

Stigmatic receptivity and hybridization in cowpea beans (*Vigna unguiculata* L. (Walp.))

Receptividad estigmática e hibridación artificial en frijol caupí (*Vigna unguiculata* L. (Walp.))



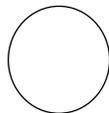
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Hybridization in cowpea beans.

Photo: H. Araméndiz-Tatis

ABSTRACT

Classic plant breeding, based on the selection of superior individuals and directed crosses, led to the need-to-know aspects of the floral biology of cowpea beans. The research was carried out at the Universidad de Córdoba, Colombia, through two experiments: in the first, stigmatic receptivity was evaluated as response time to hydrogen peroxide, under a randomized complete block design, with a 3×4 factorial arrangement (three genotypes: Caupicor 50, Missouri and BRS Milenium, and at four hours of the day: 7:00 and 9:00 AM; 3:00 and 5:00 PM) and three replications. In the second, the percentage of viable crosses was evaluated, under a randomized complete block design, with a 2×2 factorial arrangement (two crosses: Missouri × IT86 and Missouri × BRS Milenium, and two methods: 1 (morning) and 2 (afternoon), and four replications. The greatest stigmatic receptivity was recorded in the BRS Milenium and Missouri genotypes with a time of 3.28±0.07 and 2.01±0.12 min at 7:00 and 9:00 AM, while Caupicor 50, time of 1.80±0.09 min at 3:00 PM. The artificial hybridizations carried out in the morning (method 1) registered the highest viable crosses, 78.6% in Missouri × BRS Milenium and 57.1% in Missouri × IT86. Therefore, artificial hybridizations should be done in the morning due to a more favorable environment for pollen grain germination, given the greater stigmatic receptivity.



Additional key words: flower stigma; peroxidase; emasculation; anthesis; pollination.

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RESUMEN

El mejoramiento genético clásico, basado en la selección de individuos superiores y cruzamientos dirigidos, demanda conocer aspectos de la biología floral y reproductiva del frijol caupí. La investigación se llevó a cabo en la Universidad de Córdoba, mediante dos experimentos: en el primero, se evaluó la receptividad estigmática como tiempo de respuesta al peróxido de hidrógeno, bajo el diseño bloques completos aleatorizados con arreglo factorial 3×4 (tres genotipos: Caupicor 50, Missouri y BRS Milenium, y cuatro horas del día: 7:00 y 9:00 AM, 3:00 y 5:00 PM) y tres repeticiones; en el segundo, se evaluó el porcentaje de cruzamientos viables, bajo un diseño bloques completos al azar con arreglo factorial 2×2 (dos cruzamientos: Missouri \times IT86 y Missouri \times BRS Milenium, dos métodos: 1 (mañana) y 2 (tarde), y cuatro repeticiones. La mayor receptividad estigmática se registró en los genotipos BRS Milenium y Missouri, con tiempo de $3,28 \pm 0,07$ y $2,01 \pm 0,12$ min a las 7:00 y 9:00 AM, mientras que Caupicor 50, tiempo de $1,80 \pm 0,09$ min a las 3:00 AM. Las hibridaciones artificiales realizadas por la mañana registraron los mayores cruzamientos viables, 78,6% en Missouri \times BRS Milenium y 57,1% en Missouri \times IT86. Por tanto, las hibridaciones artificiales deben hacerse en horas de la mañana debido a un ambiente más propicio para la germinación del grano de polen, dada la mayor receptividad estigmática.

Palabras clave adicionales: estigma floral; peroxidasa; emasculación; antesis; polinización.

