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Floral phenology and standardization of hand pollination of Clove (Syzygium aromaticum (L.) Merr. & Perry)

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Abstract

Clove (*Syzygium aromaticum* (L.) Merr. & Perry) has a rich history and is highly valued for its culinary and medicinal uses. However, its limited genetic diversity and tendency for self-pollination pose challenges for optimizing its cultivation in India. Hybridization becomes imperative to broaden its genetic spectrum. This study, hence, focuses on refining techniques related to floral biology, pollen management and artificial hand pollination in clove. The study revealed that anthesis in clove occurs from 2:30 PM to 6:30 PM, with peak flower opening between 3:30 PM and 5:30 PM. Each flower possesses an impressive array of stamens ranging from 167 to 343 and anther dehiscence begins 36 h before anthesis and continues until the day of anthesis. Pollen grain dimensions show a polar diameter ranging from 17.06-18.22 µm and an equatorial diameter ranging from 19.40-20.70 µm. Stigma receptivity extends from the second to the sixth day of flowering, peaking on the fifth day. Emasculation is recommended 36 h before flower opening. Maximum pollen viability and fertility are observed 12 h before flower opening. Thus, pollen collection for pollination is standardized at this time. Drying pollen at 50 °C and storing it at 7 °C in a refrigerator maintain maximum viability after 60 days while drying pollen at 45 °C and storing it in a desiccator ensure maximum fertility. Artificial hand pollination results in a fruit set of 28.87 %. Artificial hand pollination results in a promising fruit set of 28.87 %, underscoring its efficacy in enhancing clove cultivation.

Keywords

Clove; floral phenology; hand pollination; hybridization; *Syzygium aromaticum*

Introduction

Syzygium aromaticum (L.) Merr. & Perry of the Myrtaceae family, commonly known as clove, is a highly esteemed spice renowned for its aromatic flavour (1) and medicinal properties (2). Cloves have long been valued in Ayurvedic medicine, celebrated for their therapeutic properties and health benefits (3). Indigenous to the Maluku Islands (the Moluccas) in Indonesia, clove has been cultivated in various parts of the world, including Zanzibar, Madagascar, Pemba, Sri Lanka and India (4). The important clove-growing regions in India include the Nilgiris, Tirunelveli and Kanyakumari districts of Tamil Nadu (765 ha); Calicut, Kottayam, Quilon and Trivandrum districts of Kerala (1123 ha), South Kanara district of Karnataka with (90 ha) and the Andaman Islands (196 ha) (Fig. 1) (2).

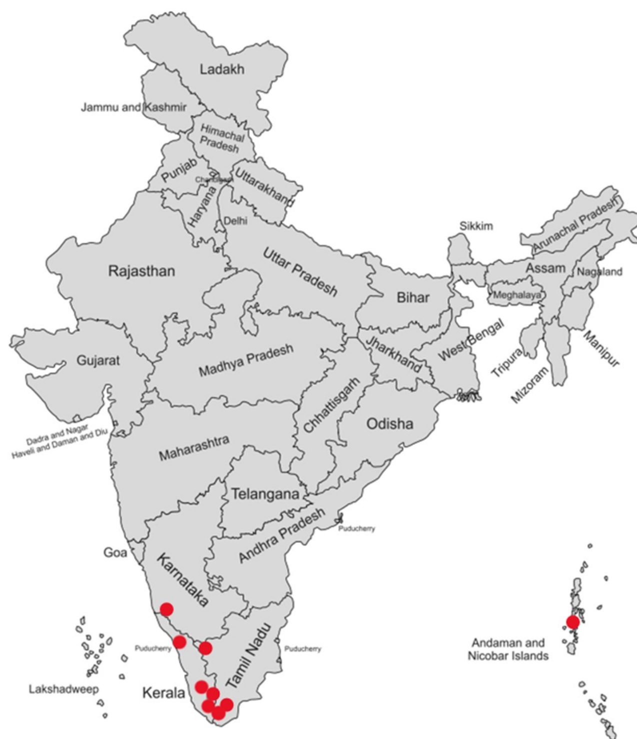

Fig. 1. Major clove growing regions in India.

The commercial clove consists of aromatic, dried, fully grown, but unopened flower buds harvested from the clove tree (5). Clove buds possess potent antioxidant properties due to their phenolic content, including compounds such as quercetin, eugenol, gallic acid (6) and significant amounts of tocopherol (7) and ascorbic acid (8). These compounds contribute to clove's free radical scavenging abilities and protective effects against metal ion-induced oxidation and biomolecules peroxidation.

Clove plants in India originated from a limited number of initial introductions, resulting in low genetic variability. A survey in the Tamil Nadu's Kanyakumari district and Kerala's southern districts revealed variability in clove tree shape, bud characteristics, yield and quality (9, 10). This inherent variability presents an opportunity to enhance clove cultivation through the selection and hybridization of diverse genotypes, potentially leading to the development of superior clove varieties. Hybridization, in particular, holds promise for increasing genetic diversity and introducing desirable traits, thereby improving crop's overall quality and productivity (11). Understanding clove's floral biology and phenology is essential to realize this potential. However, research on the floral biology and hybridization of *Syzygium* species has primarily been outdated. Clove inflorescence is a terminal, trichotomous panicle, corymbose, with a short peduncle and branching from the base, exhibiting significant variation in the number of flowers (12). A comprehensive understanding of clove's floral biology and phenology is essential to bridge this gap, forming the foundation for standardizing hybridization techniques critical for successful breeding programs. Studies on stigma receptivity in clove indicate that the stigma is most receptive on the day of anthesis, maintaining its receptivity for an additional 48 h (13). Notably, anthesis in clove has been observed to occur around 1:30 pm, with peak activity between 3:30 pm and 4:30 pm (14).

