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Morpho-phenological Variability of Flowers Traits and Hybridization of Five Inbred Lines of Cowpea [*Vigna unguiculata* (L.) Walp.] in Côte d'Ivoire

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Knowledge of the morphology and floral phenology and the mastery of a manual pollination technique in a plant species are essential for its genetic improvement.
The objective of this work will be to determine the morpho-phenological characteristics of the flowers of five self-fertilization lines of cowpea and to identify an indicated manual pollination technique.
The study was carried out in the Botanical Garden of University Peleforo GON COULIBALY, Côte d'Ivoire where 10 morpho-phenological characters of the flower were evaluated on five lines of cowpea self-fertilization. Thus, three manual pollination techniques (A, B and C) were tested by evaluating traits such as knotting rates, filling rates and maternal and paternal effects on fruit yields.
The results revealed four stages of flower development in cowpea. These are successively stage of "floral button initiation", "dark green floral button", "pale green or pale-yellow floral button" and "blooming flower". Of the three manual pollination techniques tested, technique C resulted in higher rates of knotting (45.38%) and pod filling (58.03%). Results also showed significant maternal and xenia effects on fruit yields in the cowpea.

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On the basis of the results generated on the floral biology of the cowpea, it appears that a hybridization program can now be conducted at the UPGC Botanical Garden for the creation of high-performance varieties adapted to climate change for the benefit of producers in Côte d'Ivoire.

Keywords: Cowpea; floral morpho-phenology; manual pollination; Côte d'Ivoire.

1. INTRODUCTION

Cowpea [*Vigna unguiculata* (L.) Walp.] is a seed leguminous plant belonging to the Fabaceae family, grown mainly in warm tropical regions. Cowpea has approximately 150 to 190 species. The cultivated cowpea is based on five groups or Cultigroups, namely: unguiculata, sesquipedalis, textiles, melanophthalmus, and biflora [1].

It is consumed by nearly 200 million people in tropical Africa [2] and is one of the basic food crops in the western and central areas of Africa [3]. Annual global production is approximately 5.59 million tons for cultivated areas of more than 12.61 million hectares [4]. West Africa alone produces about 83% of the world's output [4]. Its culture plays a role in reducing poverty and improving food security due to its high protein content and its socio-economic importance [5,6]. Increasing cowpea production is a priority due to the high cost of animal protein. Increasing cowpea production is a priority due to the high cost of animal protein.

In Côte d'Ivoire, although much consumed, cowpea remain a marginal culture. Production is around 36,310 tons/year, representing less than 2% of African production [4] In order to promote this culture, research must make suitable technologies available to potential producers. Knowledge of the diversity of cowpea cultivars in Côte d'Ivoire and their floral biology with a view to improving productivity would therefore be a field of study to be explored. Knowledge of floral phenology contributes to the promotion of floral synchronism between different cultivars to achieve manual hybridizations [7] However, in cowpea, it is rare for a single cultivar to have all the desired agronomic characteristics. It is therefore essential to use a reliable manual pollination technique to promote recombination of genes of interest between parental genotypes.

In addition, the floral phenology of local cowpea cultivars in Côte d'Ivoire and their typology according to the characteristics related to floral biology are not known. The objective of this work, which aim to improve the productivity of the cowpea, is to determine the morpho-phenological characteristics of the flowers of five self-fertilizing cowpea lines and to identify an appropriate manual pollination technique.

2. MATERIALS AND METHODS

2.1 Study Area

The study was conducted in the vegetable garden of the botanical garden of the University Peleforo GON COULIBALY (UPGC) in the commune of Korhogo. Korhogo is situated between latitude 9°27′41″ North and longitude 5°38 ″ 19″ West at an altitude of 360 m. The climate of the Korhogo department belongs to the dry tropical climate regime, of Sudano-Sahelian type, characterized by two major seasons: the high dry season and the high rainy season with an average annual rainfall of approximately 1200 mm [8].