Received: 09-12-2021 Accepted: 16-05-2022 Published: 24-05-2022

INTRODUCTION

Cowpea bean is a self-pollinating species native to Africa and cultivated in tropical and subtropical areas (Nameirakpam and Khanna, 2018). Cowpea bean production faces a series of biotic and abiotic constraints that affect grain and forage yield. It has been established that heat stress during the reproductive stages leads to a considerable loss of yield and seed quality (Bheemanahalli *et al.*, 2019), because heat during preanthesis and reproductive development results in a reduction in flower fertility, due to reduced pollen quantity and viability, flower abortion, slow pollen tube growth, which results in fewer seeds per pod (Bheemanahalli *et al.*, 2019; Kumar *et al.*, 2017). In addition, impaired vegetative and reproductive growth, nutrient absorption, protoplasmic movement, photosynthesis, respiration, metabolism, flower growth, fertilization, fruit maturation and seed quality (Sorkheh *et al.*, 2018).

The cowpea bean is a self-pollinating species native to Africa and cultivated in tropical and subtropical areas (Nameirakpam and Khanna, 2018); the stigma of its flower is receptive from about 12 h before anther dehiscence, a characteristic that facilitates direct crosses. Pollen adhesion and hydration are the first events of pollen stigma interactions, which allow compatible pollen to fertilize the egg cell (Yu *et al.*,

2019). For fertilization to occur, not only the pollen grains must be viable, but also the stigma must be in a receptive condition for the pollen tube to develop. Therefore, stigmatic receptivity analyses are considered essential (Silva *et al.*, 2013). A large number of studies have shown that plant stigma receptivity is closely related to flower development and morphological changes, these changes are dependent on the intensity (temperature degrees), duration and rate of the temperature increase (Giorno *et al.*, 2013; Li *et al.*, 2014) as in *Euphorbia pulcherrima* Willd. ex Klotzch (Vargas-Araújo *et al.*, 2017). Methods for determining stigmatic receptivity focus on indicating the activity of enzymes such as esterases, peroxidases, and acid phosphatases on the surface of the stigma (Dresselhaus and Franklin-Tong, 2013; Chen *et al.*, 2013), that play an important role in the adhesion, hydration, and germination of the pollen grain, pollen tube growth and fertilization success (Gupta *et al.*, 2015).

Considering that stigmatic receptivity and pod formation in cowpea beans could be affected by high temperatures in the Middle Valley region, the objective of this research was to determine the duration and times of day of greatest stigmatic receptivity to be used in breeding genetic of this species by artificial hybridization.

MATERIALS AND METHODS

Location. The research was carried out in the first semester of 2020 at the Universidad de Córdoba, in Montería (Colombia), whose geographical coordinates are 8°45'N and 75°53' W, with 15 m a.s.l. Soil conditions were loamy-clay-silty, organic matter = 1.64%, P = 17.5 mg kg⁻¹; Ca²⁺ = 13.5 mg kg⁻¹ soil; Mg²⁺ = 8.0 mg kg⁻¹ soil; K⁺ = 0.04 cmol kg⁻¹ soil and pH = 6.8 and climate conditions corresponding to tropical dry forest, with an average temperature of 27.4°C, precipitation per year of 1,346 mm, relative humidity of 85% and solar brightness of 2,108 annual solar hours (Palencia *et al.*, 2006).

Plant material. The genotypes Caupicor 50, Missouri and BRS Milenium and IT86 were used for the experiment. Caupicor 50 is a commercial variety, while Missouri, BRS Milenium and IT86 are genotypes from the cowpea collection at the Universidad de Córdoba.

Experimental design and procedures. In the first experiment, to evaluate the receptivity of the stigma a randomized complete block design was used with a 3×4 factorial arrangement (three genotypes: Caupicor 50, Missouri and BRS Milenium, and 4 h of the day: 7:00 and 9:00 AM; 3:00 and 5:00 PM, and three replications. Each cultivar was planted in two rows 10 m long, with distances between rows and plants of 0.70 and 0.50 cm, respectively, for a total of 40 plants per experimental unit. Three seeds were sown per site, the seedlings emerged 4 d later, and then two were thinned to let one per site. The agronomic management was carried out in a sustainable way in order to be friendly to the environment according to Araméndiz-Tatis *et al.* (2019).

