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Reproductive studies in commercial cocoa tree

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ABSTRACT

Theobroma cacao is a species of economic importance mainly for the food industry. The objective of the present study was to analyze pollen viability, stigma receptivity and controlled pollinations as a function of flower opening time (nine times, from 8:00 am to 4:00 pm) in selected plants of clones TSH1188 and CCN51. There was a higher average percentage of stigma receptivity except at the last collection time (4:00 pm). The clones showed a higher average percentage of pollen viability from 8:00 to 11:00 am. Fertilization percentage varied from 12.5 to 68.7% in clones throughout the flower opening time; a gradual trend in the fall in fertilization percentage was observed until 12:00 pm. Significant differences between clones for pollen viability and stigma receptivity were observed, indicating the influence of controlled pollination time on these characteristics.

Keywords: *Theobroma cacao*, stigma receptivity, pollen viability, clone TSH1188, clone CCN51.

INTRODUCTION

The cocoa tree (*Theobroma cacao* L.) is an economically important species of the Malvaceas, order Malvales.[1] It is a taxon originating from tropical and equatorial America. The cocoa tree is a monoecious and typically cauliflower species with tiny inflorescence called floral pads from which the fruits develop and are formed. Cocoa trees produce pentamerous flowers with petals, sepals, stamens and staminods or false stamens; in the pistil, the ovary has 30 to 70 ovules, however the spatial distribution of the floral parts prevents free access of pollen to the stigma.[2,3]

The cocoa tree has hermaphrodite flowers and homogamous flowers, whose main pollinating agents are microflies of the genus *Forcipomya*,[4] but from Cecidoniidae and Sciaridae families were also found in the three systems.[5] These pollinating agents are important due to reproductive self-incompatibility in *T. cacao*[6] once *T. cacao* has a complex incompatibility system under the control of several genes.[7] It is estimated that the adult cocoa tree has the capacity to produce more than 50 thousand flowers per year, however less than 5% are effectively pollinated, and 0.5 to 2% result in the production of fruits, with non-pollinated flowers falling to 10 h after anthesis.[2] Self-compatibility is also associated with productivity in plants of commercial value, a characteristic inherited from the parents used in breeding programs.[8]

The cocoa tree, especially its production system, is under biotic and abiotic stress, such as the phytopathogen that causes witches' broom (*Moniliophtora perniciosa* (Stahel) Aime & Phillps-Mora), water deficit[9] and inadequate temperatures.[10] Resistant and moderately resistant cultivars to witches' broom disease, such as clones TSH1188[11] and CCN51,[12] has been used to study plant-pathogen interactions and also to re-establish cocoa tree producing regions, as inadequate management associated with the disease leads to reproductive losses and a drop in productivity.[10]

Artificial pollination practiced manually is widely used in the cocoa tree region, mainly to verify the degree of compatibility between plants as a method of genetic improvement strategy through crossing between commercial varieties and *landraces*.[13]

Reproductive characterization in *T. cacao* is rarely reported in the literature, making it difficult to plan crosses between clones, varieties and cultivars, especially those with large genetic distances. Pollen production and viability in *T. cacao* are related to rainfall and variable temperature throughout the day but vary considerably depending on the clone analyzed.[14]

Reproductive characterization allows the evaluation of the time and season of flowering, the production and viability of pollen and stigmas, and the crossing and fertilization rates for fruit production. These data are important to define the necessary conditions for conducting crosses, as in *T. subincanum* here the highest percentage of stigma receptivity occurred between 2:00 and 10:00 am, while pollen viability was not influenced by the collection time factor.[15]

Due to the need for cross-pollination in some *T. cacao* clones because to their floral biology and self-incompatibility, studies on pollen viability, stigma receptivity and the moment at which the plant is ready for fertilization are considered informative. This work aimed to seek information regarding the best time to carry out controlled pollination as the preliminary data, in specific commercial cocoa clones that will be used in breeding programs, resistant to witches' broom.

