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ARTICLE

Optimization of Culture Medium and Conditions for *In Vitro* Pollen Germination in *Hibiscus mutabilis* Linn.

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ABSTRACT: Pollen viability plays a pivotal role in determining fertilization success, directly influencing fruit and seed production, as well as the genetic improvement and conservation of flowering plants. This study sought to establish the optimal culture medium and environmental conditions for *in vitro* pollen germination and pollen tube growth in *Hibiscus mutabilis* Linn., a species of notable ornamental and ecological significance. We employed Brewbaker and Kwack medium(BK) supplemented with varying concentrations of sucrose, H_3BO_3, $CaCl_2$, $MgSO_4{\cdot}7H_2O$, and KNO_2 to systematically evaluate the effects of these components on pollen viability. Additionally, we investigated the impact of environmental factors including temperature, light intensity, humidity, and pH. The optimal culture medium consisted of 300 g/L sucrose, 50 mg/L H_3BO_3, and 150 mg/L $CaCl_2$, yielding maximum germination rates (69.3% for red pollen, 65.7% for white pollen) and tube lengths (467.5 μm and 428.6 μm, respectively). Temperature was identified as a crucial factor, with 20°C being optimal. High humidity (100%) and slightly acidic pH (6.1) further enhanced pollen viability. Pollen germination was most successful under dark conditions, while moderate light intensity (6000–12,000 lux) facilitated optimal pollen tube elongation. This study provides a systematic investigation of pollen germination in *H. mutabilis*, offering critical insights for hybrid breeding programs and conservation efforts.

KEYWORDS: *Hibiscus mutabilis* Linn.; pollen viability; pollen germination; optimal culture medium; environmental factors

1 Introduction

Pollen viability is a crucial factor influencing the success of fruit and seed production and plays a significant role in the genetic improvement and conservation of plant species [1,2]. The morphological characteristics of pollen are key to determining the phylogenetic relationships among plant species, making them an important tool in plant taxonomy, plant protection, and genetic improvement experiments aimed at developing high-value hybrid varieties [3]. *In vitro* pollen germination is the most reliable and widely used method for assessing viability, as it closely reflects *in vivo* conditions compared to staining methods [4,5]. Successful *in vitro* germination depends on multiple factors, including the plant's nutritional status, timing and method of pollen collection, photoperiod, temperature, incubation period, and crucially, the composition of the culture medium [6]. Therefore, species-specific optimization of culture media is essential [7,8]. The composition of the culture medium plays a pivotal role in pollen germination and tube growth. Sucrose serves a dual function: it provides an energy source for tube elongation and maintains osmotic balance to

prevent pollen bursting or plasmolysis [9–11]. Boric acid (H_3BO_3) is essential for cell wall stability and pectin cross-linking, while calcium ions (Ca^{2+}) regulate tip-focused ion gradients and actin dynamics necessary for polarized tube growth [12,13]. In contrast, high concentrations of potassium (K^+) and magnesium (Mg^{2+}) can interfere with Ca^{2+}-dependent signaling and inhibit germination in some species [8,14]. Beyond medium composition, environmental factors such as temperature, light intensity, relative humidity, and pH also profoundly affect pollen viability. Temperature influences membrane fluidity and enzyme activity; extreme heat induces oxidative stress and protein denaturation, while low temperatures slow metabolic rates [15,16]. Light can have dual effects: darkness often promotes germination by reducing oxidative stress, whereas moderate light may enhance tube elongation via photosynthetic pigments [17,18]. High humidity is critical for pollen hydration and metabolic activation, while low humidity causes desiccation and irreversible loss of viability [19,20]. Similarly, pH affects cell wall expansibility and ion transport, with most species exhibiting a narrow optimal pH range [5,21].

