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STIGMATIC RECEPTIVITY AND POLLEN VIABILITY OF *Theobroma subincanum* Mart.: FRUIT SPECIES FROM THE AMAZON REGION[1]

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ABSTRACT – *Theobroma subincanum* (cupuí) is a fruit species native to the Amazon region. Fruits are enjoyed by local people and consumed both as fresh fruit such as juice, nectar or soft drinks. Reproductive biology studies provide contributions to conservation strategies and plant improvement. The present study aimed to analyze the stigmatic receptivity and pollen viability of *T. subincanum*. This study was developed in a forest fragment located in the urban perimeter of the municipality of Alta Floresta, MT. In the flowering period, flower buds or flowers were collected in seven different times, as follows: 10 pm, 02 am, 06 am, 10 am, 2 pm, 6 pm, and 10 pm. Pollen viability was estimated by reactive Alexander (1969) and stigmatic receptivity using 3% hydrogen peroxide. Pollen viability averages were submitted to analysis of variance, while stigmatic receptivity was analyzed by average percentage in each interval. Four floral stages were characterized based on flower opening, and from 6 am, fully opened flowers have already been found (stage IV). The percentage of pollen viability was not affected by collection times. In the stigmatic receptivity analysis, it was observed that in all floral stages, stigma was receptive; however, the highest percentages of stigmatic receptivity were found from 2 am to 10 am of the same day, which is the most propitious time for fertilization. Pollen collection of *T. subincanum* may be performed in any of schedules evaluated in this study, since it is held with high viability percentage.
Index terms: Cupui, pollen, fertilization, fructification, floral biology.

RECEPTIVIDADE ESTIGMÁTICA E VIABILIDADE POLÍNICA EM *Theobroma subincanum* Mart.: ESPÉCIE FRUTÍFERA DA REGIÃO AMAZÔNICA

RESUMO – *Theobroma subincanum* (cupuí) é espécie frutífera nativa da Amazônia, cujos frutos são apreciados pela população local, para consumo tanto *in natura*, como na forma de suco, néctar ou refresco. Estudos sobre a biologia reprodutiva podem fornecer informações e estratégias para a conservação dos frutos e melhoramento vegetal da espécie. O presente estudo objetivou analisar a receptividade de estigmas e a viabilidade polínica de *T. subincanum* em diferentes horários durante a antese. O estudo foi desenvolvido em um fragmento florestal localizado no perímetro urbano do município de Alta Floresta, MT. No período de florescimento da espécie foram coletados botões florais ou flores em sete horários diferentes: 22h, 02h,06h,10h, 14h, 18h, e às 22h. A viabilidade polínica foi estimada com o reativo de Alexander (1969) e a receptividade estigmática com o uso do peróxido de hidrogênio a 3%. As médias de viabilidade polínica foram submetidas à análise de variância, enquanto que a receptividade estigmática foi analisada pelo percentual médio em cada intervalo. Foram caracterizados quatro estádios florais com base na abertura floral, sendo que a partir das 6h da manhã já foram encontradas flores totalmente abertas (estádio IV). A percentagem de viabilidade polínica não foi afetada pelos horários de coleta. Na análise da receptividade estigmática foi observado que, em todos os estádios florais, o estigma encontrava-se receptivo, entretanto, os maiores percentuais de receptividade foram encontrados entre as 2h e às 10h do mesmo dia, sendo estes os horários mais propícios para a fertilização. A coleta do pólen em *T. subincanum* pode ser realizada em qualquer um dos horários avaliados neste estudo, visto que o mesmo se mantem com um alto percentual de viabilidade.
Termos para indexação: Cupuí, pólen, fertilização, frutificação, biologia floral.

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INTRODUCTION

The Amazon is considered one of the regions suitable for fruit production, with a large variety of native plants (MACHADO and RETTO JUNIOR, 1991). Among the various Amazonian fruit species, there are some of the genus *Theobroma* such as *T. cacao* L., *T. obovatum* Bern., *T. speciosum* (L.) Willd, *T. grandiflorum* (Willd. Ex Spreng.) Schum, *T. bicolor* H. & B.), and also *T. subincanum* Mart., which produce edible fruits whose seeds are used for chocolate production (VENTURIERI, 1993).

