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Effect of pollen morphology on hybridization and seed setting in hibiscus (*Hibiscus rosa sinensis*)

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ABSTRACT

Pollen studies are crucial for successful hybridization in *Hibiscus rosa sinensis*. At BCKV, West Bengal, an experiment on pollen morphometry and viability involving 10 hibiscus genotypes was conducted in 2021-22. Versicolour Pinwheel showed the highest pollen viability (95.12%), followed by Cherry Glow (92.28%). Three promising pollen parent genotypes, Brilliant, Versicolour Pinwheel, and Cherry Glow, were selected. Hybridization (line × tester) was performed, revealing Cinnamon Girl's had high compatibility with all three pollen donors. Double Peach was compatible with Versicolour Pinwheel and Agni with Cherry Glow, resulting in desirable seed set. Capsule set varied widely (0.0 - 45.67%) due to genotype compatibility, pollen viability, pollen tube growth rates, and climatic conditions. This report aids breeders in selecting suitable pollen donor parents for successful hybridization programmes.

Keywords: Acetocarmine, stigma, capsule, germplasm screening, seed set.

INTRODUCTION

The genus *Hibiscus*, belonging to the family Malvaceae (Debut *et al.*, 5), boasts numerous species, around 105 of which are commonly found in India (Janakiram and Patil, 9). Among these, *Hibiscus rosa sinensis* stands out for year-round flowering and high nutritional value. Packed with organic acids, β-carotene, vitamin C, proteins, and sugars, it holds aesthetic and medicinal significance (Janakiram and Patil, 9).

Hibiscus flowers are typically bisexual and contain calyx, corolla, androecium, and gynoecium. These vibrant flowers vary in size and colour, with single and double varieties. The stigma, where pollen are collected, features five hairy spots on the pistil (Valdoz *et al.*, 19): Pollen travels through the style, connecting the stigma to the ovary. The male part comprises filament-like structures with anthers, each containing 250 to 500 pollen grains (Salamah *et al.*, 15). Some pollen types have spines and mucilaginous substance aiding attachment, while others lack spines (Debut *et al.,* 5). Capsule-shaped fruits with five lobes house the seeds (Valdoz *et al*., 19).

The study of pollen development in hibiscus, which is significantly influenced by plant and floral morphology, provides lucid insights into seed development. This process serves as the initial stage for systematic breeding effort (Debut *et al*., 5). Despite the limited research on pollen morphology in hibiscus, especially in the Indian context, there is a focus on introducing new varieties without a comprehensive understanding of their pedigree. Therefore, a systematic breeding approach is required to improve and unveil new varieties. Improvement, domestication, and documentation efforts are essential to achieve these goals, which are hindered by insufficient information about pollen morphology and breeding techniques. This study aims to fill this gap by investigating pollen morphology and achieving successful crosses between compatible parent varieties of *Hibiscus rosa sinensis*.

MATERIALS AND METHODS

The study was conducted at Bidhan Chandra Krishi Viswavidyalaya, West Bengal (co-ordinates: 22°56′42.88″N 88°32′0.86″E, altitude: 9.75 m), in 2021-2022. It aimed to assess the pollen of ten *Hibiscus rosa sinensis* genotypes (Fig. 1). Pollen screening was done from June to December 2021, and hybridization was carried out from May to August 2022, covering varied climatic conditions (highest temp: 36.3°C in July, lowest: 11.22°C in February). The region received 140.9 cm of annual rainfall.

Pollens were collected from unopened flower buds of the ten hibiscus cultivars (enlisted below), and preserved in Carnoy's solution (4:1 ratio of absolute ethanol and acetic acid). The varieties (Fig. 1) for the study are Brilliant, Versicolour Pin Wheels, Cherry Glow, Double Peach, Cinnamon Girl, Alipore Beauty, Agni, Celia, White, and Shaker Bazaar Yellow. After 24 of fixation, buds were

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Fig. 1. Selected hibiscus genotypes used for the pollen study.

