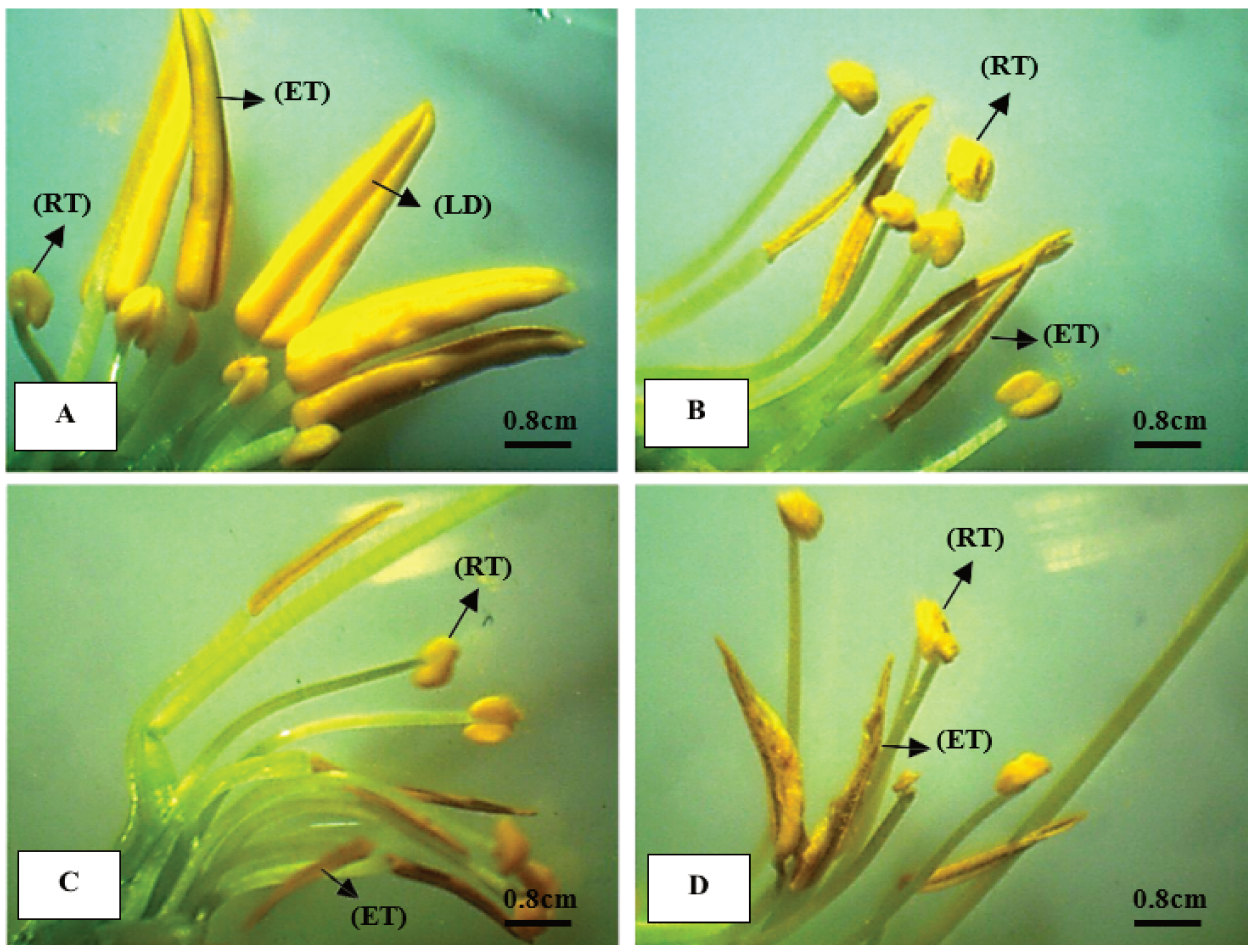
Figures

Figure 1

(A) Elongated anther of *Crotalaria juncea* L. (Leguminosae, Papilionoideae), showing teak and longitudinal dehiscence (LD) of this morphology (length = 2 mm, zoom = 400x) (B) Rounded anther, showing the teak and longitudinal dehiscence of this morphology (length = 300 µm) (C) Style (SY) surface, covered with a large quantity of trichomes (TR). (D) Stigma surface, covered with a large amount of trichomes. Note that the stigma has a hook shape, which may be connected to the pumping movement of the pollen grains by the keel blossom (KB) apex (length = 500 µm, zoom = 1200x) (E) Two petals forming the keel blossom (described by 'a' and 'b'), covered by a large amount of trichomes (length = 2 mm, zoom = 500x) (F) The trichomes of the keel blossom join at the ends, allowing the opening and exposition of the reproductive structures, during *X. frontalis* foraging (length = 50 µm, zoom = 1500x). Photos: NA Polizelli-Ricci.
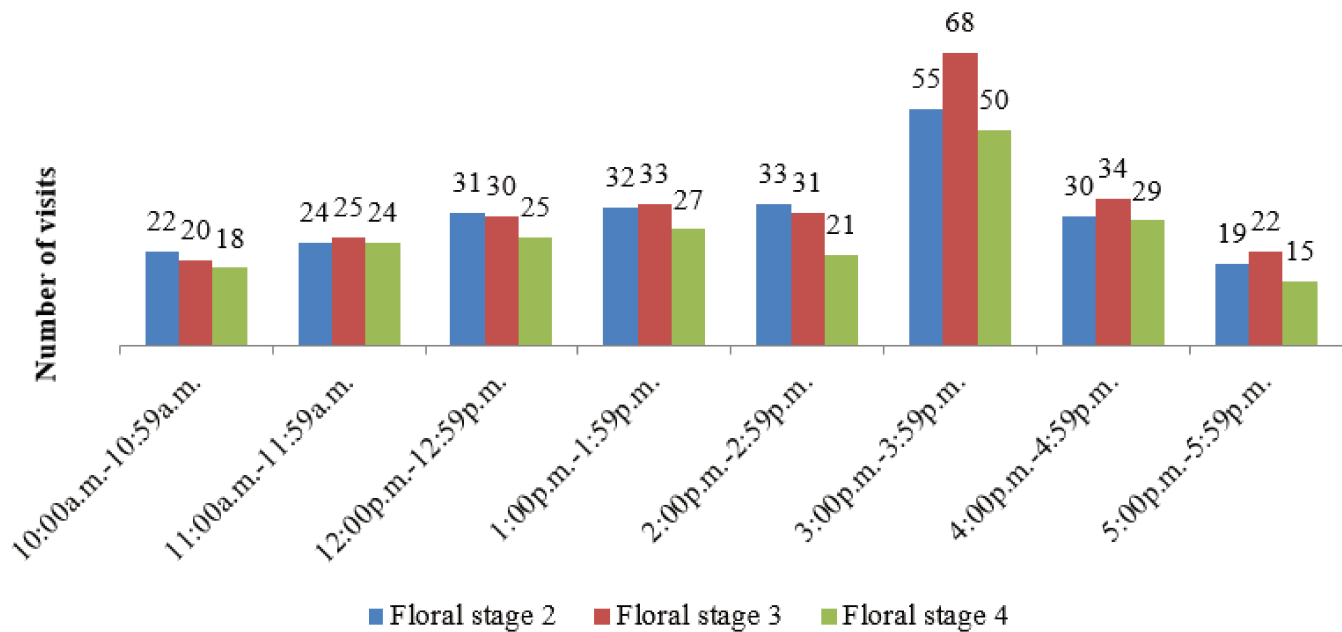

Figure 2

Crotalaria juncea L. (Leguminosae, Papilionoideae) flowers showing the different development stages of the androecium: (A) stage one: floral bud with longitudinal dehiscence (LD): fillets with rounded teaks (RT) are shorter than the length of the fillets with elongated teaks (ET), and the teaks are not yet releasing the pollen grains (B) stage two: fillets with rounded teaks are equal in length when compared with elongated teaks, and the longitudinal dehiscence between the teaks initiates the release of the pollen grains (C) stage three: fillets with rounded teaks still have the same length than fillets with elongated

teaks (D) stage four: fillets with rounded teaks extend beyond the length of the fillets with elongated teaks. ld: longitudinal dehiscence, et: elongated teaks, rt: rounded teaks. Photos: NA Polizelli-Ricci. Light microscope = 45x.

Figure 3

Number of visits of *Xylocopa* (*Neoxylocopa*) *frontalis* (Olivier, 1789) in *Crotalaria juncea* L. (Leguminosae, Papilionoideae), by day period, in the two, three and four floral stages (n = 10 flowers per stage). The period between 3:00 p.m. and 3:59 p.m had more visits of pollinators in all floral stages. Blue bars represent the second floral stage. Red bars represent the third floral stage. Green bars represent the fourth floral stage.