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BIOLOGY OF POLLINATION OF *Citrus sinensis* VARIETY 'PERA RIO'[1]

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ABSTRACT- Aspects related to the floral biology of *Citrus sinensis* 'Pera Rio' variety were studied in the present work aiming to obtain information about the pollination ecology in the local agriculture. Studies of flowering, anthesis, pollen / ovule ratio, stigmatic receptivity, pollen viability, nectar characterization and floral visitors were carried out. From the data studied, the following information was obtained: *C. sinensis* variety 'Pera Rio' flourishes in two annual periods (dry and rainy), the anthesis occurs from 9:00 AM with duration of 24 hours, during which period stigma receptivity, pollen viability and nectar secretion are highly significant, demonstrating that the species also possesses characteristics of allogenic plants. *Apis mellifera* was a floral visitor, with a greater number of individuals with a Relative Frequency (RF) of 51.1%. *Melipona scutellaris* obtained second place in visits with RR = 23.6%; Followed by *Trigona spinipes* with FR = 17.7%. The two seasons (dry and rainy) presented high similarity (Morisita index = 0.64). C. sinensis has floral biology favorable to cross pollination and bees *A. mellifera* and *M. scutellaris* are potential pollinators of this fruit in the conditions in the region of the Recôncavo Baiano. The reproductive system of *C. sinensis* is mixed, being favorable to entomophilic pollination.
Index terms: Apoidea, citricultura; pollination; bee population, orange.

BIOLOGIA DA POLINIZAÇÃO DE *Citrus sinensis* VARIEDADE 'PERA RIO'

RESUMO-Aspectos relacionados à biologia floral de *Citrus sinensis* var Pera Rio foram estudados no presente trabalho, com o objetivo de obter informações da ecologia da polinização na agricultura local. Foram realizados estudos da floração, antese, razão pólen/óvulo, receptividade estigmática, viabilidade polínica, caracterização do néctar e visitantes florais. A partir dos dados estudados, foram obtidas as seguintes informações: *C. sinensis* var Pera Rio floresce em dois períodos anuais (seco e chuvoso), a antese ocorre a partir das 9:00 da manhã com duração de 24 horas, período pelo qual a receptividade do estigma, viabilidade polínica e a secreção de néctar são altamente significativos demonstrando que a espécie também possui características de plantas alogâmicas. *Apis mellifera* se destacou como visitante floral, com um maior número de indivíduos com Frequência Relativa (FR) de 51,1%. *Melipona scutellaris* obteve segundo lugar nas visitas com FR = 23,6%; seguida por *Trigona spinipes* com FR = 17,7%. As duas estações (seca e chuvosa) apresentaram similaridade alta (índice de Morisita = 0,64). *C. sinensis* possui biologia floral favorável à polinização cruzada e as abelhas *A. mellifera* e *M. scutellaris* são potenciais polinizadores dessa fruteira nas condições do Recôncavo Baiano. O sistema reprodutivo de *C. sinensis* é misto, sendo favorável à polinização entomófila.
Termos para indexação: Apoidea, citricultura; polinização; população de abelhas, laranja.

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INTRODUCTION

In order to understand the biology of Citrus pollination, it is essential to clarify the phenomena associated with the formation of seeds and fruits (DAFNI et al., 2005; COUTO et al., 2006).

The study of floral biology is a basic condition for the analysis of the interactions between pollen and stigma, flowers and pollinators associated with the reproductive success of plant species (DAFNI et al., 2005). Size, morphology, color, odor and anthesis are floral data used in the study of agricultural crops, as they help not only the understanding of the plant-pollinator relationship, but also how the reproductive success occurs (GALETTO, BERNARDELLO, 2004; DAFNI et al. , KEVAN et al., 2005).

Another important aspect to be evaluated in studies of reproductive biology is related to floral visitors. The survey of these, determines the population of species of native bees, as well as can point out ideal strategies for the densification and management of colonies in crops of economic interest.