The staminal structure of *Syzygium* species, which includes clove, has been characterized in a previous study (15). It is noted that *Syzygium* species typically have numerous stamens, ranging from 40-50 per flower in *Syzygium balsameum* to as many as 1200 in *Syzygium metacarpal*, arranged in multiple whorls (15). In clove, the anthers dehisce longitudinally, starting 24 h before anthesis (14). This pattern of anther dehiscence has been consistently observed in clove estates in Sri Lanka, peaking immediately before flower opening (13). Similarly, in Zanzibar cloves of Indonesia, anther dehiscence occurs shortly after anthesis (16). Effective pollen storage methods ensure the availability of viable pollen for breeding programs, especially when synchronizing the flowering times of different genotypes. This is particularly important in clove, where variability in flowering periods can pose challenges for controlled pollination. In *Syzygium cuminii,* pollen can be effectively preserved for up to 9 months at 23 ºC in a dry environment (17). This instance underscores the feasibility of employing analogous storage methodologies for clove pollen, ensuring its sustained viability and availability for use as required. Also, it was observed that artificial cross-pollination in clove plays a critical role in enhancing fruit sets and ensuring genetic diversity. A study showed that artificial pollination can achieve a maximum fruit set of 30 % in clove, slightly higher than the 28 % observed under bagged conditions (13). This method involves the controlled transfer of pollen between flowers, ensuring optimal cross-pollination and seed development. It takes approximately three months for a fertilized flower in a clove plant to mature into fruit.

This study explores the reproductive biology of clove, focused on critical aspects including anthesis, floral characteristics, pollen traits, stigma receptivity, emasculation periods, pollen collection and storage, pollen viability and fertility. In addition, we developed and tested an artificial hand pollination technique to enable controlled hybridization. The primary objective was to establish a standardized hybridization method to enhance clove cultivation and support the development of new, improved genotypes. This research aims to contribute substantially to clove cultivation's agricultural and economic potential by detailing the complexities of clove's floral biology and refining hybridization techniques. The findings will provide a solid foundation for future breeding programs, ultimately leading to high-yielding creation.

Materials and Methods

Plant material and study location

Five clove plants were randomly selected for this study. These plants were aged between 30 to 35 years, as verified through primary data maintained by the estate owners at the time of planting. Four branches were selected from each of the 5 clove plants, with care taken to ensure that the branches represented the entire canopy (Fig. 2). The

Fig. 2. Clove branches tagged for study.

experiment was conducted from January to March 2020 at the Merchiston Estate, Southern Field Ventures Private Limited, in Ponmudi village, Nedumangad Taluk, Trivandrum, Kerala, India. The study sites were situated at an altitude of approximately 719 to 725 m above sea level, with coordinates ranging from 8°44’34” to 8°44’39” N latitude and 77°07’38” to 77°07’39” E longitude (Fig. 3). This region forms part of the Southern Western Ghats and experiences a moderately cool climate. The dry season spans from December to April, with March, April and May being the hottest and December and January being the coldest. The selected sites had well-drained, highland soil where pH varied from 4.8 to 5.3 (acidic). The selected plants were well-established, with uniform growth and maintained under standard agricultural practices throughout the study.

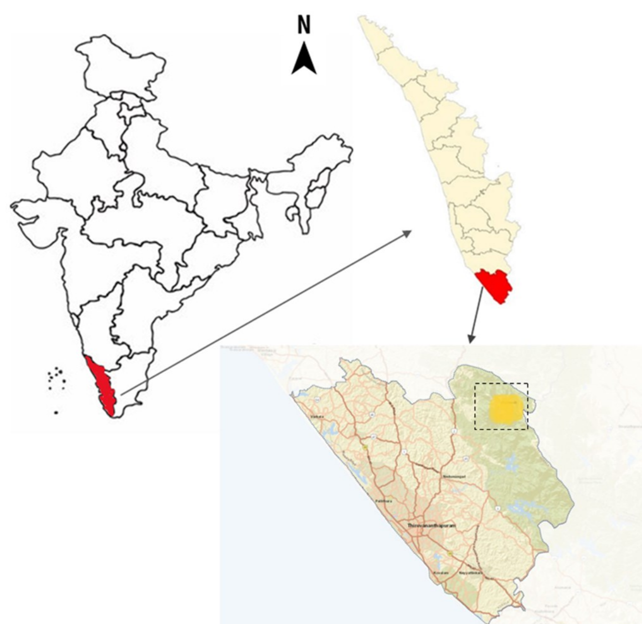

Fig. 3. Study location.

Phenology and floral biology

Anthesis time

A preliminary observation was conducted to determine the appropriate time interval for recording flower openings. Four branches with twenty mature buds were labelled on the five selected trees. A total of 400 flowers were considered. It was observed that the flowers opened between 2:30 pm and 6:30 pm. Consequently, the recording intervals were set hourly from 2:30 to 6:30 pm. The time of anthesis was determined based on the maximum number of flowers opened.

Number of stamens per flower

Four branches with 20 mature buds were labelled on the 5 selected trees and a total of 400 flowers were considered. The number of stamens in each flower was counted and the mean value was calculated to determine the average number per flower.