2.2 Plant Material

The study focused on five lines of self-fertilization of the cowpea resulting from five cycles of natural self-pollination within the collection of Peleforo GON COULIBALY University. The self-fertilization lines derive from NBO04, NKO08, NTI015, NKO03 and NFE011 encoded accessions.

2.3 Methods

2.3.1 Experimental design and collection of floral biology data

Seeds from the five cowpea self-fertilization were planted using a Fisher block experimental system. The seeding device consisted of five lines representing 10 individuals from each accession. Within the block, seeds were planted with a gap of one meter between the lines and one meter between the pots.

Characterization of the floral biology of the self-fertilization lines was performed on five randomly selected plants per line. A total of 10 quantitative traits related to floral biology were evaluated (Table 1). The data were taken according to the recommendations in the descriptor of the cow [9].

Table 1. List of traits related to flower morpho-phenology and methods of measurement

No.	Characters	Codes	Measurement Method
1	Peduncle length	PL	Average measurement made on three peduncles per plant at the beginning of maturation. Five plants were observed per block for a total of 15 plants evaluated per line.
2	Flower Length	FL	Mean measurement on three flowers per plant. Five plants were observed by block, so a total of 15 plants was measured by accession.
3	Flower Width	FW	Mean measurement on three flowers per plant. Five plants were observed per block, for a total of 15 plants evaluated per line
4	Stamen length	SL	Average measurement taken on three stamens per flower. Three flowers from each of the five plants were observed, for a total of 15 plants evaluated per line
5	Number of Flowers per Peduncle	NFP	Count the number of flowers on three peduncles per plant. Five plants were observed per block for a total of 15 plants evaluated per line
6	Flower button initiation time	FBI	Duration in days after sowing at the end of which the first flower buds appear on the plants. Five plants were observed per block, for a total of 15 plants evaluated per line
7	Duration between Burgeoning and Flower bloom	DBF	Time between the initiation of the flower bud to flower blooming. Five plants were observed per block, for a total of 15 plants evaluated per line
8	Time of appearance of the blossoming flower	TAF	Duration in days after sowing at which the plants had blooming flowers. Five plants were observed per block, for a total of 15 plants evaluated per line.
9	Duration between flower bloom and pod maturity	DFM	Time between blooming of the flower and maturity of the pod. Five plants were observed per block, for a total of 15 plants evaluated per line.
10	Maturation time	MT	Duration in days after sowing to which the plants bear mature pods. Five plants were observed per block, for a total of 15 plants evaluated per line

2.3.2 Experimental device and manual pollination data collection

The manual pollination device was installed on a plot of the garden square of the botanical garden of the University Peleforo GON COULIBALY. Pots filled with fertile soil were deposited on a plot consisting of five blocks separated by 2 m. Each block consisted of five separate lines of 1.5 m and on each line 8 pots were deposited at a distance of 1 m.

Each of the self-pollinated lines (NBO04, NKO03, NTE015, NKO08 and NFE011) was allocated to each block. Three cowpeas were planted in each pot. For the synchronization of floral phenology, the late flowering self-pollinated line (NBO04) was planted 21 days before the other four lines (NKO03, NTE015, NKO08 and NFE011). Two weeks after each semi phase, a dismating was performed to leave the most vigorous plant in the pot. In total, one self-pollinated line comprised 40 individuals (one block) and over the 5 blocks 200 individuals were involved in the different crosses.

After the appearance of the flower buds, an insecticide with the active ingredient deltamethrin was sprayed on all the plants in the plot in order to protect them from insect attacks.

The emasculation took place in the late afternoon between 4 p.m. and 6 p.m. It concerned the flower buds of the female parents to be opened the next day. The stage of evolution of the floral button as an indicator to identify those that will open within less than 24 hours was revealed in this study. emasculation, which involved grasping the button to be emasculated firmly but with delicacy, so as not to traumatize the fragile attachment point of the button and the raceme, was done according to the technique described by the manual crossing guide [7].