plunged to 70% ethanol and refrigerated (Engin and Gokbayrak, 6). Anthers were counted with a magnifying lens. For pollen production estimation, ten anthers were collected from fresh flowers of each variety pre-anther dehiscence and were stored in vials inside desiccators (Shaheen *et al.*, 17). After anther dehiscence, 1.5 ml of 1.0% Teepol-water mixture was added. Glycerine was added to even pollen dispersion. A small drop of this suspension was added to the counting chambers of a Spencer Bright line Haemocytometer. Pollen characteristics such as shape, area, diameter, and spine length were recorded for all varieties. Pollen grains were observed under a Photo-emission electric microscope (40x) (Labomed®, USA) with safranin stain (Shaheen *et al.*, 17; Arora, 2). Pollen fertility was assessed using the acetocarmine test (Xiong *et al.*, 33) and the stigmatic surface-based pollen germination method (Khanduri *et al.*, 11). Viable grains, displaying normal morphology and well-staining, were counted from ten microscopic fields. For *in-vivo* pollen germination on stigma, emasculated buds were bagged overnight and then pollinated with fresh pollen of the same variety. Styles were fixed, preserved, boiled, stained, and examined using a photoelectric microscope (40x) after gentle squashing.

For hybridization, three pollen parent varieties were selected (Table 1). Balloon-staged buds were collected, anthers detached, and placed on butter paper for dehiscence in partial shade. Pollen from selected donor varieties was stored a day before pollination. Hand emasculation and bagging of mature buds in chosen seed parents were done in the evening before pollination (Stetter *et al.*, 18). Pollination occurred in the next morning by transferring male-parent pollen to emasculated flower stigmas using a brush. Pollinated flowers were bagged and tagged with parent names and pollination dates (Anuragi, 1). After pollination, pollen tube growth on the seed parent's stigma was observed. Flowers were collected after 30 min, and pistils were isolated and preserved in acetic alcohol overnight. Styles were then separated, treated with lactophenol and stained with 1% acid fuchsin. Observations were made using a 40x magnification photoelectric microscope (Labomed®, USA), with pollen tube images captured using Diwinter Calliper Pro software.

Table 1. Details of parental genotypes used in hybridization.

Cross	Seed parent (♀)	Pollen parent (♂)
C1	Agni	Brilliant
C2	Cinnamon Girl	Brilliant
C3	Double Peach	Brilliant
C4	Agni	Versicolor Pinwheel
C5	Cinnamon Girl	Versicolor Pinwheel
C6	Double Peach	Versicolor Pinwheel
C7	Agni	Cherry Glow
C8	Cinnamon Girl	Cherry Glow
C9	Double Peach	Cherry Glow

Various parameters such as capsule development time, cross-specific capsule set, maturation duration, seeds per capsule, seed set percentage, and 10-seed weight were recorded for analysis.

The data collected from this study underwent statistical analysis using Randomized Block Design with three replicates, each consisting of three plants. The statistical analysis of the observed traits was performed utilizing both MS Excel and the OPSTAT software. The study included correlation analyses and multiple linear regression (MLR) analyses to assess the connection between hibiscus varieties and pollen morphological traits.

RESULTS AND DISCUSSION

Pollination is crucial for genetic transmission in regenerative plants. This study aims to link pollen morphology with hybridization compatibility. Pollen grain morphology of *Hibiscus* showed significant diversity in size, diameter, and spine presence (Table 2, Fig. 2). Anther count varied among hibiscus genotypes (Table 2), ranging from 82.67 to 123.67, likely influenced by genetic differences and climate (Bell, 3; Arora, 2). Pollen shape across hibiscus genotypes generally followed a spherical, 34-colporate, and polyforate structure per Erdtmans' classification (Erdtmans, 7). All 10 varieties exhibited spined pollen (Fig. 2), consistent with findings by Debut *et al*. (5).