Anther dehiscence

Based on a preliminary observation, a 12 h interval was fixed 48 h before the flower opening. Anther dehiscence starting time was observed at 48 h, 36 h, 24 h and 12 h before flower opening and 0 h, 24 h, 36 h and 48 h after flower opening. Anther dehiscence was determined by observing the anthers collected from the buds at different durations under the stereomicroscope. The percentage of anther dehiscence was calculated by observing the number of dehisced anthers out of the total number of anthers.

Pollen characteristics

Pollen morphology studies were conducted using anthers collected from buds expected to open within 12 h. These anthers were immediately preserved in 70 % ethanol. Slide preparation for pollen morphology studies was performed using the acetolysis method (18). The preserved anthers were transferred to a centrifuge tube and crushed with a glass rod. The resulting dispersion was sieved through a brass mesh with 48 divisions per cm^2 and collected in a glass centrifuge tube. After centrifuging at 2000 rpm for 5 min, the supernatant was decanted. The pollen grains were washed in glacial acetic acid and treated with an acetolysis mixture of acetic anhydride and concentrated sulphuric acid (9:1) in the centrifuge tube. A glass rod was placed in each tube, then transferred to a water bath at 70 -100 °C for 3 to 5 min until the medium turned brown. The mixture was centrifuged at 2000 rpm for 5 min, the supernatant was decanted and glacial acetic acid was added to the sediment. This was centrifuged once more and the supernatant was decanted. A sediment drop was placed on a glycerine medium in the centre of a slide and covered with a cover slip. The permanent slide prepared was then used to observe pollen shape, size and viable and nonviable pollen using a Scanning Electron Microscope.

Stigma receptivity

Ten flowers each were collected from 5 selected clove trees for 7 days from the day of anthesis. The stigmas of these flowers were immersed in 3 % hydrogen peroxide for 3 min to observe the release of air bubbles, following the method (19). The number of bubbles released from the stigma was counted each day, with the maximum number indicating the peak receptivity of the stigma.

Standardization of hand pollination

Pollen collection

Buds were expected to open in 12, 24 and 36 h and were taken to the laboratory. Petals were removed to expose the stamens and the anthers were crushed to collect pollen. The collected pollen was analyzed for viability and fertility at 12, 24 and 36 hours before anthesis. The optimal time for pollen collection was determined based on the maximum pollen viability and fertility.

Short-term pollen storage

Short-term pollen storage studies were carried out with 7 treatments, each tested in three replications.

using a Completely Randomized Design (CRD). The treatments were as follows:

T_1: Pollen dried at 40 °C for 2 h and kept at room temperature in a desiccator for 2 months

T_2: Pollen dried at 45 °C for 2 h and kept at room temperature in a desiccator for 2 months

T_3: Pollen dried at 50 °C for 2 h and kept at room temperature in a desiccator for 2 months

T_4: Pollen dried at 40 °C for 2 h and stored under refrigeration (4 °C) for 2 months

T_5: Pollen dried at 45 °C for 2 h and stored under refrigeration (4 °C) for 2 months

T_6: Pollen dried at 50 °C for 2 h and stored under refrigeration (4 °C) for 2 months

T_7: Fresh pollen

Pollen viability

Pollen viability was assessed using the Iodine Potassium Iodide (IKI) staining technique. To prepare the IKI solution, 1 g of potassium iodide and 0.5 g of iodine were dissolved in 100 mL of distilled water. The preserved anthers were transferred to a centrifuge tube and crushed with a glass rod. The pollen grains were then placed in the IKI solution for 10 min. Viability was determined by counting the dark brown or red-coloured pollen grains (15).

$$\text{Pollen viability percentage} = \frac{\text{Number of fully stained pollen grains}}{\text{Total number of pollen grains}} \times 100$$

(Eqn. 1)

Pollen fertility

Pollen stainability, a fertility index, was determined using the modified technique of acetocarmine glycerine staining (17). Anthers were placed on a clean slide and one drop of acetocarmine was added. The anthers were macerated to release the pollen grains. Debris was removed and one drop of glycerine was added. The mixture was stirred with a needle and covered with a cover slip. After 10 min, the slides were examined under a stereo microscope. The number of deep red-stained and unstained pollen grains was counted. Deep red-stained pollen grains were considered fertile, whereas unstained, undersized, partially stained and shrivelled were counted as sterile.

$$\text{Pollen fertility percentage} = \frac{\text{Number of fully stained pollen grains}}{\text{Total number of pollen grains}} \times 100$$

(Eqn. 2)

Artificial hand pollination

Artificial hand pollination was attempted on 5 selected clove plants. Twenty buds were chosen from each tree for hybridization. A modified method of artificial hand pollination, based on the standardized emasculation technique, maximum stigma receptivity and pollen storage studies, was employed following the approach outlined in another study (15).

Procedure for artificial pollination:

Selection of female and male parents: Five clove plants were selected and designated P1, P2, P3, P4 and P5. Each plant was used as a female and a male parent, resulting in 20 cross-combinations.

Identification of buds for emasculation: Buds expected to open after 36 h were selected within the inflorescence and designated female parents. The remaining buds were removed from each inflorescence, leaving only 1 or 2 chosen per inflorescence.

Emasculation and bagging: Petals were removed using forceps to expose the stamens on the selected buds. The stamens were carefully removed using a needle, scooped from their base where they attach to the calyx tube and ensured most stamens were removed. Any remaining stamens were extracted using forceps, taking care to remove them from the base to prevent anthers from falling onto the stigma. Emasculated flowers were immediately covered with butter covers and tagged for identification.

Pollination: Optimal pollination time was six days after emasculation, which equates to 5 days after anthesis. Buds expected to open after 12 h were selected and their petals were removed. Pollen was placed on the stigma of emasculated clove flowers using a brush following the removal of the butter coating.