For this purpose, an incision of approximately two-thirds (2/3) of the width of the unhatched bud with the aid of dissection scissors was made. The upper part of the wound petals was then grasped between the thumb and the forefinger in order to gently detach the incised portion. The operation exposes the upper part of the style, stigma and stamens. The 10 pollen lodges were cut with clamps or scissors (Fig. 1).

Three manual pollination techniques coded A, B and C were tested. To perform the different pollinations, the blossomed flowers of the pollen-supplying male parents were harvested in the morning between 6 a.m. and 8 a.m. and are kept in a refrigerator at 18°C. Pollen thus conserved remains viable 12 to 15 h after harvest [7]. Manual pollination was carried out in the afternoon between 4 p.m. and 6 p.m. After the anthers of the female floral button were removed, pollen from the selected male parent was transferred to the female parent's receptive stigma using the three techniques A, B and C tested.

Technique A, consisted of freeing the banner and the wing of the flower from the male parent. The keel was incised with two-thirds forceps and its outer part was removed revealing the stamens bearing the mature anthers. The set of stamens with the anthers was stained on the stigma of the pistil of the female flower. This technique is the one described in the guide for manual crossing of cowpea [7]. At the end of the operation the emasculated and pollinated flower of the female parent was covered with a small cloth bag (Fig. 2).

Technique B also consisted of brushing all the stamens with the anthers on the stigma of the pistil of the female flower. However, the upper third of the keel of the male flower which provided the pollen was used to cover the pistil of the pollinated flower in the female parent. The whole (empty keel and pollinated flower) is covered with a small fabric bag (Fig. 3).

Technique C, consisted of directly using the entire upper third of the keel of the male flower with the stamens to cover the pistil of the emasculated flower in the female parent. The whole (keel with stamens and pollinated flower) is covered with a small fabric bag (Fig. 4).

In each block, individuals from a parental line on the lines served as both a pollen donor (male parent) in a "direct crossing" and a pollen recipient (female parent) in a "reciprocal crossing". Thus, 1419 crosses were made between the five lines of self-fertilization, including 713 direct crosses and 706 reciprocal crosses. From the five parental self-pollinated lines, 20 combinations of crosses (direct and reciprocal crosses) were performed (Table 2).

To demonstrate the effectiveness of cross-pollination, 10 flowers in the flower button stage that could open the next day and 10 flowers already opened by lines of self-pollination were emasculated and not pollinated, they were each covered by a cloth bag.

Unfertilized flowers fall within 24 hours of anthesis and unfertilized ovaries may remain attached 48 hours after anthesis. Therefore, the effectiveness of the crossing can be verified three days after pollination [7]. If the crossing was successful a pod appears the next 2 or 3 days, otherwise, a fall of the female flower is observed.

Thus, two days after the pollination, the number of pollinations that led to a nesting was counted by manual pollination technique (A, B and C) and by genotype according to whether the latter was used as the male or female parent. Thus, the pollination success rate was calculated according to the formula:

$$\textbf{Success rate} = \frac{\textbf{Number of successful fecundations}}{\textbf{Total pollination achieved}} \textbf{X 100}$$

After harvest, the number of seeds contained in the pods obtained was counted by manual pollination technique (A, B and C) and by genotype according to whether the latter was

used as the male or female parent. Thus, the packing rate of the pod was calculated according to the formula:

$$\textit{Fill rate} = \frac{\textit{Mean of seed in artificial fertilization}}{\textit{Mean of seed in natural fertilization}} X\ 100$$