Theobroma subincanum, popularly known by cupuí, is a fruit species spread from the state of Para to Amazonian areas of the neighboring countries of northern Brazil. Its fruits, while having less economic importance than those of *Theobroma cacao* (cocoa) and *Theobroma grandiflorum* (cupuaçu), are appreciated by the local population and consumed both fresh as in the form of juice, nectar or soft drinks (CAVALCANTE, 2010).

Cupuí shows axillary or extra axillary inflorescences with 1-3 flowers. Its flowers are hermaphroditic, pentamerous and diclamid. Flowers have cup with yellow-ferruginous, thick-fleshy sepals and reflexed at anthesis; corolla with petals formed by two distinct regions, cogules and ligules. Ligules have dark red color, serving to attract pollinators and cogules are yellow and form a concave chamber where the androecium is located.

The species has great genetic affinity with other species of the same generic taxon, particularly with *T. grandiflorum* and *T. obovatum*, which allows obtaining interspecific hybrids (ADDISON and TAVARES, 1951). However, the reproductive organs (stamen and pistil) are isolated in the flower by two morphological barriers: the staminode crown, which isolates the stigmatic arms, and the petals (cogule) involving the anthers. These barriers prevent spontaneous contact between the two sexual parts of the flower, making autogamy very unlikely, as described by Venturieri and Ribeiro Filho (1995) for cupuaçu. According to Andrade (2009), the staminode circle forms a physical barrier which favors cross-pollination and hence increases the intraspecific genetic variability.

Determining the strategies to be adopted in a genetic plant breeding program is strongly influenced by the reproductive biology of the species (STIEHL-ALVES and MARTINS, 2008). Therefore, success in intraspecific and / or interspecific controlled crosses between selected parents requires knowledge on the pollen viability variation and the period in which the female part of the flower is receptive to the pollen grain.

It is possible to evaluate or estimate male fertility from histochemical tests using dyes, focusing on pollen viability and to assess stigma receptivity with the use of hydrogen peroxide (KEARNS and INOUYE, 1993; DAFNI, 1992).

In this context, this study aimed to analyze stigmatic receptivity and pollen viability in *T. subincanum* flowers in natural occurrence areas in different reproductive stages of this species during anthesis.

MATERIAL AND METHODS

Plant Material

During the flowering period of *T. subincanum* species in September 2014, flower buds and flowers were collected from naturally occurring individuals in an urban fragment of the municipality of Alta Floresta, MT. According to methodology proposed by Antonio (2004), with modifications, flowers and buds were identified and described in a classification of four flower development stages (Figure 1) based on the flower opening: a) stage I is characterized by fully closed and swollen flower bud b) stage II is characterized by the presence of cracks between sepals, c) and stage III is characterized by the detachment of all sepals, with petals beginning to extend, forming a hole upon the style and stigma d) stage IV when petals are in full expansion and stigma exposure.

Pollen viability via colorimetric test

Initially, flower buds in pre-anthesis were selected and marked, all in the same floral development stage, later, they were collected in 4h intervals in seven subsequent times: initial 10 pm; 02 am, 06 am, 10 am, 2 pm, 6 pm and final 10 pm, totaling 24 hours of collection during the same floral development. The collected material was fixed in Carnoy solution (ethanol: acetic acid 3: 1), kept at room temperature for 24 hours and then transferred to the freezer until the preparation of slides.

The colorimetric method was reactive Alexander (1969) using a triple solution composed of Orange G, basic fuchsin and malachite green. Basic fuchsin is a DNA specific dye, staining the cytoplasm in red; malachite green stains in green the pollen grain wall and Orange G is an enhancer. Through this test, viable pollen presents the purple color in protoplasts and green in the cellulose wall, while unviable grains stain only in green.