MATERIALS AND METHODS

The study material consisted of two commercial clones of *T. cacao* but contrasting in terms of resistance to witches' broom: clone TSH1188 which is self-incompatible and resistant to witches' broom[11] and clone CCN51 with the particularity of being self-compatible and moderately resistant to witches' broom.[12] Plants from each clone that will be used in breeding programs were selected. The clones (without a voucher number because they are part of the institution's protected area) are maintained in the Germplasm Bank of the Executive Committee of the Cacau Farming Plan (CEPLAC), Ilhéus, BA, Brazil.

Pollination procedure, experimental design and statistical analysis

A randomized block design was used, with three replications (plants) and nine treatments (collection times), in a factorial scheme two (clones) × nine (times throughout the day). The same procedure was repeated for each replication, with two clones per treatment/time. The treatments consisted of nine collection times, from 8:00 am to 4:00 pm, at one-hour intervals. Flowers close to anthesis, swollen, were protected with plastic tubes (Figure 1a - b) to ensure that they were not pollinated by insects. The following day, after the flowers opened (Figure 1c - f), they

were pollinated to assess the occurrence of fertilization or collected to carry out histochemical tests. Sampling consisted of two flowers per replication/clone/test/treatment for analysis of stigma receptivity (SR) and pollen viability (PV), and four flowers per replication/clone/test/treatment for controlled pollination. The results were submitted to ANOVA. The data were analyzed using the SAEG 5.0 computer program.

Stigma receptivity

For SR, two histochemical tests were used: a) hydrogen peroxide (HPe) (H_2O_2), which indicates the presence of peroxidase[16] through the formation of air bubbles; b) alpha-naphthyl acetate (ANA), to localize esterase activity.[17] The stigmas were transferred to glass containers containing 2.0 mL of the test solution and kept completely submerged. The stigmas were considered receptive when the five stigmatic threads reached black color and formed bubbles (test a) or reddish color with dark ends (test b). To control the effectiveness of the product, stigmas of flower buds at the beginning of development (immature stigmas) were used. To obtain reliable results, damaged stigmas or those with pollen on the surface were not used, avoiding false positive results.

Pollen viability

To test the PV, Alexander solution[18] was used, modified by Dafni.[19] The histochemical test provided reactivity of the wall/cytoplasm (blue/red, respectively), with only stained pollen grains (PG) being considered viable, with intact cytoplasm and intact exine, while abnormal/unviable PG showed absence of cytoplasm or contracted cytoplasm. Temporary blades were prepared by maceration of the anthers and observed under a bright field microscope, in which all PG were counted. All five anthers of a flower were collected per slide, two slides per clone/replication/treatment, totaling 108 slides analyzed.

Percentage of fertilization

Self-pollination was carried out in accession CCN51 and cross-pollinations in accession TSH1188, totaling 216 artificially pollinated flowers. Staminodes were removed from the flower to be pollinated using tweezers (Figure 1d), the anthers of the donor flowers were collected (Figure 1e). The anther with PG were carefully deposited on the stigma of previously protected flowers without staminodes (Figure 1f), then they were labeled and protected again until the next day. The fertilization rate was checked 15 days after pollination, with flowers whose fruits were at the beginning of development being considered fertilized.

RESULTS

Stigma receptivity

Tests for SR with peroxidase and esterase enzymes were carried out throughout the flower opening time (Figure 1g - i). For both clones there was a considerable drop in the percentage of SR only at the last collection time of the day (Table 1). It was observed at 4:00 pm that there was a reduction in SR of 7 and 33% compared to the highest percentage of SR by tests with HPe and ANA, respectively. Both clones presented similar SR values in relation to the collection time, with no difference according to the F test ($p > 0.05$), between the clones for SR using the ANA test (Table 2). The source of hourly variation revealed the existence of a difference for the test with ANA at the 8% probability level. On the other hand, there was no significant interaction between the clone and time pollination (Table 2). No source of variation was significant for the test with HPe, indicating that there are no differences in the behavior of the clones in relation to the evaluation hours or their interactions.

