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Viability and receptivity of reproductive structures in Lima bean (*Phaseolus lunatus* L.) for improvement programs

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ABSTRACT: Artificial hybridization in *Phaseolus lunatus* L. is still little used in genetic improvement programs in Brazil due to the low establishment percentages. This work aimed to evaluate pollen viability and stigma receptivity in different Lima bean genotypes throughout the day. Accessions 'Roxinha -MT' and 'Cana Longa-PB' were used in the experiment. Five flower buds were collected from each genotype at 6:00 a.m., 10:00 a.m., 2:00 p.m., and 6:00 p.m. The stigma receptivity was determined using 3% hydrogen peroxide, whereas pollen viability was determined with 2% acetocarmine colouration. The genotype and time factors acted independently on the percentage of viable and non-viable pollen grains and stigma receptivity. The genotype 'Roxinha' presented 88.54% viable pollen grains, a value 84.27% higher than that of the 'Cana Longa' genotype. This percentage varied as a function of time, with the highest means observed at 6:00 a.m. Stigma receptivity was influenced only by the time of the day, undergoing a great reduction in receptivity, from 88% in the early morning to slightly more than 40% around noon. These results indicate that 6:00 a.m. is the most suitable time to perform artificial crosses in Lima bean plants.

Key words: environment; genetic improvement; genotype; hybridization; pollen viability; stigma receptivity

Viabilidade e receptividade de estruturas reprodutivas de feijão-fava (*Phaseolus lunatus* L.) para programas de melhoramento

RESUMO: A hibridação artificial em *Phaseolus lunatus* L. ainda é pouco utilizada nos programas de melhoramento genético no Brasil devido as baixas porcentagens de pegamento. Objetivou-se avaliar a viabilidade do pólen e a receptividade do estigma em diferentes genótipos de feijão-fava ao longo do dia. Foram utilizados os acessos Roxinha -MT e Cana Longa-PB. Cinco botões florais foram coletados de cada genótipo nos horários de 06:00, 10:00, 14:00 e 18:00 horas. A receptividade estigmática foi determinada com o uso de Peroxido de hidrogênio 3% e a viabilidade do pólen com a coloração de Carmim acético a 2%. Os fatores genótipos e horário atuaram de forma independente no percentual de pólens viáveis, inviáveis e receptividade do estigma. No genótipo Rosinha foram observados 88,54% de pólen viáveis, superior ao Cana Longa com 84,27%. Este percentual variou em função do horário, com as maiores médias observadas as 6:00h. A receptividade do estigma foi influenciada apenas pelo horário do dia, sofrendo uma grande redução na porcentagem de receptividade de 88% no início da manhã, para pouco mais de 40% por volta do meio dia. Estes resultados indicam que 6:00h é o horário ideal para realização de cruzamentos artificiais em feijão-fava.

Palavras-chave: ambiente; melhoramento genético; genótipo; hibridação; viabilidade do pólen; receptividade do estigma

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Introduction

Phaseolus lunatus L., also known as Lima bean, is a legume species with great variability dispersed throughout the country, especially in the northeastern states. Its seeds are used for cooking worldwide, ranking as the second species of its genus with greater socioeconomic importance (Ormeño Orrillo et al., 2015). However, the mean yield observed in Brazil is still far from the indices obtained in other producing regions. In the United States, for example, which is considered to be one of the largest producers, mean yields above 2.500 kg ha^{-1} were easily achieved (USDA, 2018; IBGE, 2020), compared to the 313 kg ha^{-1} produced in the entire Northeast Region of Brazil in 2018 (IBGE, 2020). The low yield may be associated, among other factors, with the absence of genotypes adapted to local conditions, highlighting the importance of scientific research on genetics and improvement for the species.

Artificial crossing can be employed in the improvement of Lima bean to gather in a cultivar the agronomical traits that make this variety superior to its parents and with that obtaining greater profitability to the producer.

Furthermore, pollination is a fundamental step in the production of these plants since it allows the formation of fruits and seeds (Barbosa & Sousa, 2016). The success of pollination will depend on stigma receptivity and pollen viability, which constitute the effective beginning of the pollen-pistil interaction, ensuring the production of seeds of vigorous lineages and/or hybrids in breeding programs (Shivanna, 2003; Kaefer et al., 2016).