Eight slides were prepared for each collection

time, being recorded by the scanning method, under bright field microscopy and magnification of 400x (BEL Fhotonics and Primo Star Zeiss), 250 pollen grains per slide, totaling 2,000 pollen grains per collection time. In the preparation of slides, the technique of crushing anthers was used and during evaluation, pollen grains were classified as normal / viable (N / V) or abnormal / non-viable (A / I) according to the staining reaction (GUERRA and SOUZA, 2002).

To enable the indication of the best time in the estimation of pollen viability, the results were submitted to ANOVA and Tukey test at 5% probability with the help of the GENES statistical software (CRUZ, 2013).

Stigmatic receptivity

Flower buds or flowers were collected in seven consecutive times, as described above, to evaluate stigmatic receptivity before and during anthesis. Analyses were performed for three non-consecutive days using five flowers per collection times, and the detection of the stigmatic receptivity was performed by viewing the catalytic activity on the stigmatic surface with the use of 3% hydrogen peroxide (H_2O_2) according to methodology of Dafni (1992).

The stigmatic receptivity (%) was determined according to the amount of bubbles formed on the floral stigma surface, being classified into values of 0, 25, 50, 75 and 100% of peroxidase reaction activity. To obtain reliable results, damaged stigmas or those with pollen on the surface were not used, avoiding obtaining false positive results.

The receptivity results were submitted to ANOVA and Tukey test at 5% probability with the help of the GENES statistical software (CRUZ, 2013).

Results and discussion

Figure 1 shows the six floral stages identified and characterized during anthesis. Stage I (Figure 1A) was found in plants at 10 pm, flower buds beginning anthesis were found at 2 am, featuring stage II (Figures 1B, 1C, 1D) and stages III (Figure 1E) and IV (Figure 1F) were observed in the study population from 6 am. Anthesis in *T. subincanum* was more frequent in periods of 2 am and 6 am.

Pollen viability

The percentage of pollen viability in *T. subincanum* was not significantly affected by the collection times. However, high pollen viability was found in all times evaluated (> 98%). Similar results were found by Siqueira (2012), who analyzed the pollen viability of *Psidium guajava* in four times and found high viability (95.6%), with no statistical differences between collection times. Cabral et al. (2013) conducted a study with *T. cacao* and also found high percentages of pollen viability, above 95% on flower buds and 80% in open flowers.

Studies assessing the percentage of pollen viability at different times during flower development are important in artificial hybridization processes (COSTA et al., 2009), because they reveal the best time to collect pollen grains. However, it was observed in this study that for *T. subincanum* species, pollen grains can be collected at any time, since the pollen viability was high at all times.

Souza et al. (2002) reported that the pollen grain in the flower opening stage needs to be fully viable, since as time progresses, its viability is significantly reduced. For *T. subincanum*, there were no changes in viability rate over a day (24h). The maintenance of the viability rate of pollen grains during anthesis of flowers of a given species leaves the material more time available and facilitates the handling of artificial pollination experiments.

According to Loguercio and Battistin (2004), pollen viability studies are used to obtain results on the fertility of species and cultivars, providing a preview about plant infertility. Flowering period, environmental changes and genotypic differences may contribute to the variation in pollen viability rate (SHIVANNA and RANGASWAMY, 1992); however, in this study, these factors did not interfere in the viability rate, even with the high degree of human disturbance observed in the fragment under study.

Stigmatic receptivity

Theobroma subincanum stigmas were receptive in all evaluation times. Stigmatic receptivity analyses are essential, since fertilization will succeed only when pollen develops its pollen tube under the receptive stigmatic surface, as reported by Silva et al. (2010).

T. subincanum stigmas were already receptive in pre-enthesis flower buds at 10 pm, starting the time of evaluation; however, it was the time when species had the lowest stigmatic receptivity, with an average of 49.17% and values ranging from 45 to 52.5% (Table 1).