Pollen viability

There was a difference by F test ($p > 0.01$) between clones for PV (Table 3). However, no difference was found for the evaluation times and for the clone*time interaction (Table 3). The mean comparison test indicated a higher PV for clone CCN51, while a lower mean value was observed for clone TSH1188, corresponding to only 73.4% of the PV of CCN51.

High percentages of viable PG were observed using the PV test based on the reactivity of the pollen wall and cytoplasm (Figure 1j), with average values greater than 95% at all evaluation times (Table 1). The clone CCN51 presented a higher average percentage of PV with the highest value (99.8%) of viable PG at 10:00 am and the lowest value (98.2%) at 3:00 pm (Table 1). In clone TSH1188, the histochemical test demonstrated the highest average percentage of PV (99.9%) in PG collected at 8:00 am, while the lowest percentage (81.5%) was observed in PG collected at 2:00 pm (Table 1).

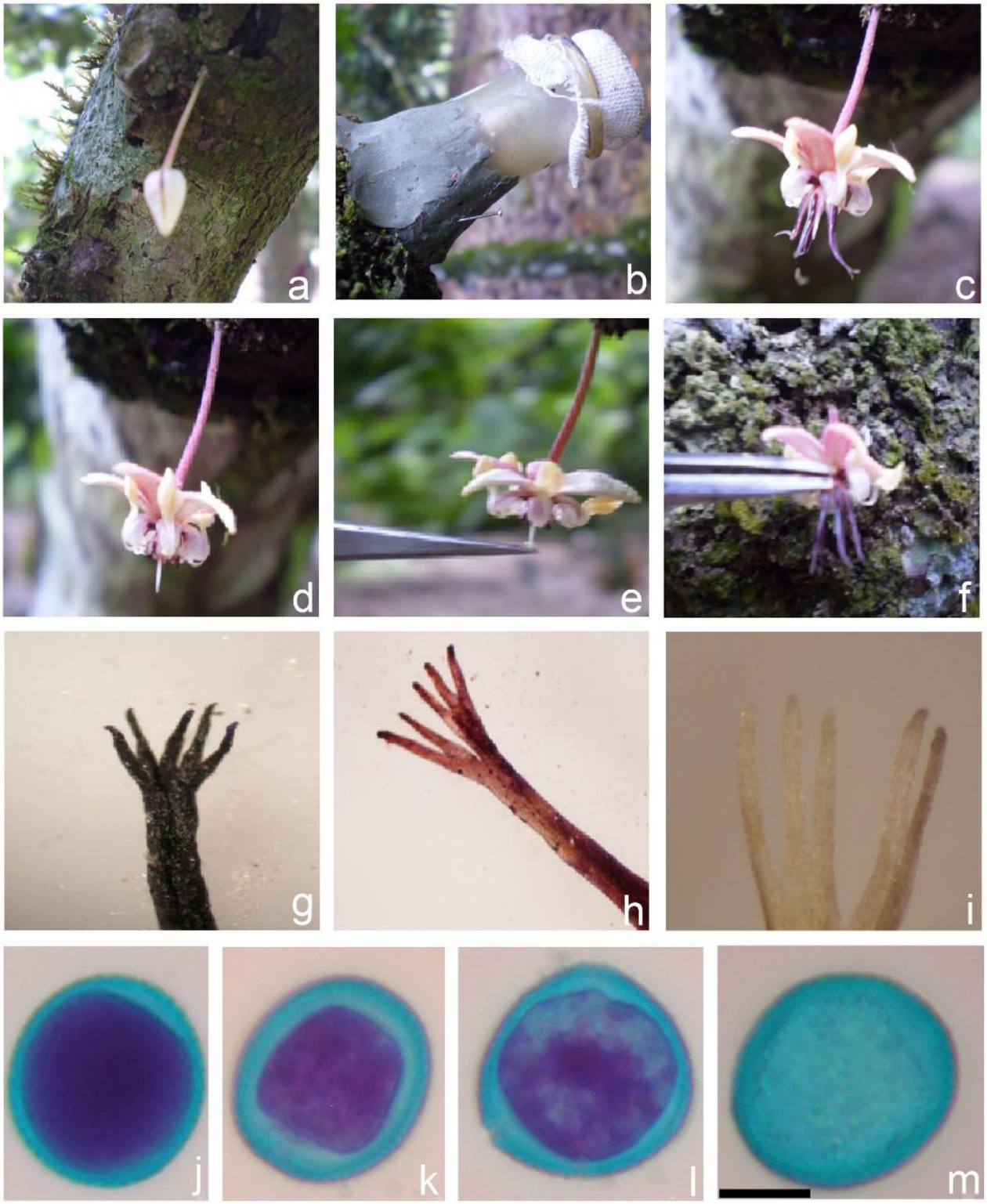

Figure 1. Pollination, stigmas and pollen grains in *Theobroma cacao*. Pre-anthesis flower bud (a); protected floral bud (b); flower in anthesis (c); flower without staminoid (d); pollen collection (donor flower) (e); pollination (f); receptive stigma by hydrogen peroxide test (g); receptive stigma using the alpha-naphthyl acetate test (h); non-receptive (immature) control stigma (I); empty unviable pollen grain (j); unviable pollen grain with contracted cytoplasm (k); unviable pollen grain with degenerating cytoplasm (l); viable pollen grain, with intact exine and cytoplasm (m). Bar: 100µm.

Table 1. Average percentage values of stigma receptivity using two histochemical tests, pollen viability and fertilization over flower opening time in *Theobroma cacao*, clones CCN 51 and TSH 1188

Clones	Collection time (hour)								