Pollen must present aptitude for success in production, with its quality being associated with its viability (Vianna et al., 2006). A stigma, in turn, is considered receptive when it can support pollen germination (Sanzol & Herrero, 2001). The duration of receptivity can vary significantly depending on the species (Dafni, 1992; Shivanna, 2003). According to Dafni (1992), stigma receptivity and pollen viability should be investigated to identify the optimal times and developmental stages of flowers for artificial pollination procedures.

Studies related to pollination, such as the viability and receptivity of reproductive structures, are still scarce for *Phaseolus lunatus* L., which may explain the lack of success in hybridizations in this species since they constitute important parameters in plant genetic improvement programs. A low percentage of fruit establishment originating from crosses has been reported for the genus *Phaseolus*, including *P. lunatus*. Segundo (2010) tested four artificial hybridization methods in *P. lunatus* and obtained less than 3% fruit establishment. In *P. vulgaris*, up to 30% of the establishment has been reported (Vaid, 1990; Carpentieri-Pípolo et al., 2001).

Assessments of pollen viability and stigma receptivity are not common practices in Lima bean breeding programs. To date, no Lima bean cultivars have originated from introduction or developed in breeding programs using the variability obtained via hybridization, protected and registered with the Ministry of Agriculture, Livestock and Food Supply - MAPA (Brasil, 2020). From this perspective, this work aimed to evaluate pollen viability and stigma receptivity at distinct times of the day and determine the best time for crossing in Lima bean genotypes.

Materials and Methods

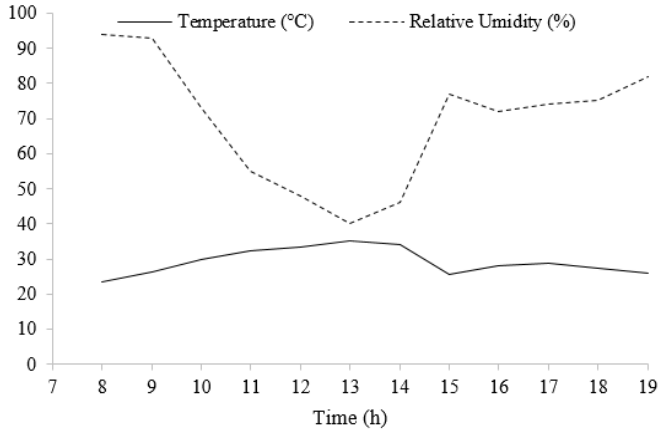
The work was conducted at the Federal University of Piauí (UFPI), Campus Professora Cinobelina Elvas (CPCE), in Bom Jesus, PI, at 09°04'28'' south latitude, 44°21'31'' west longitude, and 277 m elevation.

The municipality belongs to the semiarid region of the state of Piauí, characterized by a hot and humid climate, classified by Köppen as *Cwa* (temperate with dry winter and summer and autumn rains), with a mean rainfall varying from 900 to 1200 mm $year^{-1}$ and a mean temperature of 26.2°C, according to the data by INMET (2021). The soil of the region is classified as a Dystrophic Yellow Latosol with a predominant sandy texture.

Two Lima bean accessions belonging to the Germplasm Bank of the Federal University of Piauí were used: 'Cana Longa' – PB and 'Roxinha' – MT. The accessions were arranged in an open field experiment following a randomized block design, aiming at evaluating the agronomic performance of 10 different Lima bean genotypes under the conditions of Bom Jesus-PI.

At flowering, flower clusters were randomly selected in both Lima bean genotypes, collected at 6:00 a.m., 10:00 a.m., 2:00 p.m., and 6:00 p.m. The temperature and relative humidity data during the day are shown in Figure 1. The collection was performed using pliers to avoid damage. The material was then identified and stored in a polystyrene box containing moist cotton. After the collection process, the clusters were immediately transported to the Laboratory of Genetics of CPCE/UFPI for the analyses.

For the test of stigma receptivity, 5 unripe flower buds/time with average size were chosen (Figure 2A). The size of



Source: INMET (2021).

Figure 1. Temperature (°C) and relative humidity (%) in the city of Bom Jesus-PI during the collection of Lima bean flower buds (*Phaseolus lunatus*) for analysis of viability and receptivity of reproductive structures in February 2019.

the flower bud was determined in previous studies by the same authors, in which they compared pollen viability in different developmental stages. With the aid of tweezers, the structures that surround the stigma were removed, such as wings, banner, and keel, as well as anthers and style, leaving only the stigma attached to the peduncle, according to the sequence presented in Figure 2B.