In this context, the objective of this work was to study aspects of the pollination biology of orange tree (*Citrus sinensis*) variety 'Pera Rio', in order to identify visitors that act as potential pollinators in the region of the Recôncavo Baiano.

MATERIAL AND METHODS

The experiments were carried out at the Experimental Station of Tropical Fruit Production of the Bahia Agricultural Development Company - EBDA located in Conceição do Almeida-Bahia (12º48'45 '' S; 39º15'20''W). The climate, according to Thomthwaite, type C1, dry and subhumid; The soil is classified as yellow clay. The study was developed in an orchard of *C. sinensis* 'Pera Rio' variety of six years of age in a planted area of 5,000 m2. The study covered the following assessments:

Floral morphology: A total of 40 flowers of 300 plants were randomly selected and evaluated for morphology following the orientation of Vidal and Vidal (2006) In the dissection of the flowers and the morphological description of the details of the floral pieces.

Antese: 40 pre-anthesis buttons were selected at random at 5:00 p.m. on the day before the observations. From 6:00 a.m. on the following day, the flowers were observed hourly for the determination of the beginning of the anthesis until the senescence of the petals.

Stigma receptivity: 40 flowers were selected at random and their stigmas were evaluated To the receptivity duration every 4 hours over the duration of the anthesis. Two drops of 10% hydrogen peroxide were applied on the stigmatic surface and a peroxidase bubble formation was observed with a magnifying glass (DAFINI et al., 2005).

Pollen/ovule ratio: was determined according to Cruden's methodology (1977), consisting of maceration of the anthers, centrifugation and total counting by microscopy, of the pollen grains released. The ova were removed from the ovaries and counted with the aid of a magnifying glass. Ten replications were performed to obtain the ratio of ovulle and total pollen grains.

Pollen viability (colorimetric method): pollen of 40 flowers was removed from flowers in the initial anthesis stage, with replicates every 4 hours throughout the duration of the anthesis. Four types of dyes were tested (acetic carmine, neutral red, Evans blue and aniline blue). The analysis consisted in the visualization of pollen grains with stained (viable) and non-stained (non-viable) cytoplasm. A total of 10 grains were counted per hour, counting 100 grains of pollen randomly chosen per leaf, making a total of 1,000 grains counted and evaluated per hour (Almeida et al., 2005; DAFNI et al., 2005).

Nectar: For the development of this work, the methodology described by Dafni et al. (2005), comprising the marking of 40 flower buds one hour before the anthesis. The buttons were marked and wrapped with white voil bag to prevent interference from floral visitors. The nectar was collected with microcapillary tube with precision of 2µl and the volume was measured with a digital caliper. The Brix was evaluated using a manual refractometer, whose conversion of the refractive indices into a sugar concentration was given by the equation Y = 0.00226 + (0.00937 X) + (0.0000585 X2), where "X" is the refractometer reading and "Y" Are the mg of sugar per µl to be used in determining the mg concentration of sugars. The nectar was removed from the flowers from 9:00 am to 9:00 am the next day when senescence of the petals occurs, making a total of seven withdrawals per flower. The samples of nectar of 40 flowers corresponding to the previous schedules were spilled on Watman paper discs nº 2 to perform the evaluation of the composition of sugars, according to Galetto and Bernardello (2004) methodology. The data were submitted to the Kruskal-Wallis test (p = 0.0001).

Floral visitors: the evaluation of the floral visitors was performed through entomological network, where two transects of 100 meters each

were drawn in two rows of the orchard Each one hour, 30 minutes each, once per period (one day of each month from 06:00 to 18:00), comprising the months of June 2011 to June 2013. The Collected bees were sacrificed in a lethal chamber, assembled and sent for identification by the Laboratory of Bionomia, Biogeography and Systematics of Insects (BIOSIS) of the Federal University of Bahia, where the specimens are deposited.