	Morning					Afternoon			
	8:00	9:00	10:00	11:00	12:00	1:00	2:00	3:00	4:00
	Stigma receptivity – Alpha-Naphthyl Acetate (%)								
CCN 51	93	100	83	83	100	100	100	83	83
TSH 1188	100	83	83	100	100	100	83	83	100
Average	97	92	83	92	100	100	92	83	92
	Stigma receptivity – Hydrogen Peroxide (%)								
CCN 51	83	100	100	83	100	83	83	100	67
TSH 1188	83	100	100	83	100	100	83	100	67
Average	83	100	100	83	100	92	83	100	67
	Pollen viability – Alexander solution (%)								
CCN 51	99.3	98.2	99.8	99.6	98.4	99.0	98.9	98.2	96.2
TSH 1188	99.9	99.6	99.7	99.7	95.9	86.0	81.5	99.1	97.0
Average	99.6	98.9	99.7	99.7	97.1	92.5	90.2	98.7	96.6
	Fertilization – Pollination *in vivo* (%)								
CCN 51	18.7	43.7	43.7	18.7	12.5	37.5	31.25	18.7	18.7
TSH 1188	68.7	56.2	37.5	37.5	12.5	50.0	25.0	18.7	25.0
Average	43.7	49.9	40.6	28.1	12.5	43.7	28.1	18.7	21.8

Table 2. Summary of ANOVA for stigma receptivity using histochemical tests over flower opening time in *Theobroma cacao*, clones CCN 51 and TSH 1188

Source of variation	DF	SQ	MS	FC
	Alpha- Naphthyl Acetate			
Clone	1	46.30	46.30	0.110^{ns}
Hour	8	6481.48	810.19	1.940 *
Linear R2 = 0.13	1	840.20	840.29	2.020 **
Quadr R2 = 0.37	1	1533.35	1533.35	3.680 *
Cubic R2 = 0.37	1	50.93	50.93	0.120^{ns}
Quart R2 = 0.62	1	1648.57	1648.57	3.960 *
Quint R2 = 0.64	1	43.62	43.62	0.110^{ns}
Error	3	2364.73	788.24	1.890 *
Clone* Time	8	370.37	46.30	0.110^{ns}
Error	36	15000.00	416.67	-
Coefficient of variation (%)	22.73			
	Hydrogen peroxide			
Clone	1	0	0	0.000^{ns}
Hour	8	3703.70	462.96	1.250 **
Linear R2 = 0	1	0	0	0.000^{ns}
Square R2 = 0	1	2.41	2.41	0.010^{ns}
Cubic R2 = 0	1	0	0	0.000^{ns}
Quart R2 = 0.92	1	3409.94	3409.94	9.210 *
Quint R2 = 0.92	1	0	0	0.000^{ns}
Error	3	291.36	97.12	0.260^{ns}
Clone* Time	8	0	0	0.000^{ns}
Error	36	13333.34	370.37	
CV (%)	20.79			