At 2 am and 6 am, stigmas showed average receptivity of 81.67% and 77.50%, respectively. At 10 am, stigma was highly receptive, with average value of 83.33%, with receptivity values of up to 95%. In times of 2 pm, 6 pm and 10 pm (last

evaluation time), lower stigmatic receptivity was observed, with averages of 65.83; 57.5 and 68.33%, respectively (Table 1).

In a study by Rodrigues and Venturieri (1997) with *T. subincanum*, it was found that flowers were more receptive between 10 am and 10 pm, unlike in this study, where the greatest stigmatic receptivity occurred between 2 am and 10 am.

The initial time of 10 pm is the least suitable for conducting controlled hybridizations, since this time shows lower stigmatic receptivity percentage. On the other hand, the best times for hybridizations in *T. subincanum* are between 2 am and 10 am. According to Brasileiro and Amaral (2009), pollinations should be performed during pre-anthesis because stigmas are receptive and pollen grains have high viability. To meet this requirement, artificial pollinations should be carried out between 2 am and 6 am, whereas from 6 am, flowers are already open.

Venturieri and Ribeiro Filho (1995) studied cupuaçu individuals (*T. grandiflorum*) and observed that stigmatic branches were more receptive around 4 pm, and continued until 10 am of the next day. For cacauí flowers (*T. speciosum*), the relationship between presence of exudate and peroxidase activity indicated greater receptivity between 6 am and 10 am of the first day of anthesis (SOUZA and VENTURIERI, 2010). In other *Theobroma* species as in cocoa (*Theobroma cacao*), stigmas were more receptive between 10 am of the day of anthesis and 1 pm of the next day (SAMPAYAN, 1966). In reviews with *T. obovatum*, there is evidence that the period of greatest receptivity occurs at 6 pm of the same day of anthesis (SOUZA and VENTURIERI, 1998). This study revealed that *T. subincanum* has greater receptivity between 2 am of the day of anthesis and 10 am of the same day, and overlap between the stigmatic receptivity times in the genus *Theobroma* can be observed.

The collection of *T. subincanum* pollen can be carried out in any of the evaluation times in this study, since it remained with high viability percentage; however, to analyze the stigmatic receptivity, it is recommended that pollen is deposited on the stigma between 2 pm and 10 am of the same day (Figure 2), as these times are the most receptive, which possibly provides greater success in the process of intraspecific and interspecific artificial pollination, with *T. subincanum* as male parent.

The results of this study can be used to support experiments involving cupuí hybridization attempts (*T. subincanum*) with other species of the genus *Theobroma*. Such attempts should take into account both the period of greatest receptivity of the female part (stigmas), as the viability of pollen grains coming from the male part, quantitatively and qualitatively ensuring the supply of this pollen to breeders.