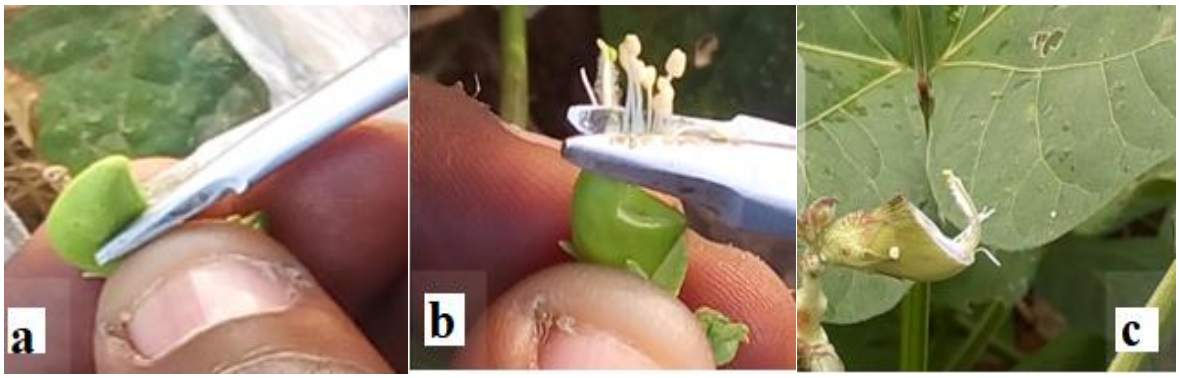

Fig. 1. Different steps of emasculation
a. Incision of the floral button at 2/3; **b**. Elimination of stamens; **c.** Emasculate floral button

Fig. 2. Manual pollination using technique A in cowpea
a. Deposit of pollen grains on the stigma of the female flower; **b.** Isolation of the pollinated flower with a cloth bag

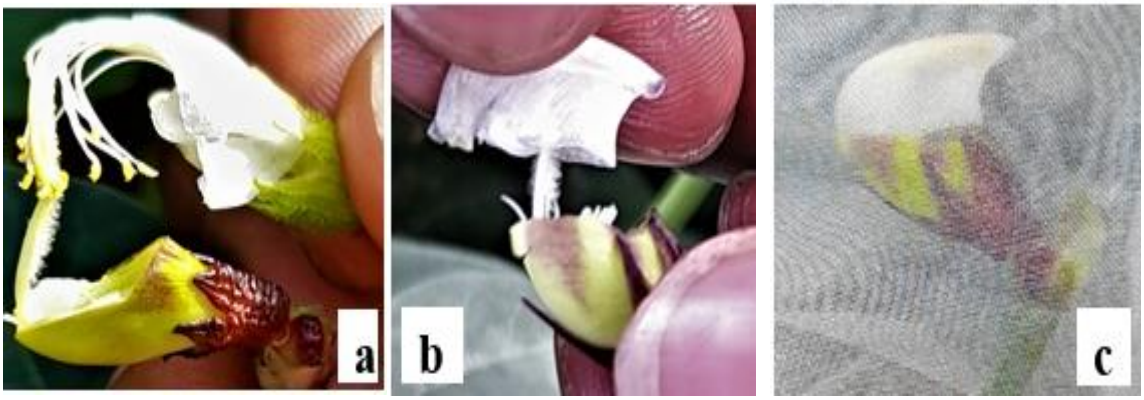

Fig. 3. Manual pollination using technique B in cowpea
a. Deposit of pollen grains on the stigma of the female flower; **b**. laying of the keel of the male flower emptied of its stamens on the pistil of the pollinated flower; **c**. Insulation of the pollinated flower covered with the empty keel using a fabric bag