Reproductive system: for the study of the reproductive system adapted The methodology of Dafni et al. (2006). 120 randomly chosen flower buds of the same width and length, measured with a digital pachymeter of 40 orchard plants. The treatments were: spontaneous self-pollination (T1); Free pollination (T2) and manual cross pollination (T3). In the T1 treatment, 40 buttons were marked and wrapped with a voile bag, the day before the anthesis. The flowers remained bagged for 48 hours. In the T2 treatment, 40 floral buds were marked and left unprotected, the day before the anthesis. In the T3 treatment 40 buttons of the same size were marked, one day before the anthesis. The flower anthers of different plants were manually removed and touched on the stigmas of the marked flowers.

These were resealed for 48 hours. All the flowers submitted to the pollination tests were evaluated after eight days to verify the formation and persistence of the fruits. The fruits were harvested 120 days after pollination to evaluate weight, diameter and seed formation. After the physical analysis (weight, diameter and number of seeds) of the fruits, the data were submitted to analysis of variance and the means compared by the Tukey test at 5% of probability. The data were analyzed by the statistical programs SISVAR 5.1 (FERREIRA, 2011) and SAS 4.1.

RESULTS AND DISCUSSION

Characterization of flowering: There was significant flowering of *Citrus sinensis* 'Pera Rio' variety in four periods: September to October 2011 and 2012 and from February to April 2012 and 2013. In these periods, flowering spread through the subsequent months due to the constant irrigation of the area. Koller (1994) defined that flowering of citrus from the same variety of the present study in tropical climates may occur during all the annual seasons. In the South of Brazil, some works that used the varieties 'Pera Rio', 'Valência' and 'Bahia' defined the month of September for flowering (TONIETTO, TONIETTO, 2005; VITTI et al., 2003). For the State of São Paulo, the flowering of the 'Pera Rio' variety was defined by Di Giorgi (1991) between the months of June and August and in the event of pluviometric variation and temperature there may be blooms outside this period. In Bahia, there are two flowering seasons comprising the months of September and April (PASSOS et al., 2010). However, they may be influenced by the years when the rainy season is longer.

Floral morphology. The floral diagnosis of *C. sinensis* variety 'Pera Rio' showed that its flowers are pedunculated, cyclic, diclamid, heteroclamidic and hermaphrodite. They have on average 27 stamens, five petals and sepals and are actinomorphic. Its corolla is deciduous and the chalice is persistent. The gynoecium is hypogynous, and the stamens are mostly dipostomone type with some gamostêmone. The branching of the fillet is simple, with free anthers and dorsifixes, with longitudinal opening. These are diteca type with introrsa dehiscence. They are also gamocarpelar type flowers, pluricarpelar, infertile ovary with terminal insertion of stylet and undivided stigma. This information corroborates the work carried out by MALERBO-SOUZA; NOGUEIRA-COUTO (2002).

Citrus sinensis is characterized as a nectariferous species, since it has a nectariferous camera with 6.5 mm diameter and presence of osmophores as likely responsible for the release of perfume. Studies have classified *C. sinensis* as a nectariferous plant, among which Kerr et al. (1987); Almeida et al. (2003); Couto and Couto (2006); Wright (2007). The anthesis occurred from 9:00 a.m. in the morning characterized by the complete opening of the petals lasting 24 hours. During this period the peroxidase test was positive until the last hour (24 hours after floral opening), characterized by the formation of bubbles in the stigmatic papillae, with a mean percentage of 95% ± 4.24. Similar flower life was found by Malerbo-Souza and Nogueira-Couto (2002) in flowers of the same species, with a record of approximately 25 hours for the three varieties of oranges studied. Throughout the daytime period of the anthesis, there were successive visits of bees, wasps, butterflies and hummingbirds in the present study.