DF = degrees of freedom, CV(%) = coefficient of variation, SQ = sum of squares, MS = mean squares, FC = F value, * Significant at 1% probability by the F test, ^{ns} Not significant by the F test.

Table 3. Summary of ANOVA for pollen viability analyzed using histochemical test over flower opening time in *Theobroma cacao*, clones CCN 51 and TSH 1188

Source of variation	DF	SQ	MS	FC
Clone	1	1041388.90	1041388.90	7.863*
Hour	8	1352547.87	169068.48	1.277 ns
Clone*Time	8	1160819.25	145102.40	1.096 ns
Error	36	4768031.50	132445.31	
Corrected total	53	8322787.53		
CV(%) =	40.21			

DF = degrees of freedom, CV(%) = coefficient of variation, SQ = sum of squares, MS = mean squares, FC = F value, * Significant at 1% probability by the F test, ns Not significant by the F test.

Despite the high PV revealed with the application of Alexander solution, unviable empty PG or with contracted cytoplasm or with a pulverized appearance were also observed (Figure 1k-m), totaling less than 5% of PG in both clones (Figure 2). The gradual increase in the number of unviable PG was observed from 12:00 am to 2:00 pm, reducing their occurrence again from 3:00 to 4:00 pm (Figure 2).

High percentages of viable PG were observed using the PV test based on the reactivity of the pollen wall and cytoplasm (Figure 1j), with average values greater than 95% at all evaluation times (Table 1). The CCN51 clone presented a higher average percentage of PV with the highest value (99.8%) of viable PG at 10:00 am and the lowest value (98.2%) at 3:00 pm (Table 1). In clone TSH1188, the histochemical test demonstrated the highest average percentage of PV (99.9%) in PG collected at 8:00 am, while the lowest percentage (81.5%) was observed in PG collected at 2:00 pm (Table 1). Despite the high PV revealed with the application of Alexander solution, unviable empty PG or with contracted cytoplasm or with a pulverized appearance were also observed (Figure 1k-m), totaling less than 5% of PG in both clones (Figure 2). The gradual increase in the number of unviable PG was observed from 12:00 am to 2:00 pm, reducing their occurrence again from 3:00 to 4:00 pm (Figure 2).

Percentage of fertilization

The analysis of variance for the fertilization percentage (FP) revealed no difference ($p > 0.05$) between the clones depending on the pollination time (Table 4). Descriptive statistical analysis indicated variation in FP from 12.5 to 68.7% (Table 1) in both clones over the flower opening time. The CCN51 clone showed the highest FP in pollinations carried out at 9:00 am (43.7%), while the TSH1188 clone showed the highest percentage at 8:00 am (68.7%); both clones showed the lowest FP at 12:00 am (12.5%), indicating a gradual downward trend until 12:00 am, but the 1:00 pm fertilization time demonstrated a sudden increase in FP (Figure 3).