Hibiscus mutabilis Linn., commonly referred to as the 'Confederate Rose', is a species native to Southeast China and has been domesticated for ornamental, medicinal, and culinary purposes for centuries [22,23]. The species is particularly prized for its large, showy flowers that undergo a dramatic color transition from white to deep red within a single day. It has been widely cultivated and naturalized across various regions globally [24]. Despite its cultural significance and horticultural potential, the reproductive biology of *H. mutabilis* remains poorly understood, especially the specific requirements for pollen germination and tube growth [25]. This knowledge gap hinders hybrid breeding programs aimed at developing new cultivars with enhanced floral traits, stress tolerance, and extended flowering periods. Within the Malvaceae family, pollen germination studies have been conducted on several Hibiscus species, but results are highly variable. For instance, *Hibiscus rosa-sinensis* (Chinese hibiscus) showed optimal *in vitro* germination at 150 g/L sucrose with 100 mg/L H_3BO_3 and 100 mg/L $CaCl_2$ [26]. In contrast, *Hibiscus syriacus* (rose of Sharon) required only 100 g/L sucrose but higher calcium (200 mg/L $CaCl_2$) for maximum pollen tube growth [6]. *Hibiscus cannabinus* (kenaf) exhibited sensitivity to herbicide stress but its basal germination requirements were not systematically optimized [27]. More recent studies on other Malvaceae members have revealed family-specific trends. For example, *Gossypium hirsutum* (upland cotton) requires 250–350 g/L sucrose for optimal germination, a concentration far higher than typical angiosperms, suggesting a conserved adaptation to high osmotic pressure [14]. Similarly, *Abelmoschus esculentus* (okra) shows optimal germination at 20–25°C with high humidity requirements [28]. These findings indicate that Malvaceae species may share certain physiological traits, but each species still requires individual optimization.

Within the Malvaceae family, pollen germination studies have been conducted on several *Hibiscus* species, including *H. rosa-sinensis* [26], *H. syriacus* [6], and *H. cannabinus* [29]. However, these studies have reported variable optimal conditions. For example, H. rosa-sinensis exhibited optimal germination at 150 g/L sucrose with 100 mg/L H_3BO_3 [26], while H. syriacus required 100 g/L sucrose and 200 mg/L $CaCl_2$ for maximum pollen tube growth [6]. To date, no published study has systematically investigated the pollen germination requirements of *H. mutabilis*, a species of significant ornamental and ecological value. This knowledge gap hinders hybrid breeding programs and conservation efforts for this species. The present study addresses this gap by systematically evaluating the effects of culture medium components (sucrose, H_3BO_3, $CaCl_2$, $MgSO_4{\cdot}7H_2O$, KNO_2) and environmental factors (temperature, light intensity, humidity, pH) on *in vitro* pollen germination and tube growth in two color varieties of *H. mutabilis*.

2 Materials and Methods

2.1 Plant Material and Overview of the Research Area

The experiments were carried out at the Chengdu Botanical Garden, Sichuan Province (30°76′ N, 104°13′ E). Two varieties of *Hibiscus mutabilis* Linn. were selected: single-petal red (Fig. 1a) and single-petal white (Fig. 1b). These varieties were transplanted from the wild and typically flowered between June and November. Flowering individuals reached heights of 2–3 m and produced an average of 74.99 ± 2.52 red flowers and 100.35 ± 3.28 white flowers per plant.

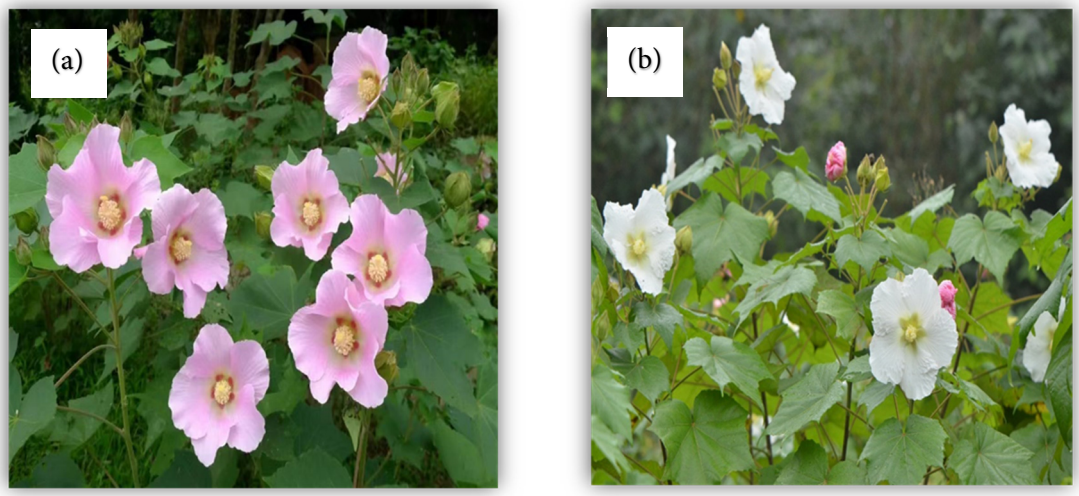

Figure 1: Blooming situation of single petal red (**a**) and single petal white turning red (**b**).