The technique described by Crispim *et al.* (2017) was used; from each genotype, three flower buds were collected in pre-anthesis at 7:00 and 9:00 AM; 3:00 and 5:00 PM. The buds were transported to the laboratory, and, after removing petals and anthers, two drops of hydrogen peroxide (3% v/v) were placed on the stigmatic papillae to observe the presence of peroxidase through the formation of bubbles as indicated by Galen and Plowright (1987) and Osborn *et al.* (1988); the reaction time of the stigma to hydrogen peroxide (TRSHP) or duration of effervescence was measured using a Weston brand stopwatch (model JS-510).

In the second experiment, to evaluate artificial hybridization, a randomized complete block design, with a 2×2 factorial arrangement (two crosses: Missouri × IT86 and Missouri × BRS Milenium, and two times of the day: morning and afternoon, and four replications.

The Missouri genotype was used as the mother and the IT86 and BRS Milenium genotypes as parents. The Missouri × IT86 and Missouri × BRS Milenium crossings were performed. Two methods of cross-breeding were used: Method 1, proposed by Zary and Miller Junior (1982), by which pollen was used from flowers in anthesis collected between 6.5 and 7.0 h. Flower buds in pre-anthesis were emasculated and pollinated at 7.0 and 9.0 h. Method 2, proposed by Zary and Miller Junior (1982), by which pollen was collected from open flowers in the morning, between 6.45 and 7.00 h, and stored in a refrigerator at 25°C, in paper bags, until its use in the afternoon when the flower buds were emasculated and pollinated at 15.0 and 17.0 h. Seven crosses were made per experimental unit due to the limited time.

To expose the anther sac, the petals were removed with forceps and pollen was deposited on the stigma of the emasculated flower, covered and closed with the wing and petals to prevent desiccation. The response variable was the percentage of viable or successful crosses, evidenced in the formation of pods with the presence of seeds 3 d after pollination. Each cross was identified with labels on the pedicel of the flower, on which the name of the parents, the date and time of pollination were recorded. Temperature and relative humidity data were taken with a Brixco brand thermohygrometer (Model 5015).

Analysis of data. For the stigmatic receptivity experiment, analysis of variance was performed according to the additive linear model of the randomized complete block design with factorial arrangement. Means were adjusted for least squares by Tukey's method at 5%, given the statistical significance of the Genotype-Time interaction, with the procedure for mixed models, considering the random blocks of the SAS software, version 9.0. A similar statistical analysis was performed for the hybridization experiment and Tukey's test was applied at 5%. The statistical model of the experimental design used in both experiments is:

$$Y_{ijk} = \mu + \rho_i + \alpha_j + \beta_k + (\alpha\beta)_{jk} + \varepsilon_{ijk} \quad (1)$$

Where Y_{ijk} is the observed response at level j of A-factor, level k of B-factor in the i -th block; μ is the mean response; ρ_i is the effect of the i -th block; α_j is the additive effect of the j -th level of A-factor; β_k is the additive effect of the k -th level of B-factor; $(\alpha\beta)_{jk}$ is the additive effect of the combination of the j -th level of A with the k -th level of B, it constitutes the effect of the interaction AB; and ε_{ijk} is the residual or random error, $\varepsilon_{ijk} \sim NI(0, \sigma^2)$.

Previously, compliance with the assumptions of the analysis of variance was verified: normality, with the Shapiro-Wilk test; homogeneity of variances and additivity, with the residual Scatter Plot versus predicted values with the model, and independence, with compliance to the basic principle of randomization.

RESULTS AND DISCUSSION

Stigmatic receptivity

The analysis of variance for the reaction time of the stigma to hydrogen peroxide (TRSHP) showed highly significant differences for each factor and the genotype-time interaction (Tab. 1). The results indicate that the flower buds showed stigmatic receptivity in the 4 h of evaluation, which agrees with that reported

by Poonia *et al.* (2018) and Boukar *et al.* (2019) in studies carried out using cowpea beans. In addition, the Genotype-Time interaction was statistically significant, suggesting that stigmatic receptivity varies with genotype and time of day. This is possible due to genetic differences, since they originate from different countries, and the environmental effect due to temperature, which in tropical conditions, above 35°C, becomes a limiting factor for production (Bacallao and Gill, 2015).