Percentage of fruit set: the success of pollination was assessed by removing the butter covers approximately 25 days after pollination. Fertilized flowers were identified by observing pronounced swelling of the calyx tube, indicating successful pollination.

$$\text{Percentage of fruit set} = \frac{\text{Fertilized flowers}}{\text{Number of flowers pollinated}} \times 100$$

(Eqn. 3)

Statistical analysis

The study results were analyzed by SPSS software Version 16.0 using analysis of variance (ANOVA) and treatment mean differences were determined using the Duncan multiple range test at a significance level of $P < 0.05$.

Results

Floral phenology of clove

The present study is unique in its comprehensive exploration of the reproductive biology of clove, focusing on critical aspects such as anthesis, floral characteristics, pollen traits, stigma receptivity and fertility. The floral phenology of clove was analysed by recording the flower opening times from 20 branches of 5 selected clove plants, totalling 403 flowers. Flower buds of clove at different stages from 1 month after initiation to the stamen fall stage are shown in Fig. 4. Our observations indicate that most clove flowers opened between 3:30 and 5:30 pm. Specifically, the highest percentage of flower openings was recorded between 4:30 and 5:30 pm, accounting for 41.62 % of the total observations. This was followed by the 3:30 to 4:30 pm time frame, encompassing 37.68 % of the flower openings (Table 1). These results suggest that the peak period for flower opening in clove occurs between 3:30 and 5:30 pm. Anthesis duration was recorded from 0 to 24 h. The flowers did not open from 6:30 pm of the current day until 2:30 pm the following day. All observed flower buds opened between 2:30 pm and 6:30 pm. Therefore, the anthesis duration was from 2:30 pm to 6:30 pm.

The average number of stamens per flower is presented in Table 2. Stamens were counted in 10 clove flowers and the mean value was calculated. The stamens ranged from 167 to 343, averaging 243.9 per flower. The rupturing of anthers started 36 h before anthesis and continued till the opening of the flowers. Anther dehiscence peaks at 12 h before the flower opens on the day of anthesis. Therefore, the anther dehiscence occurs 36 h before anthesis to the day of anthesis. The percentage of anthers dehisced in clove is presented in Table 3. Anther dehiscence at different stages is presented in Fig. 5.

The pollen grains were monad, radially symmetrical, triangular, trizonosyncolporate and exhibited exine ornamentation (Fig. 6). The polar diameter of pollen grains ranged from 17.08 to 18.02 μm and the equatorial diameter ranged from 19.04 to 20.7 μm (Table 4).

Stigma receptivity was observed for 7 days from the day of flower opening and is presented in Table 5. The hydrogen peroxide test revealed that stigma receptivity lasted up to six days from anthesis. Bubbles started appearing 2 days after anthesis, with maximum stigma receptivity observed on the fifth day of anthesis, indicated by the highest number of bubbles (30.40 %). A stereomicroscopic image of the stigma through the hydrogen peroxide test is shown in Fig. 7.

Standardisation of hand pollination in clove

Emasculation period

The emasculation period for clove was determined based on the timing of anther dehiscence and stigma receptivity. Anther dehiscence commences 36 h before anthesis, with no pollen remaining in the anthers 2 days post-anthesis. Stigma receptivity begins on the second day after anthesis, peaks on the fifth day and continues until the sixth day. Consequently, emasculation should commence 48 h before the flower bud opens, marked by the initial separation of the petals. Despite anther dehiscence concluding immediately after anthesis, the stamens falling off 2 days later, and stigma receptivity initiating 2 days post-anthesis, emasculation of buds may be unnecessary. However, research in this area remains scant.

Pollen collection

The pollen collected at various intervals before anthesis (12, 24 and 36 h) from clove plants was tested for viability and fertility. Table 6 presents the pollen viability and fertility results, showing that pollen collected 12 h before anthesis had the highest viability and fertility at 94.02 % and 82.83 % respectively, followed by pollen collected 24 h before anthesis at 92.16 % and 80.84 % respectively. Viability decreased as the time to anthesis increased. Since viability and fertility were highest for pollen collected 12 h before anthesis, this time was standardized for pollen collection. The stainability of clove pollen was 81 %, with larger stained pollen grains exhibiting higher germination rates (4). Fig. 8 illustrates pollen collection at different bud stages.

Short-term pollen storage

Short-term pollen storage was standardized by evaluating various storage methods to determine which maintained the highest viability and fertility.

The two-way mean of the percentage of pollen viability of clove by IKI method: The percentage of pollen viability in clove, determined by the IKI method, revealed that T6 (pollen dried at 50 °C and stored in a refrigerator) had the highest viability at 84.92 % (Table 7). This result significantly differed from the other treatments regardless of the storage duration. The other treatments-T1 (desiccator at 40 °C), T2 (desiccator at 45 °C), T3 (desiccator at 50 °C), T7 (fresh pollen stored at room temperature, control), T5 (refrigerator at 45 °C) and T4 (refrigerator at 40 °C)-had viabilities of 82.46 %, 82.01 %, 81.90 %, 81.75 %, 80.32 % and 80.25 % respectively and

Fig. 4. Flower buds of clove at different stages (1 month after initiation of bud to stamens fall stage).

Table 1. Flower opening time in clove.