Pollen/ovule ratio. The total production of pollen per flower of *C. sinensis* variety Pera Rio was estimated at 25,488 pollen grains the number of ovule was estimated at 90 per flower. Thus, the pollen / ovule ratio is 2,832: 1. According to Cruden's (1977) classification, the pollen / ovule ratio is an indicator of the reproductive system of plants and suggests that if this ratio is high, as found in the present study, it would be indicative of allogenic plants. According

to Cruden (2000), the long duration of floral anthesis and stigmatic receptivity is directly associated with the large dispersion of pollen and the high pollen ovule ratio.

Pollen viability. The dyes tested were adequate in the estimation of pollen viability (Table 1), although the use of the acetic carmine dye showed a higher average of viable pollen grains. Pollen viability is important for obtaining information to support plant breeding programs. Shivanna and Johri (1992) observed that estimates of pollen viability may vary between species and even between samples of the same species or individual. In the specific case of Citrus, Soost and Roose (1996) reported that pollen viability may vary among species, but not in the same plant or species. Cavalcante et al. (2000) found variances of 9 to 98% in the estimation of the viability of the pollen of tangeleiros, using propionic carmine as dye.

Domingues et al. (1999) found viability of up to 78.2% in pollen in the 'Common Caipira' variety of oranges. Brugnara et al. (2011) reported values varying from 42% to 70% of viability in mandarins (*Citrus* sp.) And sweet oranges (*Citrus* sp.).

Nectar. The mean volume of potential nectar per 'Pera-Rio' flower was 9.52 ± 1.56μL / flower. On the other hand, there were significant differences between the means of the nectar available per hour by the Kruskal-Wallis test (p = 0.0001), with values varying between 20.2 ± 1.2 μL (10:30) and 1.3 ± 1.1 μL (3:30 p.m.). In the first few hours after the anthesis, a greater availability of nectar was observed.

This availability fell during the subsequent hours of cabbage Eta (15:50 h) and secretion was resumed at 17:30 h and 24 h after anthesis (Table 2). Nectar secretion occurred up to 24 hours after anthesis, along with positive data related to pollen viability, stigma receptivity and bee visitation. These constant visits during the day can cause continuity of the nectar production, only ending when senescence of the petals. Even in this long period after floral opening, the total soluble solids concentration was 240 Brix, characterized by the composition of 44.6% sucrose, 44.5% fructose and 10.9% glucose (Table 2). According to Galetto and Bernardello (2004), these sugar concentrations are ideal or preferred by bees.

These values were higher than the hours of the highest temperature of the day, since the sugar concentrations of the nectar are influenced by the climatic conditions mainly during the period when the temperature is higher. The sugar concentration (Brix) presented significant differences for the nectar Instantaneously available, observing higher concentrations in the hours of 15:30 h; 17:30 h and 24 hours after the anthesis and a lower concentration in the closing hours of the anthesis (10:30 a.m. and 1:30 p.m.).

The same occurred for mg of sugars and percentages of sucrose, fructose and glucose present in the nectar (Table 2). Santos (1956) considered that the production of nectar by citrus is relatively large, which is why it is highly sought by several bees. This author has carried out a study on the concentration of sugars in several species of botanical families, among them *C. aurantifolia* (Persia lime), where it found concentration of up to 380 Brix, close to noon. In the present work, an increase in the sugar concentration was observed from the second hour of nectar collection, reaching the maximum peak at 17:30 h, continuing until the next day, with an increase in the concentration of sucrose and glucose .

There was no increase in fructose concentration. For Galetto and Bernardello (2004) the relation pollinator plant is associated with the volume changes and concentrations of sugars in the nectar of the flowers of a certain plant, which promotes foraging fidelity. Chalcoff et al. (2006) suggested that entomophilic pollination is attracted by the quantity of sugars present in the nectar produced. Malerbo-Souza et al. (2003) when collecting nectar present in *A. mellifera* worker' bees after foraging on *C. sinensis* 'Pera-Rio' flowers, did not find divergent values in the concentration of sugars in five hours from the anthesis.