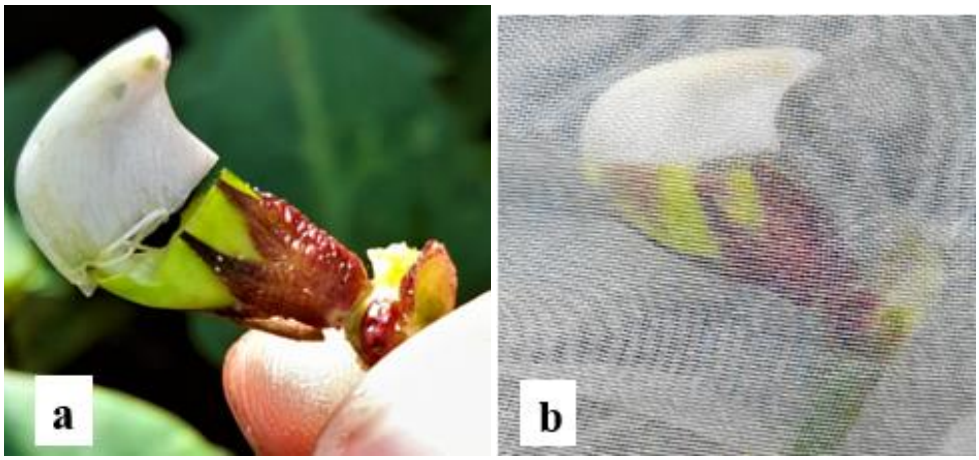

Fig. 4. Manual pollination with technique, C in the cowpea
a. Cover of pistil with keel containing male flower stamens **b.** Insulation of the pollinated flower covered with the keel carrying the stamens using a fabric bag

Table 2. Number of direct and reciprocal crosses from five cowpea parental lines

Male (1) / Female (2)	NBO04	NKO08	NTE015	NKO03	NFE011
NBO04		77	66	76	75
NKO08	58		83	58	65
NTE015	73	63		52	69
NKO03	62	73	98		92
NFE011	64	75	72	68	

Number of crosses performed using genotypes as a male (direct crosses)
Number of crosses performed using genotypes as a female (reciprocal crosses)

2.4 Statistical Analysis of Collected Data

To determine the morpho-phenological characteristics of the flowers of the different self-fertilization lines, a variance analysis (ANOVA) was performed to evaluate the discriminating power of each of the quantitative traits studied, at a threshold of 5%. When the ANOVA test was significant *(P*<0.05), a post-ANOVA test by Student Newman Keuls (SNK) was performed to classify the lines studied. In the hybridization trials between cowling lines, the ANOVA and Student Newman Keuls post-ANOVA tests were performed at the 5% threshold to rank the effectiveness of hand pollination techniques or self-fertilization lines involved in crosses. Statistical analyzes were performed using SPSS software version 20 (IBM Corporation, USA).

3. RESULTS AND DISCUSSION

3.1 Stages of Flower Bud Evolution in Cowpeas

The stages of evolution of the flower bud are summarized in four phases: stage 1 corresponding to the initiation of the flower bud, stage 2 to the flower bud with a dark green color, stage 3 to the flower bud with a pale green or pale-yellow color suitable for emasculation and the last stage, stage 4 corresponding to the blossomed flower (Fig. 5). These different floral stages could be a generality in cultivated cowpeas.

3.2 Morphology of Male and Female Reproductive Organs of the Cowpea

The reproductive system consists of 10 stamens welded together at the base and a pistil. Each stamen consists of a net topped with an anther containing pollen grains. The pistil consists of a stigma, a style with a beard at the top and an ovary containing the eggs (Fig. 6).

3.3 Variability in Flower Morphology of the Studied Cowpea Self-fertilization Lines

Three flower morphology traits (flower width, flower length, and stamen length) significantly differentiated the self-fertilization lines.

Stage 1: Flower bud initiation Stage 2: Dark green floral button

Stage 3: Pale green floral button Stage 4: blooming flower

Fig. 5. Evolution of the floral button showing the favorable stage for emasculation