DISCUSSION

The clones TSH1188 and CCN51 are, respectively, resistant and moderately resistant to the phytopathogen that causes witches' broom, and both have been widely used in cocoa tree improvement strategies and in the recovery of crops and fruit production in Brazil.[20,21] Both clones are useful for disease resistance studies, in planning aimed at increasing productivity and characterizing vegetative and reproductive morpho-agronomic descriptors.[21]

Variations in SR were observed, which continue throughout the day also depending on the evaluation method. For SR, the activities of peoxidase and esterase enzymes indicated a gradual reduction in SR with minimum values in the late afternoon, in both clones, revealing similar behavior. This fact is attributed to the longtime anthesis and exposure of the stigma to environmental conditions, mainly temperature and humidity, and not to the reproductive variation (compatibility) between both clones. In *T. subincanum* the SR lasts a long time, being receptive to pollen throughout the day, and the stigma remains receptive even in pre-anthesis.[15] SR is important because PG requires specific receptors and inducers on the surface of the stigma for pollen tube emission and subsequent ovule fertilization.[22]

The reduction in SR was 7 and 33% at 4:00 pm compared to the highest percentage of SR by the HPe and ANA tests, respectively, maybe indicating that greater weight of fruit can be obtained by pollination carried out in the early hours of the day. The histochemical test with ANA

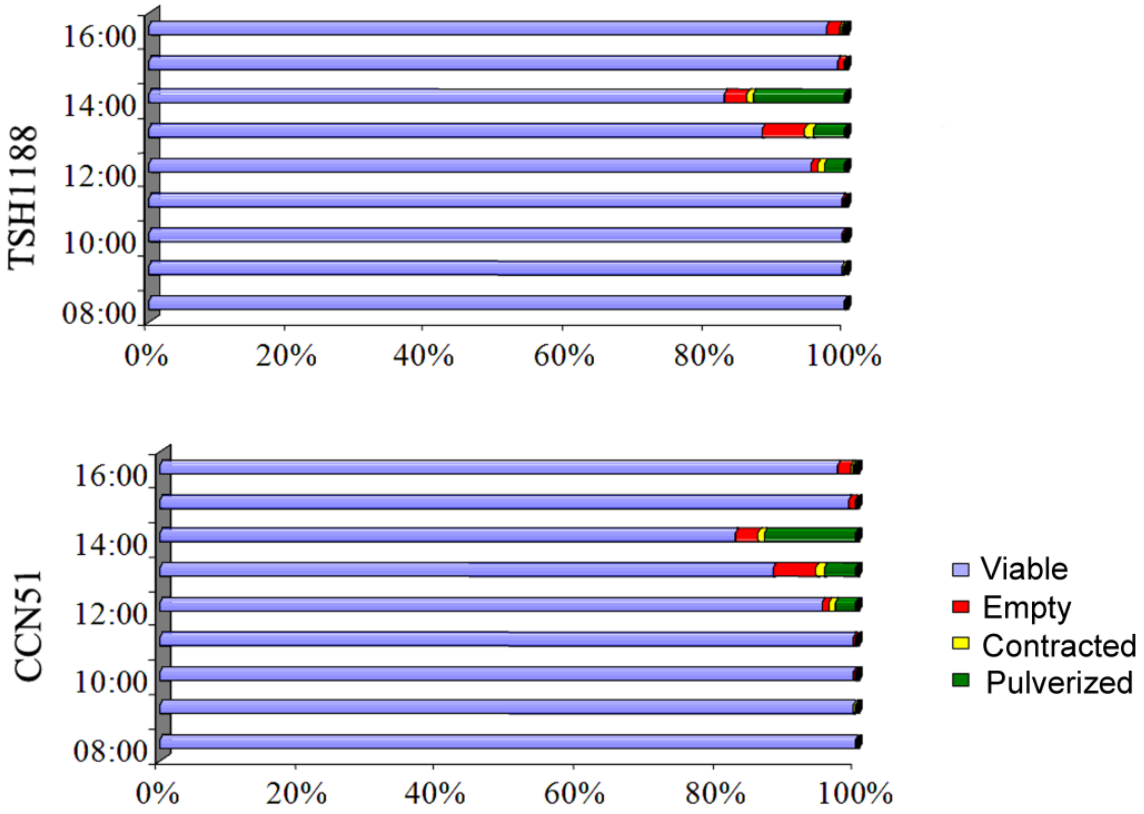

Figure 2. Percentage of viable and unviable pollen in clones TSH1188 and CCN51 evaluated as a function of collection time over the flower opening time in *Theobroma cacao*.