2.2 Optimization of Culture Medium for in Vitro Pollen Germination

All experiments were performed with three biological replicates per treatment. For each replicate, three microscopic fields were examined, with a minimum of 300 pollen grains counted per field. The experiment utilized Brewbaker and Kwack's medium for *in vitro* pollen germination and pollen tube growth. To identify the optimal composition for *H. mutabilis*, an orthogonal design was employed. The study assessed the effects of various concentrations of mineral salts, including seven different sucrose concentrations (0 g/L, 50 g/L, 100 g/L, 150 g/L, 200 g/L, 250 g/L, 300 g/L, and 350 g/L), calcium chloride ($CaCl_2$) concentrations (0 mg/L, 50 mg/L, 100 mg/L, 150 mg/L, 200 mg/L, 250 mg/L, 300 mg/L, and 350 mg/L), and boric acid (H_3BO_3) concentrations (0 mg/L, 50 mg/L, 100 mg/L, and 150 mg/L). Fresh pollen grains were collected from dehisced anthers of flowers that had opened between 9:00 and 10:00. Pollen was carefully mixed using tweezers, and a sample was obtained with a fine hair (213 ± 4 microns). This pollen sample was placed into a 0.6 mm groove of a concavity slide (25 mm height × 76 mm diameter), which was then mixed with the prepared culture medium. The slides, along with the pollen samples, were placed in Petri dishes (15 mm height × 90 mm diameter) containing moist filter paper to maintain a humidity level of >95% and incubated at 25°C for various durations. After three hours of incubation, pollen germination and pollen tube length were assessed.

Further optimization was performed to identify other ions affecting pollen germination, with potassium nitrite (KNO_2) concentrations (0 mg/L, 10 mg/L, 20 mg/L, 50 mg/L, 100 mg/L) and magnesium sulfate ($MgSO_4{\cdot}7H_2O$) concentrations (0 mg/L, 10 mg/L, 20 mg/L, 50 mg/L, 100 mg/L) being tested based on the optimized culture medium. The concentration of each substance was varied while keeping others constant.

2.3 Optimization of in Vitro Pollen Germination Conditions

To determine the optimal environmental conditions for pollen germination, freshly collected pollen was used. Based on the previously optimized culture medium, the study investigated the effects of different temperature (10°C, 15°C, 20°C, 25°C, 30°C), humidity (40%, 50%, 60%, 70%, 80%, 90%, and 100%), light intensity (0 Lux, 3000 Lux, 6000 Lux, 9000 Lux, 12,000 Lux), and pH levels (4.5, 5, 5.5, 6.1, 6.5, 7, 7.5, 8.0). Temperature, humidity, and light were controlled using Boxun and LVBO artificial climate incubators (BSG-250, LRG-500Y), while pH was adjusted using a pH meter (TRANS WIGGENS pH610).

2.4 Pollen Germination and Tube Growth

Pollen germination was quantified by counting the number of germinated grains, with pollen germination defined as the growth of the pollen tube exceeding the diameter of the pollen grain. Pollen tube length (μm) was measured using a Leica Axio Imager A2 (Germany) and analyzed with ZEN 3.3 software (blue edition). All data were presented as the mean of three replicates, with the standard deviation calculated.

$$\text{Pollen germination (\%)} = \frac{\text{No. of germinated pollen grains per field}}{\text{Tota no. of pollen observed per field}} \times 100$$

2.5 Data Analysis

Prior to ANOVA, data were tested for normality using Shapiro-Wilk test and homogeneity of variances using Levene's test. One-way ANOVA was performed followed by Duncan's multiple range test for post-hoc comparisons. Significance was set at $p < 0.05$. All statistical analyses were conducted using SPSS 20.0, and graphs were generated using Origin 2018 (64-bit).