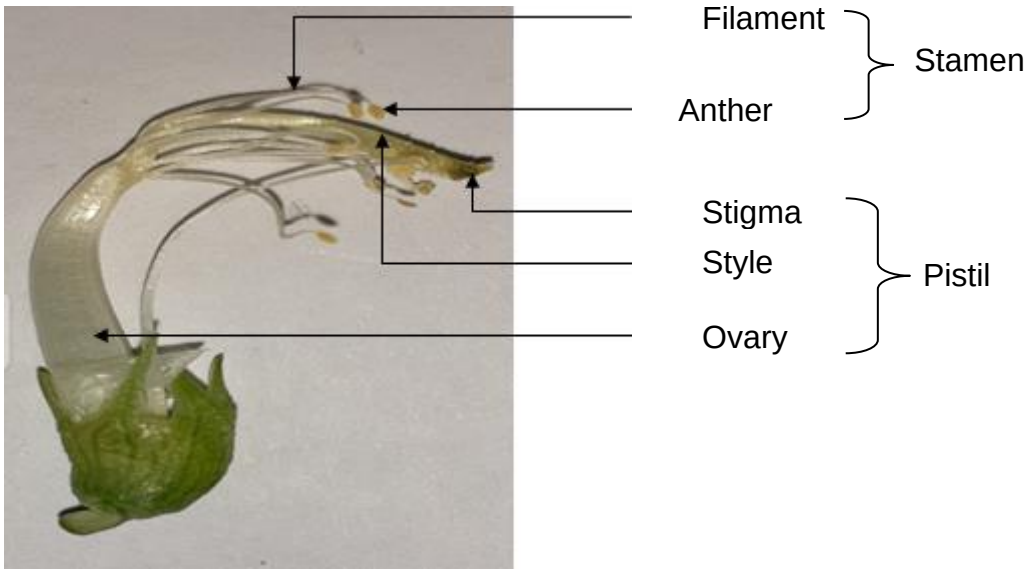

Fig. 6. Male and female breeding organs of the hermaphrodite flower of Cowpea

Thus, for the flower width (F = 34.423; *P* < .001), the NFE011 line with a mean of 2.9 cm and the NBO04 line with a mean of 2.4 cm represented the broad-flowered self-fertilization lines and the small-flowered genotype, respectively.

In terms of flower length, the NBO04 genotype had the least flower length (2.56 ± 0.05 cm), while the NKO08 (3 ± 0.11 cm), NFE011 (2.96 ± 0.15 cm), NTE011 5 (2.84 ± 0.11 cm) and NKO03 (2.8 ± 0.07 cm) had substantially identical values (F = 11.687; *P* < .001).

Stamen length differentiated the five self-fertilization lines into 2 subsets (F = 9.16; *P* < .001). These are the relatively short stamen lines NBO04 (2.32 ± 0.45), NTE015 (2.4 ± 0.12 cm), NKO08 (2.5 ± 0.22 cm) and NKO03 (2.5 ± 0.07 cm) and a long-stamen line (2.76 ± 0.05 cm) (Table 3). However, two traits did not significantly differentiate the five lines of self-fertilization. This is the peduncle length character (F = 0.251; *P* = 0.906) and the number of flowers per peduncle character (F = 1.16; *P* = 0.208) (Table 3).

3.4 Floral Phenophases of the Five Cowpea Self-fertilization Lines Studied

From semi to flower bud initiation, from semi to flowering flower appearance, and from semi to pod maturity, the five self-fertilization lines studied were divided into two groups (Table 4). These are four self-fertilization lines (NFE011, NKO03, NTE015 and NKO08) with an early cycle of which the initiation stage of the flower bud varied from 29.2 to 30.2 days after semi, the time of onset of the blooming flower varied from 40.6 to 42.8 days after semi and the maturation time from 60.4 to 63 days after semi and the line NBO04 with late cycle whose initiation stage of the flower bud was 48.2 days after semi, the time of appearance of the blooming flower 62.4 days after semi and the maturation time of 84.2 days after semi. [10] attributed the difference in flowering days between inbreeding lines to the fact that the trait depends on a complex of minor genes. [11] observed a tendency for early flowering dominance in cowpeas. It should also be added that environmental conditions could also influence flowering. These results corroborate those of [12] in Ghana and [13] in India. Also the observed differences in duration between flower bud initiation and flower blooming were significant between inbreeding lines. The shortest time interval was noted in line NFE011 (10.4 ± 1.14 days) and line NBO04 had the longest time interval (14.2 ± 1.64 days). However, the time interval between flower blooming (flowering) and pod maturity was identical for the five self-fertilization lines. The mean duration is 20.79 ± 1.69 days after semi (Table 4). This time interval can therefore be estimated between 20 and 21 days (approximately 3 weeks) after semi [14] stated that the time of flowering determines the time of harvest of mature pods.