Pollen grain count per anther varied from 62.5 to 236.00 among the varieties (Table 2, Fig. 3). Cherry Glow had the highest count (236.00), followed closely by Shaker Bazaar Yellow, while Agni showed the lowest count (62.5). Larger pollen size and more anthers per flower may have contributed to lower counts per anther, as observed in previous studies (Salamah *et al*., 15). Regarding pollen area (Fig. 3), Versicolour Pinwheel had the maximum (2.03 mm²), with Alipore Beauty closely behind (1.98 mm²), while Agni exhibited the minimum (1.12 mm²) (Table 2). These findings differ from pollen size data in other studies for *H. rosa sinensis* (Naggar, 12), suggesting study-dependent variation likely due to different *hibiscus* genotypes being analyzed. Table 2 reveals significant pollen diameter variation in the hibiscus genotypes (Fig. 3). Versicolour Pinwheel had the largest diameter (0.51 mm), along with Alipore Beauty (0.51 mm), and White Satin the smallest (0.30 mm). Earlier reports on *H. rosa sinensis* also highlighted diameter differences (Naggar, 12), typically ranging from 124 to 165 µm.

Versicolour Pinwheel had the most pollen spines (61.50), followed by White Satin (57.33), while Celia had the fewest (5.0). The longest spines were in Versicolour Pinwheel (0.06 mm), similar with Brilliant (0.06 mm), with Celia and Agni having the shortest (0.02 mm each). Taxonomically significant variations in Malvaceae pollen grain spines have been noted (Naggar, 12).

Table 2 indicates that the Versicolour Pinwheel had the highest Cherry Glow (92.28%), which was significantly higher than the others. Conversely, Agni displayed the lowest viability (62.85%). This aligns with findings by Khanduri *et al.* (11). Table 2 and Fig. 3 illustrate pollen growth on stigmatic surfaces among selected varieties. Versicolour Pin Wheel had the highest growth (9.24 mm), followed

Table 2. Pollen morphology of 10 *Hibiscus rosa sinensis* genotypes.

Genotypes	Anther flower	Pollens/ anther	Pollen shape	Pollen area (mm²)	Pollen dia. (mm)	Spine/ pollen	Spine length (mm)	Pollen growth on stigma (mm)	Pollen viability (%)
Brilliant	84.67	114.00	Spherical	1.43	0.42	54.00	0.06	6.34	85.01(67.21)
Versicolour Pinwheel	108.67	175.00	Spherical	2.03	0.51	61.50	0.06	9.24	95.12(77.21)
Cherry Glow	104.33	236.00	Spherical	1.56	0.41	50.67	0.03	9.04	92.28(73.76)
Double Peach	109.33	174.00	Spherical	1.76	0.49	14.33	0.04	8.69	76.19(60.73)
Cinnamon Girl	113.33	200.00	Spherical	1.41	0.43	52.00	0.05	6.73	71.22(57.54)
Alipore Beauty	113.67	167.00	Spherical	1.98	0.51	45.67	0.06	6.62	78.28(62.17)
Agni	82.67	62.50	Spherical	1.12	0.37	26.00	0.02	5.17	62.85(52.83)
Celia	123.67	134.00	Spherical	1.65	0.42	5.00	0.02	5.09	81.17(64.23)
White Satin	84.67	145.00	Spherical	1.47	0.30	57.33	0.05	5.47	81.42(64.45+)
Shaker Bazaar Yellow	101.33	211.00	Spherical	1.39	0.46	51.00	0.04	7.72	90.63(72.15-)
$CD_{0.05}$	11.531	44.795		0.436	0.072	10.821	0.014	1.518	9.139
SE m±	3.881	15.077		0.147	0.024	3.642	0.005	0.511	3.076