Time period	Flowers opened on each day (%)								Total
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	
6:30 - 2:30 pm	0	0	0	0	0	0	0	0	0
2:30 - 3:30 pm	3.2	2.48	4.46	0.99	1.48	0.66	0.49	0.24	14
3:30 - 4:30 pm	9.18	8.93	4.96	3.72	4.71	4.21	1.73	0.24	37.68
4:30 - 5:30 pm	7.9	10.9	5.46	3.72	4.96	3.97	3.72	0.99	41.62
5:30 - 6:30 pm	0.49	1.24	1.73	0.74	1.48	0.24	0.49	0	6.41
Total	20.77	23.55	16.61	9.17	12.63	9.08	6.43	1.47	100

Table 2. Mean number of stamens per flower in clove

Sl. No.	Number of stamens/ flower
1	167
2	169
3	243
4	293
5	343
6	287
7	267
8	258
9	198
10	214
Mean	243.9

Table 3. Percentage of anthers dehisced in clove.

Time period	Replication 1	Replication 2	Replication 3	Replication 4	Replication 5	Mean
48 h	0	0	0	0	0	0
36 h	17.96	18.93	16.46	13.31	14.28	16.18
24 h	89.8	75.65	81.3	85.85	92.52	85.02
12 h	95.5	94.59	97.34	94.33	95.76	95.50
0 h	98.29	98.15	98.36	98.26	98.59	98.33

Table 4. Polar and equatorial axis diameter of pollen grains of clove.

Sl. No.	Polar axis diameter (μm)	Equatorial axis diameter (μm)
1	17.24	20.12
2	17.06	19.40
3	18.22	20.70
4	18.15	20.10
5	17.29	20.30
Mean	17.59	20.12

Table 5. Stigma receptivity through hydrogen peroxide test of clove.

Days from anthesis	Bubbles per flower										Bubbles Number (Mean)	%
	1	2	3	4	5	6	7	8	9	10		
1st day	0	0	0	0	0	0	0	0	0	0	0.00	0.00
2nd day	16	15	14	15	16	13	14	16	17	14	15.00	15.20
3rd day	17	15	18	16	14	17	19	15	20	17	16.80	17.12
4th day	23	24	23	26	21	28	24	25	22	25	24.10	24.56
5th day	28	35	29	30	32	28	35	31	29	37	31.40	32.00
6th day	14	8	6	9	13	10	12	12	11	13	10.80	11.00
7th day	0	0	0	0	0	0	0	0	0	0	0.00	0.00

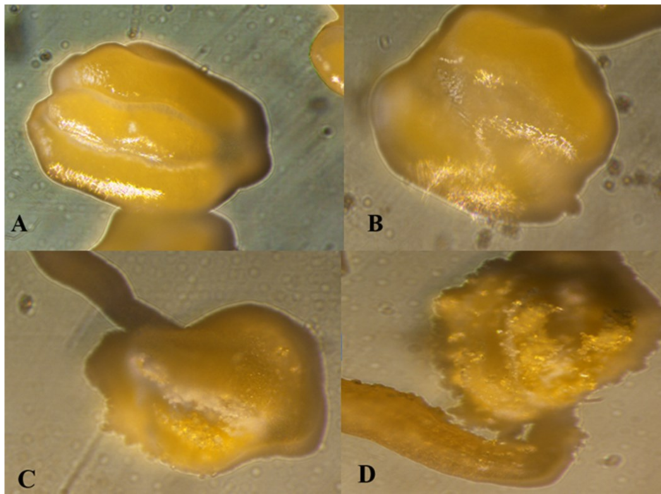

Fig. 5. Anther dehiscence at different stages **A.** anther at 48 h before anthesis **B.** anther at 36 h before anthesis **C.** anther at 24 h before anthesis **D.** anther at 12 h before anthesis.

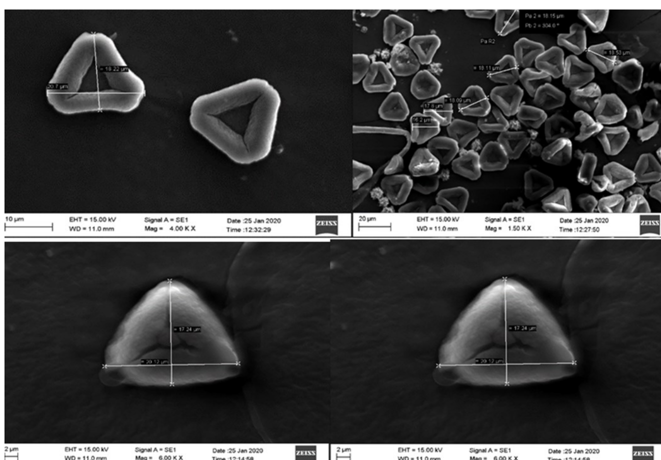

Fig. 6. Equatorial and polar diameter of pollen grains of clove.

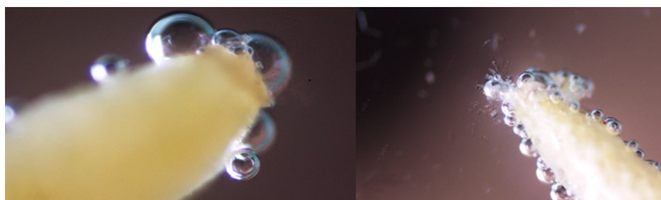

Fig. 7. Stigma receptivity on the fifth day from anthesis.