3 Results

3.1 Optimization of the Culture Medium

Pollen germination of *H. mutabilis* required the simultaneous presence of sucrose, $CaCl_2$, and H_3BO_3. Complete absence of any of these three components resulted in no germination: no germination occurred when sucrose was absent (Table 1, Nos. 2–8), when $CaCl_2$ was absent (Nos. 11, 21, 31, 38, 48, 50, 60), or when H_3BO_3 was absent (Nos. 9, 17, 25, 33, 41, 49, 57). In addition, very low concentrations of these components also failed to support germination (e.g., Nos. 10, 12–14, 16, 18–19, 22–24, 26, 28–30, 32, 36–37, 39–40, 42–43, 45–47, 51–53, 55–56, 58–59, 61–64, all showing 0% or very low germination rates below 5%). One-way ANOVA revealed significant differences in germination rates and tube lengths across the 64 treatment combinations for both red pollen (germination: $F_{63,128} = 34.67$, $p < 0.001$; tube length: $F_{63,128} = 28.43$, $p < 0.001$) and white pollen (germination: $F_{63,128} = 31.22$, $p < 0.001$; tube length: $F_{63,128} = 26.15$, $p < 0.001$). The optimal culture medium was 300 g/L sucrose, 150 mg/L $CaCl_2$, and 50 mg/L H_3BO_3 (Treatment No. 54), yielding maximum germination rates (red: 69.32 ± 2.48%; white: 65.73 ± 2.22%) and tube lengths (red: 467.50 ± 16.53 μm, Fig. 2a; white: 428.63 ± 19.37 μm, Fig. 2c). Microscopic observation also revealed a distinct morphological difference between the two varieties: red pollen grains exhibited multiple germination pores (Fig. 2b), while white pollen grains possessed fewer pores (Fig. 2d). This structural difference may contribute to the higher germination rate observed in red pollen, although both varieties achieved high viability under optimal conditions.

Table 1: Optimization of the culture medium of sample *H. mutabilis*. Each value represents mean ± standard error (ANOVA, $p < 0.05$).