3.5 Effect of Pollination Technique

All emasculated control flowers at the flower button stage without being pollinated dropped all between day 2 and day 3. However, 45/50 or 90% of the flowers already blossomed before being emasculated gave pods. This reflects the fact that pods obtained after pollination are actually due to the input of foreign pollen. Thus, the formation of pods in almost all newly blossomed and emasculated flowers without pollen input confirmed the work of [15]. According to these authors, the fertilization in the cowpea occurs a few hours before the flower opens.

Three manual pollination techniques (A, B and C) were tested. These techniques significantly influenced the fruit set rate (F = 50.28; *P* <.001) as well as the pod filling rate (F = 7.002; *P* = .010), (Table 5). Statistical analyzes showed that the first two techniques (A and B) used gave identical fruit setting rates (17.45% and 16.92% respectively) and pod filling rates (40.68% and 41 , 34% respectively) also identical. These fruit set rates corroborate the estimate made by the cowpea manual crossing guide [7] which places the success rate between 10 and 20%. The third pollination technique (technique C) described in this study gave a higher success rate (45.38%) and an even higher pod filling rate (58.03%). This could be explained by the fact that in this technique, the keel being completely cut with all the stamens to cover the pistil, still contains a lot of pollen to fertilize the eggs of the pistil. The high pollen load around the pistil would favor its pollination. This method could therefore be recommended in breeding programs for cowpeas and for certain plants which have the same floral morphologies.

3.6 Effect of Female and Male Genotypes (Pollen Source)

The female genotype significantly influenced the fruit setting rate (F = 3.241; *P* = .042) as well as

the filling rate (F = 5.476; *P* = .06) (Table 6). The NBO04 genotype used as a female was more successful. Indeed according to [7], the receptivity of the stigma is selective; some self-fertilization lines therefore turn out to be better female progenitors than others.

Pollen source significantly influenced the knotting rate (F = 26.079; *P* < .001). The NTE015 genotype gave the highest knotting rate when used as a male parent (54.64 ± 1.24%) (Table 7). This was confirmed by the work of [16] which showed high pollen viability in certain self-fertilization lines of cowpea. Other authors [17,18] have attributed the poor pod formation of cowpea to poor pollen viability and dehiscence of anthers. However, in our study, no significant difference was noted in the filling rate using different sources of pollen. However, pollen source did not significantly influence pod fill rate (F = 1.542; p = 0.241), (Table 7).

Table 3. Means± standard Error of Morphological characteristics of flowers of five cowpea self-fertilization lines

Self-fertilization lines	PL (cm)	FL (cm)	FW (cm)	SL (cm)	NFP
NBO04	18.00 ± 3.39 a	2.56 ± 0.07 b	2.40 ± 0.05 d	2.32 ± 0.45 b	2.20 ± 0.45 a
NKO08	19.80 ± 4.55 a	3.00 ± 0.10 a	2.70 ± 0.14 b	2.50 ± 0.22 b	3.00 ± 0.71 a
NTE015	19.60 ± 5.41 a	2.84 ± 0.04 a	2.74 ± 0.11 b	2.40 ± 0.12 b	2.60 ± 0.89 a
NKO03	18.00 ± 2.55 a	2.80 ± 0.05 a	2.56 ± 0.07 c	2.50 ± 0.07 b	3.20 ± 0.84 a
NFE011	18.60 ± 2.41 a	2.96 ± 0.07 a	2.90 ± 0.15 a	2.76 ± 0.55 a	3.20 ± 0.84 a
F	0.251	11.687	34.42	9.16	1.621
P	.906	<.001	<.001	<.001	.208

PL: Peduncle length; FL: Flower Length; FW: Flower Width; SL: Stamen length; NFP: Number of Flowers per Peduncle; cm: centimeter. The means followed by the same letter, in the same column, are statistically equal according to the Newman-Keuls post-ANOVA test at the 5% threshold