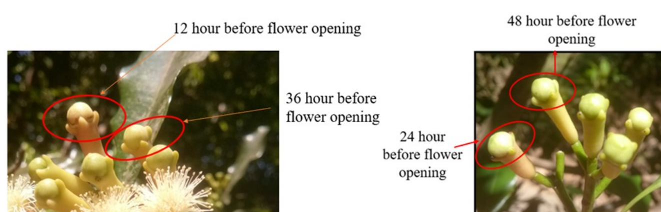

Fig. 8. Pollen collection at different flower bud stages.

were statistically comparable. Among all treatments, the most effective was pollen dried at 50 °C and stored in the refrigerator. At 0 days after storage, the viability was highest at 90.52 %, followed by 84.86 % at 15 days, 78.07 % at 30 days and 74.32 % at 60 days after storage.

Two-way mean table of percentage of pollen fertility in clove by acetocarmine glycerine method: T3 (pollen dried at 50 °C and stored in a desiccator) had the highest pollen fertility at 83.39 %, which was comparable to T5 (refrigerator at 45 °C), T4 (refrigerator at 40 °C), T2 (desiccator at 45 °C) and T6 (refrigerator at 50 °C). The mean pollen fertility for the different treatments was as follows: T1 (desiccator at 40 °C) at 80.59 %, T2 at 81.00 %, T4 at 82.18 %, T5 at 83.29 % and T6 at 80.64 %. T7 (fresh pollen stored at room temperature, control) had the lowest fertility at 77.70 %. Among the treatments, the most effective was pollen dried at 50 °C and stored in a desiccator. Table 8 presents a 2-way mean table of pollen fertility in clove by the acetocarmine glycerine method. Pollen on the collection day had the highest fertility at 89.34 %, followed by 85.24 % at 15 days after storage, 77.59 % at 30 days and 74.52 % at 60 days after storage.

Artificial hand pollination

The data presented in Table 9, which shows the mean percentage of fruit set from various plant combinations during the crossing, highlights significant variability in the reproductive outcomes. The range of fruit set percentages, spanning from 15 % to 42.5 %, suggests a considerable influence of genetic compatibility and environmental factors on the success of these crosses. The average fruit set from all the cross combinations was 28.87 %. Graphical presentation of hand pollination in clove is mentioned in Fig. 9.

Discussions

Floral phenology of clove

The anthesis phase is crucial in the reproductive cycle of plants, marking the period when the flower crown and its reproductive organs fully open and mature (20). This phase, which signifies the maturity of the reproductive organs, can occur before or after the flowers bloom, depending on the species. Our study suggests that the peak period for flower opening in clove occurs between 3:30 and 5:30 pm, contrasting with observations in Indonesia, where clove blossoms typically open at dawn (21). In our study area, the late afternoon may provide optimal conditions for flower opening, differing significantly from those in Indonesia. Previous research

Table 6. Pollen viability by iodine potassium iodide and pollen fertility by acetocarmine method in clove.

Treatments	Pollen viability	Pollen fertility
P_1-12 h before anthesis	94.02[a]	82.83[a]
P_2-24 h before anthesis	92.16[a]	80.42[ab]
P_3-36 h before anthesis	72.47[b]	77.02[b]
CD (0.05)	3.522	4.421