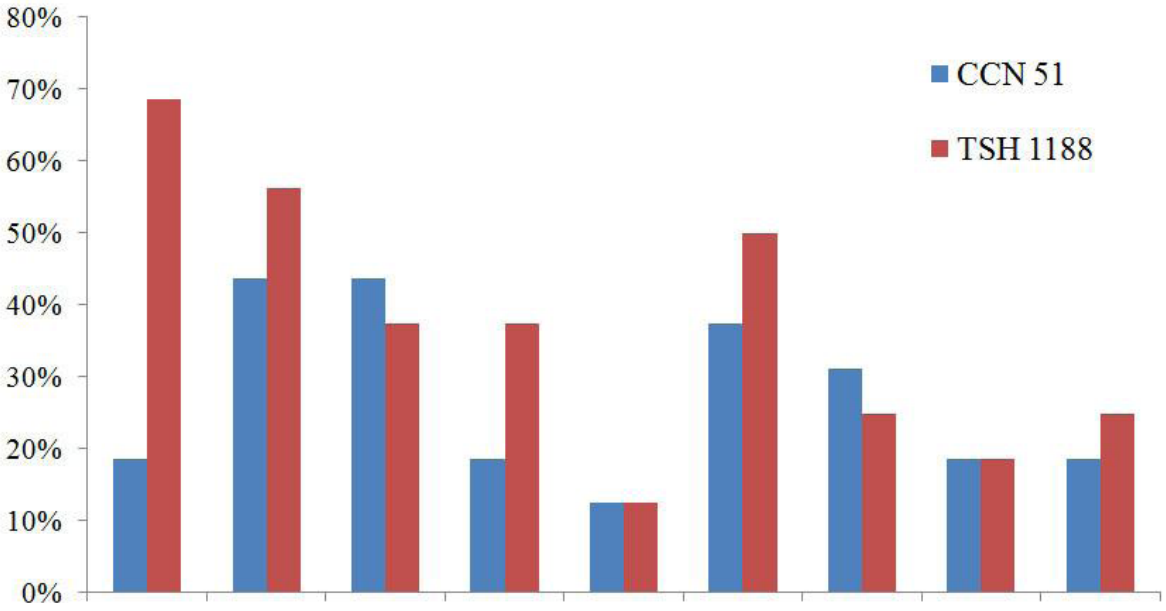

Figure 3. Average percentage of fertilization rate of clones CCN51 and TSH1188 after 15 days of controlled artificial pollination throughout the flower opening time (from 8:00 am to 4:00 pm) in *Theobroma cacao*.

proved to be more sensitive in detecting the reduction in SR at 4:00 pm. In clones CCN51 and THS1188, the absence of significant difference by the F test ($p > 0.05$) between the clones for SR using ANA suggests that both germplasms are equally ideal for crossing each other, at the same time of collection pollen and controlled artificial pollination. The significant differences in the time of evaluation with the ANA test reinforce the influence of the time for field practices involving artificial pollination.[23]

The lack of significant interaction between clone*time does not suggest differences in relation to stigma reproductive behavior between the clones analyzed and the evaluation times, even though both are contrasting in terms of compatibility. The similarities in reproductive terms between the clones is reinforced by the lack of significance in the differences between them using the HPe test.

In angiosperms, stigma behavior varies considerably between different botanical groups. In *Centrosema pubescens* Benth. the receptivity of the stigma was verified throughout the flower opening time, with the stigmas being receptive throughout the entire time analyzed.[24] In *Acacia mearnsii* De Wild. the stigmas became receptive one day after complete floral opening and remained so for around 48 h.[25] In fact, variations between species in terms of SR are due to botanical, ecological and evolutionary particularities, such as, for example, the performance of the pollinating agent or mode of pollination, habit and habitat, genetic and environmental factors.