Table 4. Means± standard Error of Phenological Characteristics of Five Cowpea self-fertilization Lines

Self-fertilization lines	FBI (DAS)	DBF (Days)	TAF (DAS)	TFEMa (Days)	MT (DAS)
NBO04	48,20 ± 3,63 a	14,20 ± 1,64 a	62,40 ± 2,88 a	21,80 ± 0,84 a	84,20 ± 3,35 a
NKO08	29,40 ± 1,67 b	13,00 ± 1,41 ab	42,40 ± 1,67 b	21,40 ± 1,52 a	60,80 ± 1,52 b
NTE015	29,8 ± 1,30 b	12,00 ± 1,48 ab	41,80 ± 1,48 b	21,20 ± 2,05 a	63,00 ± 2,55 b
NKO03	29,2 ± 1,67 b	12,80 ± 1,92 ab	42,00 ± 0,71 b	19,60 ± 1,67 a	61,60 ± 1,82 b
NFE011	30,20 ± 3,19 b	10,40 ± 1,14 b	40,60 ± 2,41 b	19,80 ± 1,48 a	60,40 ± 2,07 b
F	49,832	4,041	109,883	2,025	74,621
P	<.001	.015	<.001	.130	<.001

FBI: Flower Button Initiation time; DBF: Duration between Burgeoning and Flower bloom; TAF: Time of Appearance of the blossoming Flower; DFM: Duration between Flower bloom and pod Maturity; MT: Maturation time; DAS: Days After Sowing. The means followed by the same letter, in the same column, are statistically equal according to the Newman-Keuls post-ANOVA test at the 5% threshold

Table 5. Effect of the pollination technique on the rate of knotting and pod filling in cowpeas

Techniques	Knot Rate (%)	Fill rate (%)
A	17.45 ± 4.5 b	40.68 ± 10.29 b
B	16.92 ± 4.5 b	41.34 ± 7.55 b
C	45.38 ± 7.38 a	58.03 ± 6.64 a
F	50.275	7.00
P	<.001	.01

The means followed by the same letter, in the same column, are statistically equal according to the Newman-Keuls post-ANOVA test at the 5% threshold

Table 6. Effect of female genotype on cowpea knotting and pod filling

Female genotypes	Knot Rate (%)	Fill rate (%)
NBO04	56.63 ± 3.32 a	66.67 ± 3.81 a
NFE011	44.50 ± 3.86 bc	55.56 ± 1.15 b
NKO03	37.23 ± 2.04 c	53.33 ± 8.30 b
NKO08	42.28 ± 9.66 bc	53.03 ± 5.40 b
NTE015	47.73 ± 9.73 bc	58.20 ± 0.95 b
F	3.241	5.476
P	.042	.006

The means followed by the same letter, in the same column, are statistically equal according to the Newman-Keuls post-ANOVA test at the 5% threshold

Table 7. Effect of male genotype (pollen source) on knotting rate and pod filling

Male genotypes	Knot Rate (%)	Fill rate (%)
NBO04	41.29 ± 1.28b	56.58 ± 2.53a
NFE011	35.61 ± 4.78b	53.97 ± 8.69a
NKO03	49.31 ± 2.69b	56.79 ± 9.63a
NKO08	48.01 ± 2.97b	53.82 ± 5.48a
NTE015	54.64 ± 1.25a	65.62 ± 9.88a
F	26.079	1.542
P	<.001	.241

The means followed by the same letter, in the same column, are statistically equal according to the Newman-Keuls post-ANOVA test at the 5% threshold

4. CONCLUSION

Control of floral morpho-phenology and artificial pollination techniques in a species is an important asset in the genetic improvement of the species. This study, based on five different lines of self-fertilization of cowpeas in northern Côte d'Ivoire, revealed the phenophase of these lines of self-fertilization and some parameters that may influence the success of cross-pollination in cowpeas. Three manual pollination techniques were described and one (technique C) was found to have a high success rate compared to the others. Finally, it was shown in this study that the genotype of the cowpea could influence the success of manual pollination.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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