No	Sucrose (g/L)	H_3BO_3 (mg/L)	$CaCl_2$ (mg/L)	Red Germination (%)	Tube Length (μm)	White Germination (%)	Tube Length (μm)
1	0	0	0	0.00	0.00	0.00	0.00
2	0	10	40	0.00	0.00	0.00	0.00
3	0	20	50	0.00	0.00	0.00	0.00
4	0	30	10	0.00	0.00	0.00	0.00
5	0	40	150	0.00	0.00	0.00	0.00
6	0	50	30	0.00	0.00	0.00	0.00
7	0	100	20	0.00	0.00	0.00	0.00
8	0	150	100	0.00	0.00	0.00	0.00
9	50	0	100	0.00	0.00	0.00	0.00
10	50	10	10	0.00	0.00	0.00	0.00
11	50	20	0	0.00	0.00	0.00	0.00
12	50	30	40	0.00	0.00	0.00	0.00
13	50	40	20	0.00	0.00	0.00	0.00
14	50	50	100	0.00	0.00	0.00	0.00
15	50	100	150	5.18 ± 1.78	180.50 ± 1.31	3.59 ± 1.55	195.71 ± 5.39
16	50	150	30	0.00	0.00	0.00	0.00
17	100	0	150	0.00	0.00	0.00	0.00
18	100	10	30	0.00	0.00	0.00	0.00
19	100	20	20	0.00	0.00	0.00	0.00
20	100	30	100	18.98 ± 1.97	211.13 ± 6.36	16.15 ± 2.87	218.08 ± 7.09
21	100	40	0	0.00	0.00	0.00	0.00
22	100	50	40	22.58 ± 1.21	217.00 ± 5.86	3.30 ± 1.01	190.93 ± 3.74
23	100	100	50	0.00	0.00	0.00	0.00
24	100	150	10	0.00	0.00	0.00	0.00
25	150	0	20	0.00	0.00	0.00	0.00
26	150	10	100	27.48 ± 1.46	232.33 ± 9.08	18.38 ± 3.10	206.56 ± 7.32
27	150	20	150	38.11 ± 3.00	264.92 ± 7.56	34.52 ± 3.41	234.50 ± 10.72
28	150	30	30	25.13 ± 3.02	223.16 ± 7.39	28.97 ± 4.69	240.93 ± 13.64
29	150	40	50	23.13 ± 3.91	222.97 ± 8.02	17.61 ± 2.78	233.37 ± 9.21
30	150	50	10	16.53 ± 1.37	197.37 ± 3.37	12.93 ± 3.54	218.67 ± 5.63
31	150	100	0	0.00	0.00	0.00	0.00
32	150	150	40	0.00	0.00	0.00	0.00
33	200	0	30	0.00	0.00	0.00	0.00
34	200	10	150	38.59 ± 2.88	312.69 ± 19.03	31.38 ± 2.81	314.08 ± 12.14
35	200	20	100	31.22 ± 2.63	289.34 ± 14.01	39.78 ± 3.04	282.05 ± 9.98
36	200	30	20	31.34 ± 2.75	248.97 ± 9.77	29.82 ± 1.60	235.78 ± 9.24
37	200	40	40	20.53 ± 2.34	217.85 ± 3.37	34.20 ± 2.28	217.26 ± 9.34
38	200	50	0	0.00	0.00	0.00	0.00
39	200	100	10	20.21 ± 2.77	218.02 ± 6.07	27.11 ± 2.02	261.36 ± 11.46
40	200	150	50	16.98 ± 3.16	225.30 ± 7.17	21.16 ± 1.70	240.96 ± 9.30
41	250	0	100	0.00	0.00	0.00	0.00
42	250	10	20	25.09 ± 2.33	231.85 ± 16.12	22.44 ± 2.31	253.70 ± 10.51
43	250	20	30	32.10 ± 4.31	247.57 ± 7.48	27.00 ± 2.53	270.27 ± 9.12
44	250	30	150	32.41 ± 2.29	322.71 ± 20.00	44.21 ± 2.19	360.70 ± 13.88
45	250	40	10	23.39 ± 3.74	230.33 ± 7.23	27.27 ± 2.26	322.49 ± 18.85
46	250	50	50	22.24 ± 1.42	309.59 ± 18.26	28.76 ± 2.17	337.39 ± 16.43
47	250	100	40	17.05 ± 2.58	240.64 ± 9.69	18.97 ± 1.42	329.44 ± 14.05
48	250	150	0	0.00	0.00	0.00	0.00
49	300	0	40	0.00	0.00	0.00	0.00
50	300	10	0	0.00	0.00	0.00	0.00
51	300	20	10	26.67 ± 2.59	273.25 ± 10.02	27.57 ± 1.36	288.96 ± 14.91
52	300	30	50	32.30 ± 2.18	308.78 ± 12.41	36.08 ± 2.50	340.92 ± 15.97

Table 1: *Cont.*

No	Sucrose (g/L)	H_3BO_3 (mg/L)	$CaCl_2$ (mg/L)	Red Germination (%)	Tube Length (μm)	White Germination (%)	Tube Length (μm)
53	300	40	30	21.90 ± 3.15	285.17 ± 10.82	38.17 ± 3.25	349.49 ± 20.25
54	300	50	150	69.32 ± 2.48	467.50 ± 16.53	65.73 ± 2.22	428.63 ± 19.37
55	300	100	100	23.59 ± 2.63	295.75 ± 16.67	36.43 ± 3.21	290.86 ± 19.02
56	300	150	20	17.70 ± 2.62	268.71 ± 10.07	21.19 ± 1.24	253.89 ± 9.93
57	350	0	10	0.00	0.00	0.00	0.00
58	350	10	50	35.78 ± 1.80	290.79 ± 15.16	36.76 ± 2.19	325.35 ± 15.56
59	350	20	40	39.94 ± 2.81	288.70 ± 16.25	42.99 ± 2.59	273.17 ± 15.80
60	350	30	0	0.00	0.00	0.00	0.00
61	350	40	100	42.65 ± 2.70	325.60 ± 9.95	45.93 ± 2.06	363.84 ± 17.19
62	350	50	20	33.18 ± 2.43	291.00 ± 11.61	34.95 ± 2.52	255.16 ± 11.39
63	350	100	30	26.33 ± 1.68	249.01 ± 9.49	33.82 ± 2.32	304.56 ± 13.21
64	350	150	150	28.59 ± 1.52	253.26 ± 11.33	34.20 ± 1.92	284.84 ± 17.90
p	-	-	-	0.00	0.00	0.00	0.00

Note: Each value represents mean ± standard error (ANOVA, $p < 0.05$).