The already opened buds were taken to a stereomicroscope (10x) on Petri dishes, where with the aid of a liquid dropper, one drop of 3% hydrogen peroxide was used on the stigmatic surface of each flower bud. The stigmatic receptivity (ER) was estimated by observing the amount of bubbles formed on the surface of the stigma at 0, 30, 60, and 100%.

For the pollen viability test, emasculation of 5 flower buds/time was performed in each of the genotypes by removing the anthers and placing them on microscope slides. Each anther was opened for the removal of pollen grains. A drop of 2% acetic carmine was used in each slide, and the slides were immediately taken for microscope counting using a 4x objective lens. A total of 500 pollen grains were counted for each flower bud, estimating the percentage of viable (PV) and non-viable grains (PIV) by the presence (viable) or absence (non-viable) of carmine coloring.

The experiment was analyzed in a completely randomized design in a 2 x 4 factorial scheme (genotypes x evaluation times) with 5 replications (flowers). The data were subjected to analysis of variance, and when detecting significant differences, they were compared by the F-test. Regression models were adjusted for the time variable. Spearman's correlation coefficient between the percentage of viable pollen grains and stigma receptivity was also estimated, and the significance was tested by Student's t-test. All analyses were performed in R software, version 3.5.3 (R Core Team, 2019).

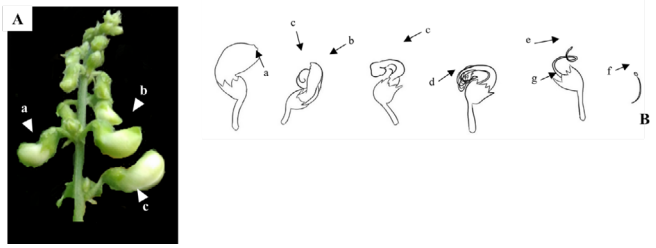


Figure 2. A: Flower buds of *Phaseolus lunatus* L. Arranged in a cluster. a: small (≤ 2.9 mm); b: average (3 to 3.5 mm); c: large (> 3.5 mm). B: flower before anthesis; a: wing, b: banner; C: flower bud; c: keel; d: reproductive structures (stigma and anthers); e: stigma; f: anther; g: peduncle.

Results and Discussion

No significant interaction was observed ($p \leq 0.05$) between the genotypes and times analyzed (Table 1), meaning that the factors act independently in the determination of pollen viability and stigma receptivity. This result is important for breeding programs since a determined time can be used to perform crosses in more than one genotype. There was a significant difference between the levels of the genotypic factors and the time levels ($p \leq 0.05$) for the PV variable and consequently for the PIV. The ER was influenced only by the times.

Table 1. Summary of the analysis of variance and mean main effect of the genotypes for pollen viability and stigma receptivity of Lima bean genotypes (*Phaseolus lunatus* L.), at different times.

Source of variation	PV	PIV	ER
Genotype	181.902*	181.902*	10.0 ns
Time	265.912*	265.912*	4750.0*
G x H	49.565^{ns}	49.505^{ns}	163.3^{ns}
CV %	5.77	36.67	28.28
Genotype	**PV (%)**	**PIV (%)**	**ER (%)**
Roxinha – MT	88.54 a [1]	11.46 b	62.00 a
Cana Longa – PB	84.27 b	15.73 a	63.00 a

PV – Percentage of viable pollen; PIV – Percentage of non-viable pollen; ER – Stigmatic receptivity.
*, ns Significant and non-significant at 5% by F test.
[1]Means followed by the same letter in the same column do not present significant differences at a 5% level of probability by Tukey's test.

Viable and non-viable pollen grains were observed in both genotypes (Figure 3A), with the 2% acetocarmine solution being efficient in determining pollen viability in *P. lunnatus* L. The coloration occurs due to the plenitude of cellular structures, such as the nucleus (Munhoz et al., 2008). Little stained and deformed pollen grains were also observed and classified as non-viable (Figure 3A).