In the present study, the reduction in SR was verified with greater sensitivity by the histochemical test with ANA, in relation to the use of HPe. This fact can be attributed to the varying levels of the esterase enzyme on the stigma surface. On the other hand, the peroxidase test using HPe solution has been effective in other plant groups. In *Psychotria spectabilis* Steyrm., the peroxidase test carried out every two hours from the opening of the flower until the end of the afternoon revealed that there was a greater SR between 9:00 and 11:00 am and continued to be receptive until 2:00 pm. with a subsequent decline in SR.[26] In *Alpinia zerumbet* (Pers.) B. L. Burtt & R. M. Sm., the average percentage of SR in the period from 7:00 to 8:00 am was 20%, from 12:00 am to 1:00 pm was 40% and between 6:00 and 7:00 pm was 70%. SR showed significant differences in the three collection periods.[27] Despite the high stigmatic receptivity, the attachment rate was considered low for the clones, indicating that factors such as ovary development, emission of the pollen tube by the pollen grain, among others, may be acting to prejudice the reproductive process of these clones.

The existence of significant differences between the CCN51 and TSH1188 clones analyzed in the present study indicates that the clones differ from each other for PV with a higher percentage of PV for CCN51. Different rates and percentages of PV are common among cultivars and varieties of different plant species.[14,15] The main reproductive characteristic that differentiates clones CCN51 and THS1188 is the compatibility, which in *T. cacao* is governed by the gametophytic and sporophytic systems.[28] However, there was no relationship between this characteristic and PV. Unviable PG were observed in the present work in both clones. Unviable PG with empty, contracted, or pulverized cytoplasm are generally the result of meiotic abnormalities that can occur both in meiosis, mainly due to the formation of unbalanced gametes, and in post-meiotic events.[29]

Although both clones present differences in the percentages of PV, the greater occurrence of unviable GP observed during the hottest times of the collection days was the same pattern observed in both clones. In the region where the study was carried out, at 12:00 am there are considerable increases in ambient temperature and solar irradiance, lasting with high values until 2:00 pm. In *T. cacao*, temperature and other environmental factors such as precipitation and temperature have been considered to influence flowering, also indicating that these environmental characteristics qualitatively and quantitatively reduce the number of PG and PV.[14]

Therefore, in the present study, the gradual reduction in PV from 12:00 am to 2:00 pm in both clones, detected by the presence of unviable PG, can be attributed mainly to an increase in temperature at these times. For this reason, it is not recommended to carry out crossings or self-pollination in *T. cacao* between 12:00 am and 2:00 pm, where the ambient temperature rises together with solar irradiance. However, tests carried out with various doses of gamma radiation did not reduce the PV of *T. cacao* evaluated by *in vitro* germination.[30]

The variation in FP observed between clones can be attributed to the contrasting reproductive system between them. However, time was not observed as an important factor in fruiting. This fact can be attributed to the high PV (> 80%) and the high SR throughout the evaluation times. Temperature has been considered one of the main environmental factors associated with fertilization and

fruiting of species of the genus *Theobroma*, however humidity is also a factor considered relevant.[14] The species *T. cacao* have origin and primary center of distribution in the forests of tropical America, but precisely the Amazon forest, whose humidity is annually high, which may have favored the reproduction of the species in humid environments.[31]

CONCLUSIONS

Considering the receptivity rates of the stigma, controlled pollination can be carried out in clones TSH1188 and CCN 51, throughout the period under study (from 8:00 am to 4:00 pm), since these presented rates between 80 and 100%.

The best percentage of fertilization, using artificial pollination, occurred at 11:00 am for clone TSH 1188 and at 10:00 am for CCN 51, while the lowest value was obtained with pollination at 3:00 pm for both clones, with 9.4% fertilization.

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The authors have no relevant financial or non-financial interests to disclose and report there is no conflict of interest to declare.

DATA AVAILABILITY STATEMENT

The datasets generated during and/or analyzed during the current study are publicly in this article.

AUTHOR CONTRIBUTIONS

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Funding acquisition: Margarete Magalhães Souza.

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Visualization: Cláusio Antônio Ferreira de Melo, Ronan Xavier Corrêa.

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