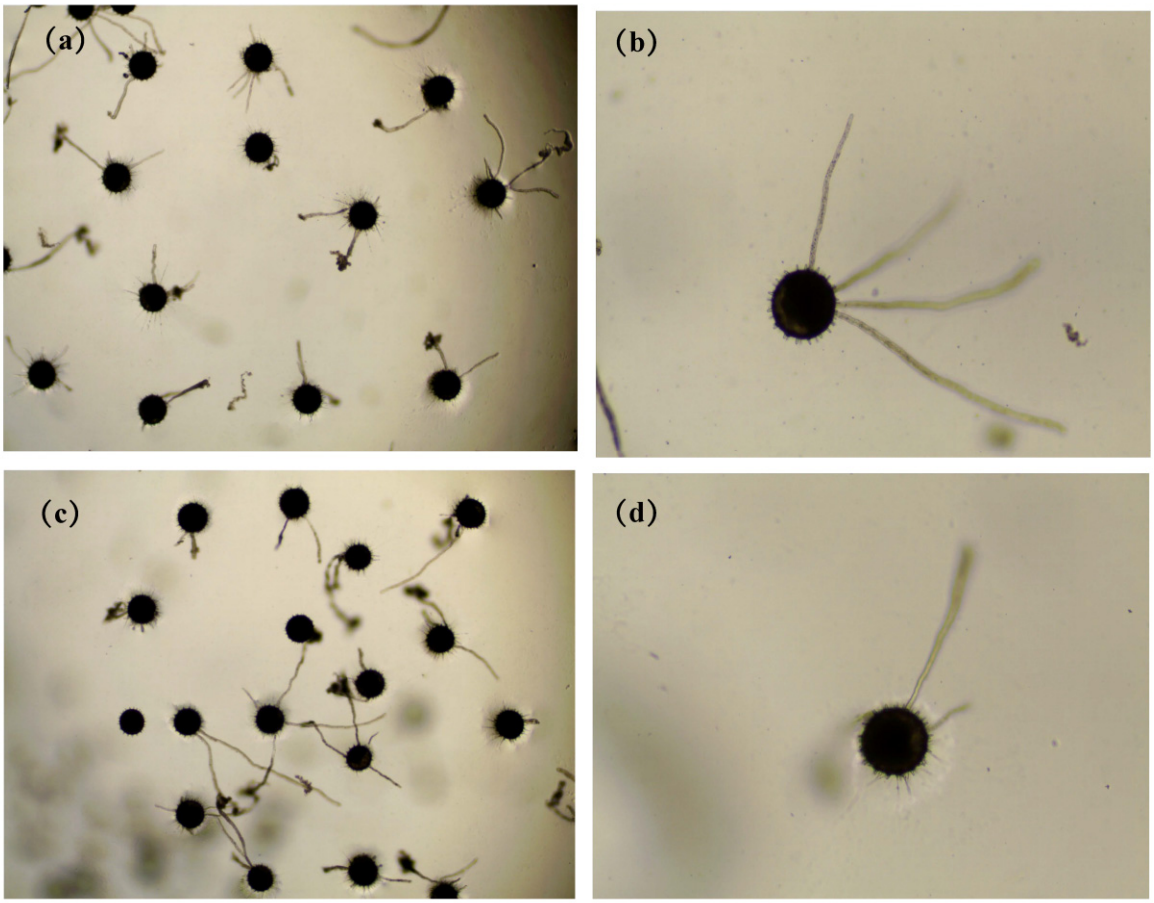

Figure 2: Pollen germination and pollen tube growth of single petal red (**a**,**b**) and single petal white (**c**,**d**). (**a**) Red pollen tube elongation; (**b**) Red pollen grains showing multiple germination pores (arrows); (**c**) White pollen tube elongation; (**d**) White pollen grains showing fewer germination pores (arrows). Scale bars = 50 μm.