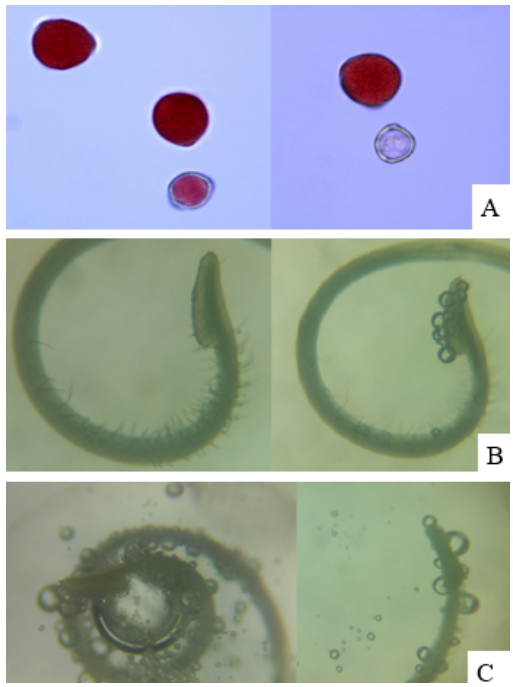


Figure 3. A) Pollen grains of *Phaseolus lunatus* L. stained with acetocarmine (viable) and not stained and/or slightly stained pollen grains with anomalous (non-viable) B) Stigma of *Phaseolus lunatus* L. removed from flower buds without hydrogen peroxide and stigmatic surface with bubbling at 60% receptivity. C) Degeneration of the stigma and style indicated by the activity of peroxidases in a pollinated stigma and non-pollinated stigma presenting bubbling in all its structures.

The genotype 'Roxinha' obtained a higher number of viable pollen grains (8,854 out of 10,000), corresponding to 88.54%, differing statistically from the 'Cana Longa' genotype, with 84.27% (Table 1). In a work performed by Jesus et al. (2018) testing pollen viability in nine accessions of *Phaseolus lunatus* L., values ranging from 77% to 95% were verified for this variable, concluding that genotypes of the same species may present variability regarding pollen viability. In this manner, in addition to agronomical traits, pollen viability studies should be performed to aid in the selection of parents in breeding programs. The percentage of inviable pollen grains is complementary to the percentage of viable pollen grains, presenting mean percentages of 11.46% and 15.73% for genotypes 'Roxinha' and 'Cana Longa', respectively.

Pollen viability also varied as a function of time (Figure 4). The quadratic behavior reveals that the highest means of pollen viability are observed in the early hours of the day, with a gradual reduction, reaching the lowest percentage at 1:14 p.m., which corresponds to approximately 81.4% of viable pollen grains (Figure 4). Kaefer et al. (2016) also reported a reduction in the viability of maize pollen grains throughout the day. Pollen grain viability is influenced not only by intrinsic factors, such as the physiological maturation stage, origin, and genetic and nutritional traits but also by extrinsic factors, such as temperature and humidity (Stanley & Linskens, 1974; Almeida et al., 2002). Temperature and humidity follow a normal variation throughout the day (Figure 1). It is believed that the time effect on pollen viability is directly related to the variation in temperature and humidity, such as that observed for the maize crop, in which a reduction in pollen viability was verified in the hottest hours of the day, with lower humidity, between 11:00 a.m. and 1:00 p.m. (Kaefer et al., 2016). Higher temperatures and lower humidity values were expected late in the morning and in the early afternoon (Figure 1), and the lowest viability percentages were observed at this time, corroborating the data of this experiment.

Although pollen viability was reduced at the above-mentioned time, the values obtained varied from 93.46% earlier in the day (6:00 a.m.) to 86.87% at the end of the day (6:00 p.m.). Pollen viability values above 70% are considered high according to the criterion by Souza (2002). Therefore, the percentages observed in this work are satisfactory for the breeding program at any of the times.

In addition to pollen viability, flower receptivity plays a crucial role in the dynamics of pollination, reproductive success, and consequently fruit production since the stigma is the first surface of the pistil to come into contact with the pollen grain (Carpenedo et al., 2020). In this region, the formation of bubbles resulting from contact with hydrogen peroxide is observed in response to several enzymes that characterize the receptive stigma (Figure 3B), such as dehydrogenases and esterases (Shivanna & Rangaswamy, 1992; Zulkarnain et al., 2019). A variation in bubble production was observed at the different times of the day, evidencing the time influence on the determination of stigma receptivity in Lima beans.

A quadratic behavior was also observed to explain stigma receptivity as a function of time (Figure 4). It was verified that the best receptivity occurs at 6:00 a.m., reaching approximately 88%. Throughout the day, the receptivity was drastically reduced, with the minimum value observed at 12:41 p.m., corresponding to only 40.12% of receptivity, a reduction of more than 50%. At the end of the day (6:00 p.m.), an increase of approximately 72% is observed in the receptivity.