These authors concluded that the sugars present in the nectar are concentrated during the day, and because of this sugar concentration, bees prefer to be easier to dehydrate in the transformation into honey. This observation was also made by Santos (1956) and by Almeida et al. (2003). From the 17:30 h the visits are minimal and the bees return to an intense forage in flowers of the previous day from the 7:00 am of the following day which is perfectly explained with the analysis of the composition of sucrose and glucose More concentrated at that time.

Floral visitors. The community of bees at the study site was identified in a total of 5.050 specimens collected from floral visitors of *C. sinensis* which belong to five families. Of this total, the Apidae Family stood out with 99.00%, while the other Families (Andrenidae, Halictidae and Megachilidae) with percentages lower than 1%. In the present study, we observed a high association between species of the Apidae family and the species under study, with percentages similar to those observed in the present study (MALERBO-SOUZA

et al., 2003; NASCIMENTO et al., 2011).

Apis Mellifera was represented by total of 2.580 specimens. Bodlan; Armad (2015) found bees of the genus *Apis* in greater abundance when studying floral visitors of a species of Citrus. During their visits to the flower, behavior was registered preferentially for collecting nectar, and in more than 90% of their visits it was observed that the bee touched the reproductive structures. The relative frequency (51.1%) and its behavior in the flowers of *C. sinensis* suggests that this species may be a potential orange pollinator. Malerbo-Souza et al. (2003) found a relative frequency of 66% of *A. mellifera* in an orchard of the same species, considering it as an effective pollinator of the crop in some municipalities of São Paulo. Nascimento et al. (2011) also found a high frequency of *A. mellifera* in orchards of mandarin and sweet orange (77% and 50%, respectively).

Apis mellifera is a generalist bee with regard to foraging activity predominating over other species in most agricultural crops, except those requiring a pollinated agent Or specific due to its aspects related to the floral biology (AGUIAR, MARTINS, 2002, MALERBO-SOUZA et al., 2003).

In the study area, *Melipona scutellaris* was the second most frequent species, with 1.340 specimens, frequency of 23.6%, being also the majority of the visits characterized by the forage of the nectar and rarely pollen. Certainly this number had influence of the meliponary installed inside the orchard, what reinforces the possibility of handling this species in programs of pollination of *Citrus sinensis* var. 'Pera Rio' in the region.

Trigona spinipes was represented by 892 specimens with 17.7% frequency. In most of their visits, no contact of the bee's body with the reproductive structures of the flowers was observed, since the majority of the collections happened indirectly through a hole opened by them in the flower still in the bud stage (plunder of nectar). This bee was considered dominant, with a frequency of 34%, in the studies conducted by Malerbo-Souza et al. (2003) in orange blossoms. There was also dominance of *T. spinipes* (1,078 individuals and frequency of 21.37%) in the work of Nascimento et al. (2011) on C. sinensis flowers. The remaining visitors did not have a representative number of individuals and were considered as an accidental species by frequency and constancy.

The two dry seasons (months of August to February) and rainy (months of March to July) showed high similarity, As evidenced by the Morisita index (0.64), mainly due to the predominance of *A. mellifera* and *M. scutellaris* in comparison to the other species. Nascimento et al. (2011) studying three varieties of *Citrus* sp in Salinas, MG, found high similarity between two orchards in full bloom with seasons similar to those of the present work. The authors stated that the causes of similarity are due to the large population of *A. mellifera.*

Reproductive system. Free pollination (T2) promoted the highest percentage of fruit glue (78%); Followed by manual cross-pollination (T3), with the formation of 65% of fruits. And the self-pollination test (T1) resulted in the lowest glue (42%). Similar results were found by Malerbo-Souza et al (2003), the authors observed percentage of fruit glue of 63.80% in the free pollination test. The results obtained demonstrate that the action of several species of pollinators is important in Citrus production, corroborating the previous data of this same study.