The simple effects of the time factor for each genotype indicate that for the Caupicor 50 genotype, the pollen collection time did not influence its stigmatic receptivity, measured as effervescence time when applying hydrogen peroxide to the stigma. In the Missouri genotype, the greatest stigmatic receptivity was observed at 9:00 AM, while in the BRS Milenium genotype at 7:00 AM; for this genotype the lowest receptivity was observed at 3:00 PM (Tab. 1).

The simple effects of the genotype factor for each time of evaluation of pollen viability indicate that, at seven hours, the greatest receptivity of stigma was estimated in the BRS Milenium genotype; at 9:00 AM, in the BRS Milenium and Missouri genotypes; at 3:00 PM in Caupicor 50 and, at 5:00 PM in BRS Milenium without differing statistically from Caupicor 50 (Tab. 1). In general, a greater stigmatic receptivity was evidenced in the morning hours in the BRS Milenium and Missouri genotypes, while Caupicor

Table 1. Analysis of variance and comparisons of the reaction time of the stigma to hydrogen peroxide (TRSHP) in cultivars of cowpea beans evaluated at different times of the day.

Source	DF	Mean square	F-value	Pr > F
Block	2	0.82903889	2.85	0.0794
Genotype	2	1.53783611	10.57	0.0006
Time	3	0.84296574	5.79	0.0045
Genotype × Time	6	1.36412130	9.38	<0.0001
Error	22	0.14549823		
R^2	0.820365			
CV (%)	22.30294			
Genotype	Time (h)			
	7:00 AM	9:00 AM	3:00 PM	5:00 PM
'Caupicor 50'	1.22 ± 0.08 aB	1.37 ± 0.08 aB	1.80 ± 0.09 aA	1.79 ± 0.04 aAB
'Missouri'	1.39 ± 0.10 bB	2.01 ± 0.12 aAB	1.15 ± 0.03 bB	1.30 ± 0.06 bB
'BRS Milenium'	3.28 ± 0.07 aA	2.33 ± 0.09 bA	0.91 ± 0.03 cB	1.96 ± 0.10 bA

Means with the same letters estimated by least squares and adjusted by Tukey's test, do not differ significantly. Lowercase letters correspond to horizontal comparisons and uppercase letters to vertical comparisons.

50 presented greater stigmatic receptivity at 3:00 PM, when the flower buds have been exposed to the highest temperatures and low relative humidity, which could suggest a greater adaptation to extreme conditions. These results have reproductive implications, since the success of the pollen grain depends on how long the receptive stigma remains. Likewise, the growth and development of the plant depends on the accumulated heat units which supply energy needs conducive to the formation of quality seeds (Maity *et al.*, 2019).

In relation to the time of effervescence, Thimmaiah *et al.* (2018), stated that the stigmas are considered receptive when the effervescence time is greater than two minutes due to the release of oxygen, which reflects the potential for receptivity and seed formation (Gupta *et al.*, 2015). In this research, the BRS Milenium and Missouri genotypes presented effervescence values equal to or greater than 2 min in the morning hours, while Caupicor 50 was close in the afternoon.

The correlations of temperature, relative humidity and reaction time of the stigma to hydrogen peroxide (TRSHP) are shown in table 2. There was a positive and significant correlation at 10% type I error probability ($P < 0.10$) between TRSHP and RH, while temperature and relative humidity were highly negatively correlated ($P < 0.01$). The temperature and relative humidity conditions fluctuated between 27.8 and 36.1°C and 47.0 and 77.0%, respectively. The TRSHP was lower at higher temperatures, possibly heat stress affects the germination and growth of pollen tubes. Sage *et al.* (2015) argued that temperatures

above 30°C affect both pollen viability and stigmatic receptivity; as well as the growth and development of the pollen tube, resulting in failures in the formation of pods (Priya *et al.*, 2019); the opposite occurs when the relative humidity increases, indicated by a greater activity of peroxidase, in the face of better conditions for pollen grain germination. It has been reported that the main function of the synergids is the attraction and reception of the pollen tube towards the entrance of the micropyle for the fertilization of the ovule (Sage *et al.*, 2015; Skinner and Sundaresan, 2018).