*mean with different letters are significantly different

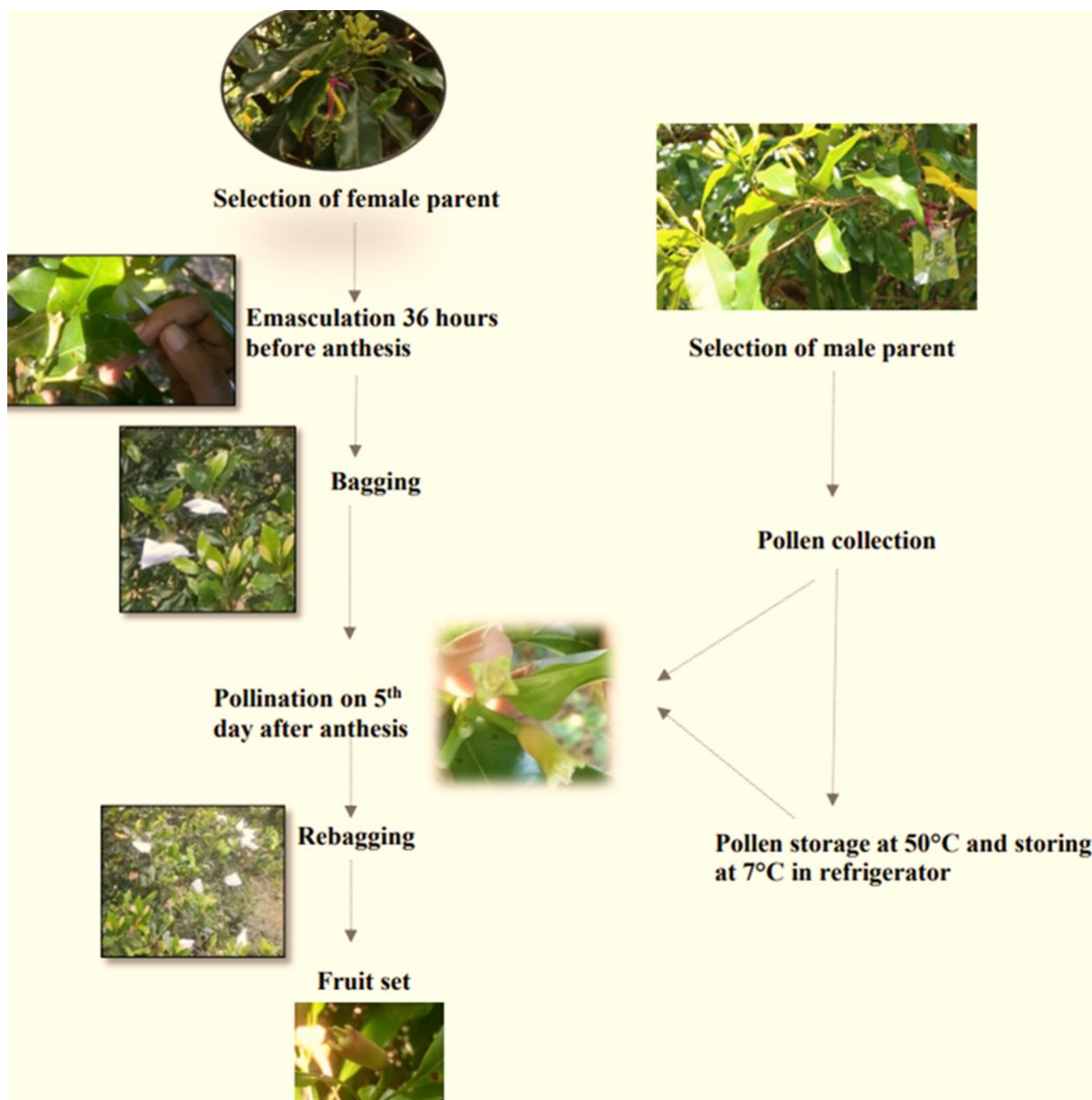

Fig. 9. Procedure of hand pollination in clove.

has shown that anthesis in clove typically occurs in the afternoon around 1:30 pm, peaking between 3:30 pm and 4:30 pm (5). This discrepancy may be attributed to climatic conditions, geographical locations and genetic variations among clove populations (22). Environmental factors such as temperature, humidity and light influence daytime flower openings by affecting the timing and extent of the flowering process (23).

A study on the floral biology of *Syzygium myhendrae*, a wild endemic tree of the Southern Western Ghats in India, observed that anthesis started on the nineteenth day after the emergence of the floral bud and lasted up to 10 days. This prolonged anthesis period might be an evolutionary adaptation to increase reproductive success by extending the timeframe for pollination (24). The present study observed a mean number of stamens of 243.9 stamens per flower. The number of stamens varies widely in the *Syzygium* genus, typically ranging from 40-50 in *Syzygium balsameum* to as many as 1200 in *Syzygium megacarpum* (14). Significant variation in stamen counts among different *Syzygium* species has been reported, with *Syzygium samarangense* having the highest count at 397.50 stamens per flower and *Syzygium jambosa* the lowest at 224 (25). Similarly, *Syzygium aromaticum* shows considerable variability in stamen numbers, ranging from 167 to 343 per flower. Numerous stamens have also been reported in *Syzygium myrtifolium* (26). In contrast, the stamen count in *Syzygium cumini* was recorded at 72.11 per flower (17). The study reveals that anther dehiscence in clove begins 36 h before anthesis and peaks around 12 h before the flower opens on the anthesis day, contrasting with previous studies. For instance, research in Indonesia observed that anther dehiscence in clove flowers occurs within a few hours of flower opening (21).

Table 7. Two-way mean table of percentage of pollen viability of clove by IKI method

Treatments (Factor A)	Days (Factor B)				Treatment Mean A*
	0 DAS	15 DAS	30 DAS	60 DAS	
T_1-Desiccator (40 °C dried pollen)	90.24	83.107	80.833	75.663	82.46^b
T_2 –Desiccator (45 °C dried pollen)	89.107	83.817	78.483	76.64	82.01^b
T_3 –Desiccator (50 °C dried pollen)	90.31	81.833	78.333	77.127	s81.90^b
T_4- Refrigerator (40 °C dried pollen)	90.24	85.623	73.813	71.333	80.25^b
T_5- Refrigerator (45 °C dried pollen)	89.107	83.983	75.373	72.84	80.32^b
T_6 –Refrigerator (50 °C dried pollen)	90.31	87.9	84.737	76.747	84.92^a
T_7-Fresh pollen stored at room temperature (Control)	94.367	87.78	74.973	69.903	81.75^b
Days Mean B	90.52^a	84.86^b	78.07^c	74.32^d	
Treatment Mean A CD (0.05)			1.743		
Days Mean B CD (0.05)			1.317		
Factor (A x B) CD (0.05)			3.485		

*mean with different letters are significantly different

Table 8. Two-way mean table of percentage of pollen fertility in clove by acetocarmine glycerine method.

Treatments (Factor A)	Days (Factor B)				Treatment Mean A
	0 DAS	15 DAS	30 DAS	60 DAS	
T_1-Desiccator (40 °C dried pollen)	89.63	88.31	75.83	68.60	80.59^b
T_2 –Desiccator (45 °C dried pollen)	88.67	79.72	78.51	77.10	81.00^b
T_3 –Desiccator (50 °C dried pollen)	89.28	83.99	82.12	78.19	83.39^a
T_4- Refrigerator (40 °C dried pollen)	89.63	86.85	76.50	75.75	82.18^{ab}
T_5- Refrigerator (45 °C dried pollen)	88.67	85.96	80.26	78.30	83.29^a
T_6 –Refrigerator (50 °C dried pollen)	89.28	81.69	76.73	74.86	80.64^b
T_7-Fresh pollen stored at room temperature (control)	90.18	78.69	73.17	68.82	77.70^c
Days Mean B	89.34^a	85.24^b	77.59^c	74.52^d	
Treatment Mean A CD (0.05)			1.802		
Days Mean B CD (0.05)			1.362		
Factor (A x B) CD (0.05)			1.362		