Castañer (2003) stated that the number of seeds formed in the fruits can vary due to the genetic viability of the gametes, independently of the amount of pollen deposited on the stigma in the different forms of pollination, this being a peculiar characteristic of Citrus. However, the analysis of variance and the mean comparison test did not present statistical differences among the treatments regarding the number of seeds according to the table (Table 3).

In the other hand, statistical differences related to fresh mass and fruit diameter were observed. Fruits from free pollination presented the highest fresh mass. Malerbo-Souza et al. (2003) obtained higher fruit yields of the same variety of the present study when they were submitted to the free pollination, corroborating the results obtained in this study. However, Azevedo et al. (2013) found greater fresh mass of fruits in tests of manual cross-pollination for *C. sinensis*. In free pollination there is a greater possibility of pollen distribution under the stigma, which causes higher carpel fertilization and thus, a higher fruit yield.

TABLE 1- Pollen viability of *Citrus sinensis* var. 'Pera Rio' by means of four types of dyes in Conceição do Almeida, Bahia, 2011-2013.

Dyes	Schedules						
	9:00	13:00	17:00	21:00	1:00	5:00	24 h
Carmine Acetic	70,8 a	55b	50c	46d	44d	38e	22f
Evans Blue	56 a	53b	47c	43d	41e	38,2e	30g
Neutral red	45b	52 a	32c	41c	37d	34d	37d
Aniline blue	62 a	55b	61 a	54b	54b	45c	38d

Means followed by equal letters on the same line do not statistically differ according to the Scott-Knott test α = 0.05 probability.

TABLE 2- Volume (µL), concentration (Brix), mg sugar and percentage of sucrose, fructose and glucose of the nectar instantaneously available in the flowers of *Citrus sinensis* var. 'Pera Rio' in function of five collection times in Conceição do Almeida, Bahia, 2011-2013.

Variables	Schedules				
	10:30	13:30	15:30	17:30	24 h*
Volume (µL)	20,3 a	4,0c	1,3 d	4,3 c	17,7b
Brix (0)	21c	21c	22b	25 a	24 a
Concent. (%)	20,1 a	2,55c	0,84d	2,69c	11,1b
Mg of sugar	4,52 a	0,62c	0,229d	0,74c	2,929b
Sucrose	55,7b	61,7b	44,5c	72 a	44,6c
Fructose**	37,7b	29,4c	51,7a	19,8c	44,5b
Glucose**	6,6c	8,9b	3,8d	8,2b	10,9 a

Means followed by equal letters in the same row do not statistically differ according to the Kruskal-Wallis test, α = 0.05 probability. *24 hours after anthesis; ** Corresponding to the percentage of each sugar from the mg found previously.

TABLE 3- Characteristics of the fruits of *Citrus sinensis* 'Pera Rio' variety depending on the type of pollination. Conceição do Almeida-BA 2011-2013.

TREATMENTS		VARIABLES		
		Weight (g)	Diameter (cm) Nº	Seeds
Spontaneous self-pollination	T1	136,0 b	5,9 b	4,0 a
Free pollination	T2	223,8 a	6,9 a	5,4 a
Manual Cross-Pollination	T3	103,6 b	5,3 c	4,8 a
CV%		18,08	4,5	50,51

Means followed by the same letter in the column do not differ statistically from each other by the Tukey test at 5% probability.

CONCLUSION

In the conditions of the Recôncavo of Bahia, the anthesis of *C. sinensis* var. Pera-Rio occurs from 9:00 a.m. extending up to 24 hours after opening; Pollen dispersion, pollen / ovule ratio, pollen viability, nectar secretion and reproductive biology tests characterize this orange variety as an alternative allogeneic plant or mixed system. *Apis mellifera* and *Melipona scutellaris* are potential pollinators of the *C. sinensis*. Free pollination provides higher percentages of fruiting and commercial characteristics in fruits.

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