Artificial hybridization

The analysis of variance for the percentage of viable crosses (PVC) showed that there is no significant interaction between the crosses and the hybridization methods, so the effects of both factors are independent. Only the Method factor was highly significant ($P < 0.01$). Method 1 was superior with values of 42.9 and 78.6% at 7:00 and 9:00 AM, respectively, which suggests that in the morning greater success in hybridization is achieved, which agrees with Nameirakpam and Khanna (2018) in cowpea bean, who carried out pollination in the morning hours immediately after emasculation, obtaining success rates between 54.55 and 70.59%.

Method 2 was less efficient with values of 7.1 and 0% at 3:00 and 5:00 PM, respectively, a result similar to that reported by Nunes *et al.* (2010), with the same method but with a success rate of 10.52%. This

Table 2. Descriptive statistics and Spearman's correlation coefficients for TRSHP, temperature (°C) and relative humidity (%) during the evaluation of the stigmatic receptivity of cowpea beans.

Variable	Mean	Minimum	Maximum
TRSHP	1.71±0.71	0.78	3.62
Temperature	31.23±3.17	27.80	36.10
Relative humidity (RH)	65.00±11.27	47.00	77.00
Spearman's correlation coefficients, $n=36$			
	TRSHP	Temperature	Relative humidity (RH)
TRSHP	1	-0.2537 $P=0.1355$	0.2991 $P=0.0764$
Temperature		1	-0.8000 $P<0.0001$
Relative humidity (RH)			1

The upper bold value in each box is the correlation coefficients and the lower value is the P -value.

response is possibly related to the plant's ability to produce viable pollen, the duration of pollen viability and temperature, which at 3:00 PM was 36°C, an approach consistent with Ting *et al.* (2014), Kaushal *et al.* (2016) and Jiang *et al.* (2019).

For Ting *et al.* (2014), the success of hybridization depends on the ability of the donor plant to produce viable pollen and the duration of pollen viability. Jiang *et al.* (2019), stated that with viable pollen grains, the post-pollination and fertilization steps may fail when high temperatures are recorded, causing deterioration of the pollen grain in microgametogenesis, in tissues such as tapetum, epidermis, endothecium and stomium (Giorno *et al.*, 2013). Addition, the heat stress impairs plant growth and development with marked alterations in phenology, morphology, physiology, biochemistry and gene expression that eventually inhibit the production potential of affected crops, by a possible reduction of γ -aminobutyric acid in leaves and anthers (Priya *et al.*, 2019).

The heat sensitive stages are the receptivity of the stigma to pollen, the retention of pollen grains on the surfaces of the stigma, pollen hydration and

germination of the pollen tube (Kaushal *et al.*, 2016; Sita *et al.*, 2017). In the present investigation, during the crosses made at 3:00 PM, temperatures around 37.5°C were recorded and it is possible that this had a negative influence on the germination of the pollen grain and the growth of the pollen tube (Tab. 3). Likewise, authors such as Giorno *et al.* (2013), reported that even with successful fertilization from pollen and viable eggs, an embryo can still abort under conditions of heat stress. Boukar *et al.* (2015), showed that, although emasculation and pollination can be carried out throughout the day, cowpea hybridization is less effective when high temperatures prevail. Therefore, it has been suggested that pollination should take place in the morning between 7:00 and 9:00 AM.

The Missouri \times IT86 cross, with time 1, registered a higher percentage of viable crosses than with method 2 (Tab. 3), the same happened with the Missouri \times BRS Milenium cross. Results similar to those obtained with time 1 between Missouri \times BRS Milenium were reported by Rangkhom and Khanna (2018), in cowpea hybridizations, with success between 60.0 and 76.2% and Tondonba *et al.* (2018), in hybridizations

Table 3. Analysis of variance and comparisons of the percentage of viable crosses (PVC) in cowpea beans carried out in the field, using two hybridization methods. Montería, 2020.