Fig. 2. Pollen micro-morphology in 10 varieties of hibiscus

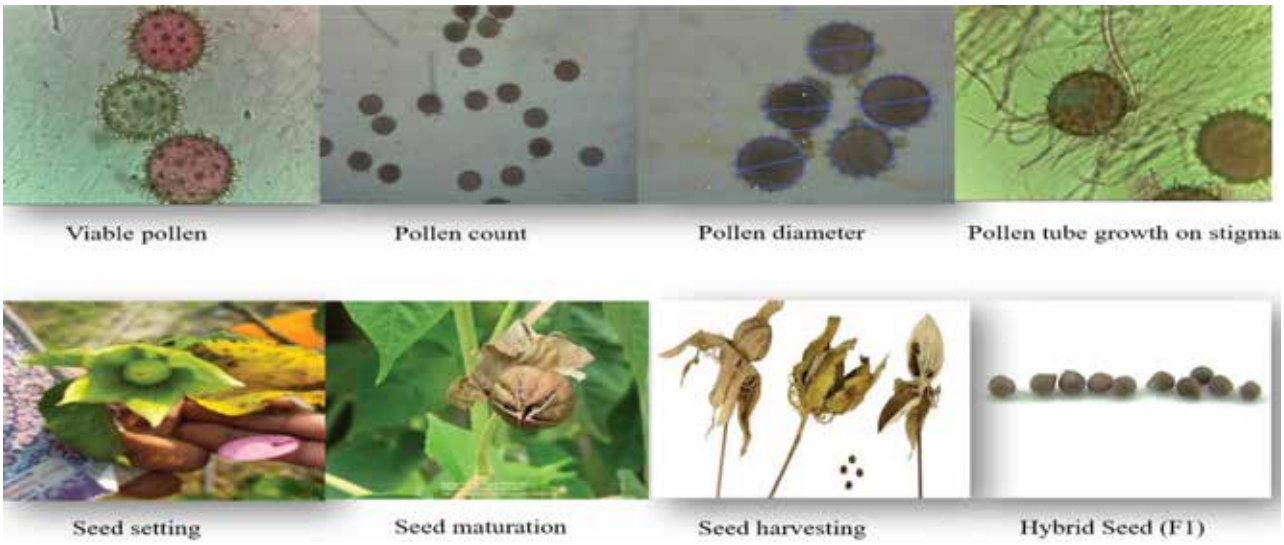

Fig. 3. Observations during hybridization and post-hybridization in *Hibiscus*.

by Double Peach (9.05 mm), while White Satin had the lowest (5.09 mm). Differences in pollen germination and tube growth on stigma among hibiscus varieties have been discussed by Anuragi (1). Patil *et al.* (13) observed high pollen germination rates (80–90%) in *Abelmoschus* spp. shortly after anther opening, suggesting barriers rather than pollen vigour may cause germination failure. As in the methodology, a correlation study assessed variable relationships via a correlation matrix (Fig. 4). The heatmap simplifies these correlations visually, aiding in identifying key variables and avoiding duplication. The dendrogram highlights three clusters, with Cherry Glow, Versicolour Pinwheel, and Brilliant in separate clusters. Positive correlations are denoted as red, and negative correlations are denoted as blue. Versicolour Pinwheel shows strong positive correlations with various pollen traits, as does Brilliant. Shaker Bazaar Yellow, conversely, exhibits negative correlations with pollen traits. Similar analyses were performed by Kabre *et al.* (10) for hibiscus diversity analysis using heatmaps.

Hybridization blends traits from different germplasms to create desired offspring, which may become novel varieties or foundational lines for future breeding. In hibiscus, flowers are known

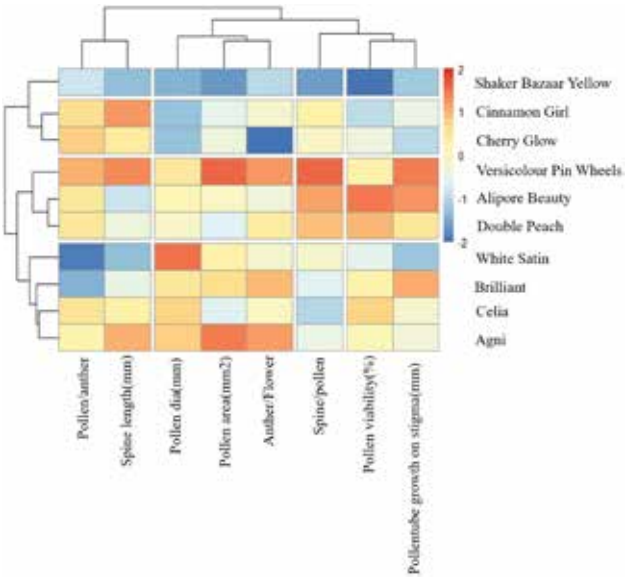

Fig. 4. Heatmap depicting correlations between expression patterns of ten selected hibiscus genotypes and pollen morphology.