*mean with different letters are significantly different

Similarly, other studies reported that anther dehiscence in the clove begins 24 h before anthesis, with dehiscence typically peaking immediately afterward (11, 16). Additionally, anther dehiscence has been noted to occur shortly after anthesis in other studies (15). The pollen of Myrtaceae is distinctive, typically syncopate or para syncopate (27). Previous studies have shown significant variation in pollen grain size within the genus *Syzygium*. For instance, *Syzygium megacarpum* was found to have the longest pollen grains, measuring 18.4 µm, nearly twice the size of those in *Syzygium foxworthianum* and *Syzygium jasminifolium*, which had the smallest pollen grains at 7.7 µm (28). In the current study, the pollen grain size ranged from 17.59 to 20.12 µm, closely matching that of *Syzygium megacarpum* and significantly larger than those of *Syzygium foxworthianum* and *Syzygium jasminifolium.* This study found that stigma receptivity lasted up to 6 days post-anthesis, with maximum receptivity observed on the fifth day. It differed from previous studies on clove. It is reported that stigma receptivity in clove was highest on the day of anthesis and remained receptive for an additional 48 h (13). Another study observed that stigma receptivity did not commence until three days after flower opening, peaking on the fifth day and continuing for 2 more days (13). The timing of stigma receptivity is crucial as it directly influences the effective pollination period, which impacts yield (29). The highest stigma receptivity in *Syzygium cumini* occurred one day after anthesis (17).

Table 9. Fruit set percentage of different plant combinations during crossing in clove.

Plant combinations	Mean percentage of fruit set (%)
P_1 X P_2	27.50
P_1 X P_3	25.00
P_1 X P_4	35.00
P_1 X P_5	20.00
P_2 X P_1	40.00
P_2 X P_3	30.00
P_2 X P_4	22.50
P_2 X P_5	15.00
P_3 X P_1	25.00
P_3 X P_2	32.50
P_3 X P_4	42.50
P_3 X P_5	37.50
P_4 X P_1	42.50
P_4 X P_2	30.00
P_4 X P_3	27.50
P_4 X P_5	32.50
P_5 X P_1	20.00
P_5 X P_2	25.00
P_5 X P_3	27.50
P_5 X P_4	20.00
Mean	28.87

Standardisation of hand pollination in clove

Pollen viability and fertility are crucial indicators of pollen quality (30, 31). In this study, pollen viability and fertility were maximum at 12 h before anthesis. Similarly, in *Syzygium leymannii*, peak pollen germination was reported 4 days after anthesis, which coincided with 2 days after stamen emergence and anther dehiscence (32). Pollen viability can decline under low humidity due to rapid moisture loss or conversely, under high humidity due to increased physiological activity (27). However, pollen viability during storage is also affected by moisture levels, storage temperatures as well as physiological and genetic factors (33).

Standard methods for pollen storage involve reducing water content and maintaining low temperatures to minimize fluctuations. In *Syzigium cuminii*, successful pollen storage for up to 9 months was achieved at 23 °C in a dry atmosphere (17). The present study suggests optimizing storage conditions, such as timing, temperature, humidity and storage duration, can improve pollen viability. Maximum pollen viability and fertility are observed 12 h before flower opening, making this the ideal time for pollen collection. Once collected, drying pollen at 50 °C and storing it at 7 °C in a refrigerator preserves maximum viability for up to 60 days. Alternatively, drying pollen at 45 °C and storing it in a desiccator ensures the highest fertility levels.

The range of fruit set percentages, spanning from 15 % to 42.5 %, suggests a considerable influence of genetic compatibility and environmental factors on the success of these crosses (34, 35). The observed average fruit set percentage of 28.87 % provides a valuable benchmark for evaluating the overall reproductive performance of the different plant combinations. Environmental conditions during the crossing period, such as temperature, humidity and soil fertility, could also play a significant role. Additionally, the method and timing of pollination might affect the fruit set outcomes, suggesting that optimizing these variables could improve the success rates of future crossings. Also, cross-pollination success rates depended on the genotype of the pollen donor, irrespective of the genetic distance between the donor and the recipient (36). The highest fruit set percentage observed, 42.5 %, indicates the potential for achieving higher reproductive success with certain combinations. Development of an artificial hand pollination technique for controlled hybridization represents a novel and valuable contribution to clove research, as it has not been widely explored in existing studies. Also, identifying and understanding the characteristics of these successful crosses could provide valuable insights for breeding programs.

This technique is crucial for overcoming the genetic limitations associated with the low variability in clove populations, particularly in regions like India, where clove plants are derived from a limited number of initial introductions. By enabling the controlled crossing of diverse genotypes, this method provides a reliable means to enhance genetic diversity, improve yield and introduce desirable traits such as disease resistance and better adaptation to environmental conditions. Ultimately, this innovation lays the foundation for advancing breeding programs that aim to develop high-performing, high-yielding clove varieties, offering substantial improvements in the quality and productivity of clove cultivation.

Conclusion

The floral biology of clove exhibited notable differences from prior literature, showing regional variations. Optimal pollen viability and fertility were observed when collected 12 h before anthesis. Pollen dried at 45 °C to 50 °C showed promise for extended storage durations. Significant differences were found among various pollen storage methods in terms of both viability and fertility. A modified approach to artificial hand pollination also resulted in fruit set percentages ranging from 15 % to 43 %. These findings provide significant insights into clove floral biology, effective pollen collection and storage techniques and the successful implementation of hand pollination methods to enhance fruit sets in clove cultivation.

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Authors' contributions

RJB carried out the experiments, SGS planned the experiment, RP wrote the manuscript, DSN – Head of the department

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