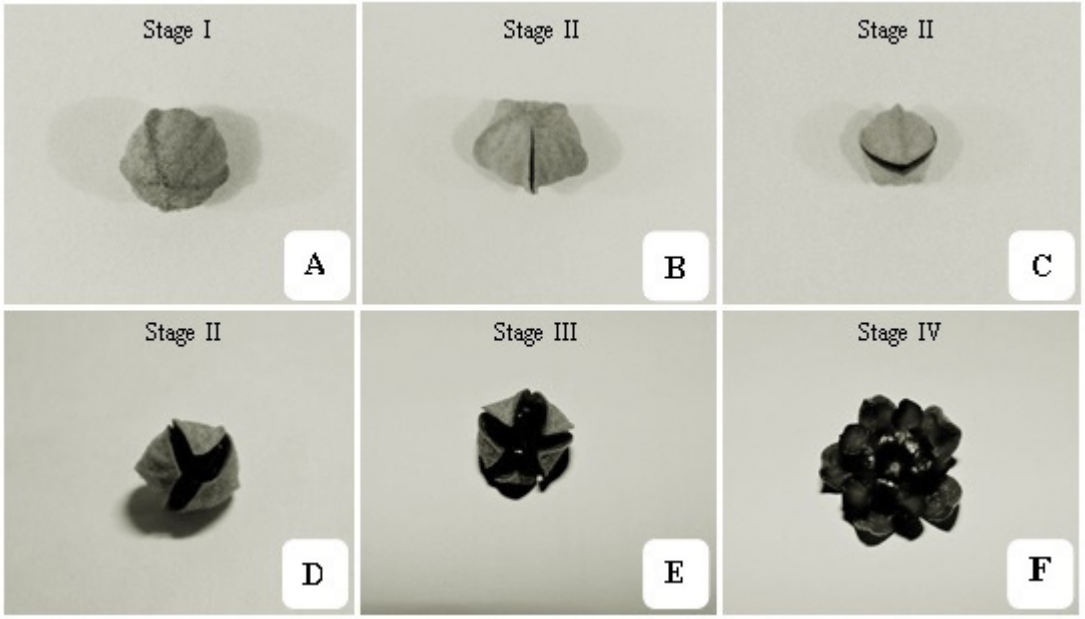

FIGURE 1- Floral development stages featured on this study for *Theobroma subincanum* species. A) Swollen bud; B, C and D) Bud with small cracks between sepals; E) Bud with detached sepals and appearance of hole and F) Open flower with detached sepals, expanded petals and stigma exposure.

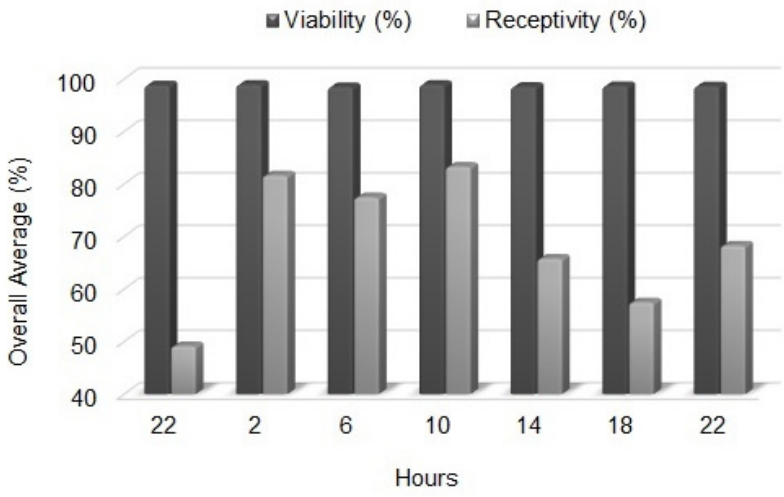

FIGURE 2- Pollen viability and stigmatic receptivity in *Theobroma subincanum* in seven different evaluation times.

TABLE 1- Stigmatic receptivity values in *Theobroma subincanum* genotypes collected in seven different times.

Stigmatic receptivity (%)				
Times	**First Assessment**	**Second Assessment**	**Third Assessment**	**General Average**
Initial 10 am	50.00 b	52.50 ab	45.00 d	49.17 c
2 am	82.50 a	77.50 a	85.00 a	81.67 a
6 am	85.00 a	67.50 ab	80.00 ab	77.50 a
10 am	95.00 a	70.00 ab	85.00 a	83.33 a
2 pm	72.50 ab	65.00 ab	60.00 bcd	65.83 abc
6 pm	72.50 ab	47.50 b	52.50 cd	57.50 bc
Final 10 pm	70.00 ab	62.50 ab	72.50 ab	68.33 ab

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

CONCLUSIONS

Pollen viability is greater than 95% at all times of the day. Flowers continued receptive throughout the observation period, with lower receptivity at 10 pm (flower buds at pre-anthesis) and greater receptivity percentage between 2 am and 10 am, which are the most recommended times to carry out controlled pollinations.

Pollen viability remains high regardless of the time analyzed and flowers. The best times to perform controlled pollination are between 2 am and 10 am due to higher stigmatic receptivity.

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