for their hypoglycemic (Janakiram and Patil, 9) and hypotensive effects (Bell, 3). *Hibiscus rosa-sinensis* exhibits rich vegetative and floral traits, enhancing genetic diversity through self-crossing and crossbreeding with other varieties (Kabre *et al*., 10). Pollen tube growth on the stigmatic surface of seed parents in one-line crosses showed significant variation (Table 3, Fig. 3). Notably, Cherry Glow pollen on Cinnamon Girl (C8) led to rapid tube growth (6.78 mm), while the slowest growth was in Double Peach with Cherry Glow and Brilliant pollens (0.68 mm and 0.67 mm, respectively) in C9 and C3. In *Hibiscus*, Chachalis *et al.* (4) linked tube growth variation to flower morphology in *Hibiscus trionum*, while Arora (2) attributed differences to pollen competitiveness in varied environments. Patil *et al*. (13) found normal pollen tube growth in both directions of the *Abelmoschus esculentus* × *A. caillei* cross, implying genetic enhancement potential.

In the crosses, C5 (Versicolour Pinwheel × Cinnamon Girl) had the highest capsule formation at 46.2% (Table 3, Fig. 3). However, some crosses like Agni × Brilliant (C1), Double Peach × Brilliant (C3), Agni × Versicolour Pinwheel (C4), and Double Peach × Cherry Glow (C9) didn't form capsules (Table 4). Lack of seed development might be due to issues like endosperm degeneration (Shaheen *et al*., 17) or early style and stigma abscission (Fakir *et al*., 8).

Capsule formation varied from 4.67 to 6.67 days after pollination. Certain crosses like Agni × Brilliant (C1), Double Peach × Brilliant (C3), Agni × Versicolour Pinwheel (C4), and Double Peach × Cherry Glow (C9) didn't result in capsule formation. The longest time was in Cinnamon Girl × Cherry Glow (C8) at 6.67 days, followed by Double Peach × Versicolour Pinwheel (C6) at 6.33 days. Capsule maturation duration aligned with findings by Fakir *et al.* (8). C7 (Agni × Cherry Glow) displayed the highest 10-seed weight at 2.25 mg. At the same time, some crosses produced no seeds Agni × Brilliant (C1), Double Peach × Brilliant (C3), Agni × Versicolour Pinwheel (C4), and Double Peach × Cherry Glow (C9). Physiological maturity, marked by maximum dry weight in seeds, is highlighted by Raifa *et al.* (14) in hibiscus.

This study offers insights into pollen viability, aiding hibiscus breeding. Versicolour Pinwheel, Brilliant, and Cherry Glow were found to be potential pollen donors (male parent candidates). The study

Table 3. Observations recorded after hybridization in *Hibiscus* genotypes.

Crosses (♀ × ♂)	Pollen tube growth on stigma (mm)	Capsule set (%)	Days to capsule set	Wt. of 10-seeds (mg)
C1 (Agni × Brilliant)	1.67	0.00 (0)	0.00	0.00
C2 (Cinnamon Girl × Brilliant)	4.00	38.67 (38.35+)	4.67	1.33
C3 (Double Peach × Brilliant)	0.67	0.00 (0)	0.00	0.00
C4 (Agni × Versicolour Pinwheel)	2.00	0.00 (0)	0.00	0.00
C5 (Cinnamon Girl × Versicolor Pinwheel)	5.33	46.20 (42.82)	5.33	2.07
C6 (Double Peach × Versicolour Pinwheel)	4.83	34.33 (35.85)	6.33	1.00
C7 (Agni × Cherry Glow)	3.50	29.33 (32.77)	5.33	2.25
C8 (Cinnamon Girl × Cherry Glow)	6.78	36.67 (37.23)	6.67	1.68
C9 (Double Peach × Cherry Glow)	0.67	0.00 (0)	0.00	0.00
$CD_{0.05}$	1.34	3.405	1.562	0.613
S.Em ±	0.447	1.136	0.521	0.204

guides breeders and cultivators toward successful hibiscus choices, contributing to breeding strategies and future efforts in the field.

AUTHORS' CONTRIBUTION

Conceptualization (JM), Methodology (PKTN, JM), Investigation (PKTN), Data curation and Formal analysis (PKTN, JM), Writing original draft (PKTN), Resources, Software, Validation (TK and KA), Writing, review and editing (PKTN, TK and KA).

DECLARATION

The authors declare that they do not have any conflict of interest.

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