It is worth noting that the high presence of bubbles at times from 10:00 a.m. to 2:00 p.m. (Figure 3C) does not occur due to the viability of the stigma but rather to some degeneration process, characterized by the presence of bubbles in the whole pistil. According to Preczehnak et al., (2011), stigma tissue begins a degeneration stage after fecundation, forming small necroses and reacting to hydrogen peroxide in its entire structure, causing the production of large amounts of bubbling. Bubble formation was also observed in the entire structure of Lima bean stigmas after pollination and fecundation (Figure 3C). It is believed that high bubbling is also associated with the damage caused by the high temperatures and low humidity at these times (Figure 1); thus, bubbling was only counted on the stigmatic surface. Stigma receptivity was much more influenced by the times when compared to pollen viability. This behavior was also observed in *Theobroma subincanum* (Arenas de Souza, 2016). Pollen viability was greater than 95% throughout the day, although stigma receptivity was higher between 2:00 a.m. and 10:00 a.m., with a reduction from 85% to less than 69% at the end of the day (10:00 p.m.). In this manner, for the Lima beans, as well as for *T. subincanum*, stigma receptivity is the parameter that must guide the most suitable times to perform pollinations in breeding programs since this pollen-stigma interaction is the initial condition to initiate the remaining processes that involve pollination and fecundation.

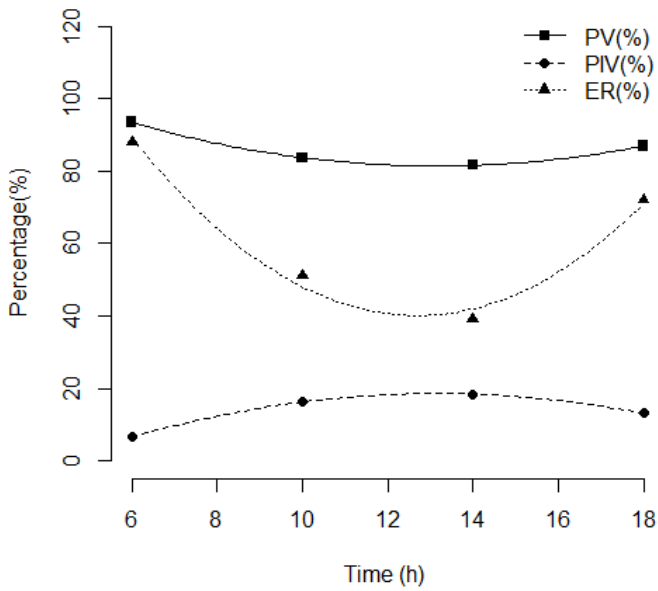


Figure 4. Percentage of viable pollen grains (PV), non-viable pollen grains (PIV), and stigma receptivity (ER) in Lima bean flowers (*Phaseolus lunatus*) at different times of the day.

The early hours of the day are, therefore, the most suitable for Lima bean pollination, at 6:00 a.m. time being recommended since it provides a high percentage of viable pollen grains, and the stigma is also receptive. The positive (0.52) and significant correlation ($p<0.001$) reaffirms that the stigma is receptive at the same time that the pollen is viable. In genetic improvement, it is important to consider this effect since in addition to facilitating autogamy, the coinciding pollen maturation and receptivity of the stigma surface increased the self-pollination ability in *Phaseolus lunatus* L. (Bi et al., 2005), it is crucial to define hybridization strategies.

Conclusion

Pollen viability is influenced by Lima bean genotypes.

The time-of-day influences pollen viability and stigma receptivity in Lima beans.

The 6:00 a.m. time must be used to perform crosses and self-pollination in Lima beans.

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Compliance with Ethical Standards

Author contributions: Conceptualization: PAB; Data curation: PAB, AMM, MPLS; Formal analysis: PAB, AMM; Investigation: MPLB, KLSB, SCS; Methodology: PAB, SCS, AMM; Project administration: PAB, SCS; Resources: PAB, SCS, MPLS; Supervision: PAB, SCS; Validation: PAB, SCS; Visualization: PAB, MPLS, KLSB, AMM; Writing – original draft: PAB, MPLP; Writing – review & editing: AMM, SCS.

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