Source	DF	Mean square	F-value	Pr > F	
Block	3	594.52417	2.06	0.1761	
Genotype	1	203.06250	0.70	0.4234	
Time	1	11481.12250	39.76	0.0001	
Genotype \times time	1	203.06250	0.70	0.4234	
Error	15	288.74194			
R^2	0.840273				
CV (%)	55.96512				
Crossing	Method	PVC (%)	Temp (°C)	RH (%)	
Missouri \times IT86	1	7:00 AM	42.9 a	29.9	78.5
		9:00 AM	57.1 a	39.4	45.5
	2	15:00 PM	0.0 b	37.5	48.0
		17:00 PM	7.1 b	27.5	78.0
Missouri \times BRS Milenium	1	7:00 AM	50.0 a	29.9	78.5
		9:00 AM	78.6 a	39.4	45.5
	2	15:00 PM	7.1 b	37.5	48.0
		17:00 PM	0.0 b	27.5	78.0

DF: degree freedom; R^2 : determination coefficient; CV (%): coefficient of variation. For the method factor, percentages with the same letter do not differ significantly, according to the Tukey test at 5%. Temp: temperature; RH: relative humidity, Method 1: emasculation and pollination in the morning; Method 2: emasculation and pollination in the afternoon (pollen collected in the morning).

between *Vigna mungo* genotypes, achieving successes of 58.33 and 70.58%, performing pollination immediately after emasculation.

The results above show that hybridizations in Method 1: emasculation and pollination in the morning, produced superior effectiveness in crosses, with respect to the results obtained with Method 2: emasculation and pollination in the afternoon, with pollen from flowers collected in the morning. In this sense, Thuzar *et al.* (2010) and Khattak *et al.* (2009), in works on *Vigna radiata* reported that high temperature can cause drying of the stigma and ovary or disturb the viability of the anthers, so hybridization can fail causing the shedding of flowers without initiating the pod. It is important to note that the changes that proceed and accompany pollen germination are rapid and dramatic (Gill, 2014) and may be associated with microsporogenesis or microgametogenesis (Giorno *et al.*, 2013).

Carrying out artificial hybridization processes requires prior knowledge of relevant aspects in the floral biology of the species, such as: anthesis start time, stigmatic receptivity, and pollen viability, as well as taking into account the prevailing environmental conditions such as temperature and relative humidity. Parrotta *et al.* (2016) and Snider *et al.* (2009) pointed out that heat stress leads to reduced carbohydrate content in pollen grains along with low energy reserves in the pistil, which in turn reduces the energy available to the growing pollen tube, producing negative effects on fertilization. Moderate temperature and increased humidity appear to increase the percentage of established pods after hand-emasculated crosses. In general, the rate of pod formation varies greatly with environmental conditions, genotype, and handling techniques (Boukar *et al.*, 2015). In this sense, plants treated with γ -aminobutyric acid can partially protect plants from high-temperature stress, significantly improved in terms of pollen germination, pollen viability, stigma receptivity and ovule viability, according to studies carried out on *Vigna radiata* L (Priya *et al.*, 2019).

The development process of pollen and gynoecium, and the synchrony of the stamen and fertilization are very sensitive to high temperatures (Thuzar *et al.*, 2010). Khattak *et al.* (2009) while working on *Vigna radiata* reported that high temperature can cause drying of the stigma and ovary or disturb the viability of the anthers, so hybridization can fail causing the shedding of flowers without initiating the pod.

CONCLUSIONS

The highest stigmatic receptivity was recorded in the BRS Milenium and Missouri genotypes, in the morning hours, but in the afternoon, it was Caupicor 50.

Controlled pollinations carried out between 7:00 and 9:00 AM registered a higher percentage of viable crosses, so their application is recommended to achieve a high percentage of success.

Conflict of interest: The manuscript was prepared and reviewed with the participation of the authors, who declare that there is no conflict of interest that puts the validity of the results presented at risk.

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