Based on the optimization of the culture medium for *H. mutabilis* (comprising 300 g/L sucrose, 150 mg/L $CaCl_2$, and 50 mg/L H_3BO_3), we investigated the effects of varying sucrose concentrations (0 g/L, 50 g/L, 100 g/L, 150 g/L, 200 g/L, 250 g/L, 300 g/L, 350 g/L), while keeping the concentrations of $CaCl_2$ and H_3BO_3 constant (150 mg/L and 50 mg/L, respectively). One-way analysis of variance (ANOVA) revealed that the highest pollen germination percentage was observed at 300 g/L sucrose (Fig. 3a, $p < 0.05$). However, there was no significant difference in pollen tube growth across the sucrose concentrations tested (Fig. 3b, $p > 0.05$). At 300 g/L sucrose, the pollen germination rates were 64.30 ± 4.14% for single petal red and 68.96 ± 1.83% for single petal white. The corresponding average pollen tube lengths were 413.94 ± 11.85 μm for single petal red and 487.58 ± 24.28 μm for single petal white.

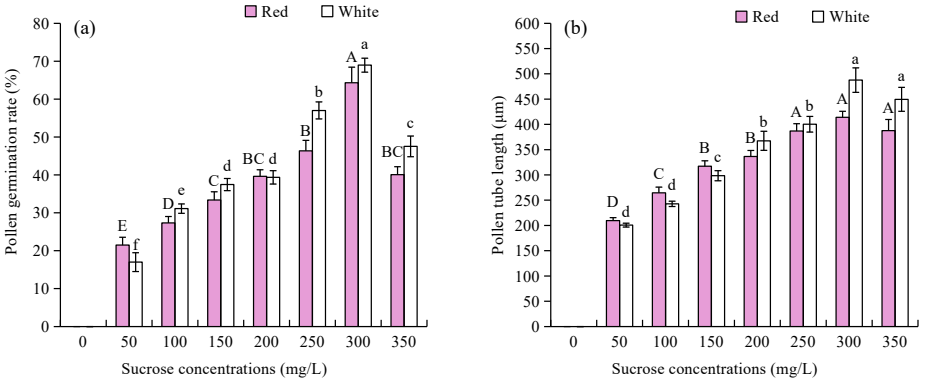

Figure 3: Effects of sucrose concentration on pollen germination rate (**a**) and pollen tube length (**b**). Uppercase letters represent significant differences among red pollen groups, and lowercase letters represent significant differences among white pollen groups; different uppercase or lowercase letters indicate significant differences at the ($p = 0.05$) level, whereas identical letters denote no significant difference.

Based on the optimization of the culture medium for *H. mutabilis* (300 g/L sucrose, 150 mg/L $CaCl_2$, and 50 mg/L H_3BO_3), the highest pollen germination percentages were observed in the absence of KNO_2 and $MgSO_4{\cdot}7H_2O$ (Fig. 4a). Specifically, in the absence of KNO_2, the pollen germination rates were 65.99 ± 2.25% for single petal red and 69.55 ± 2.00% for single petal white, with corresponding pollen tube lengths of 392.39 ± 11.85 μm and 374.72 ± 18.85 μm, respectively (Fig. 4b). In the absence of $MgSO_4{\cdot}7H_2O$, the pollen germination rates were 69.54 ± 2.13% for single petal red and 69.19 ± 2.09% for single petal white, with pollen tube lengths measuring 400.46 ± 11.85 μm and 415.26 ± 20.32 μm, respectively (Fig. 4c,d).

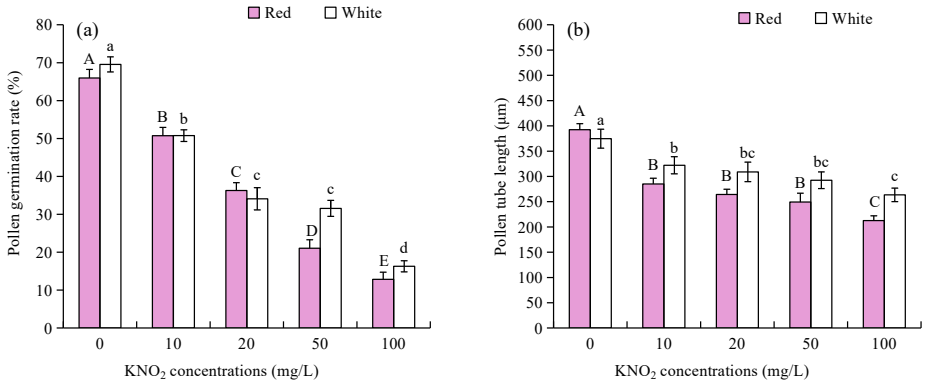

Figure 4: *Cont.*

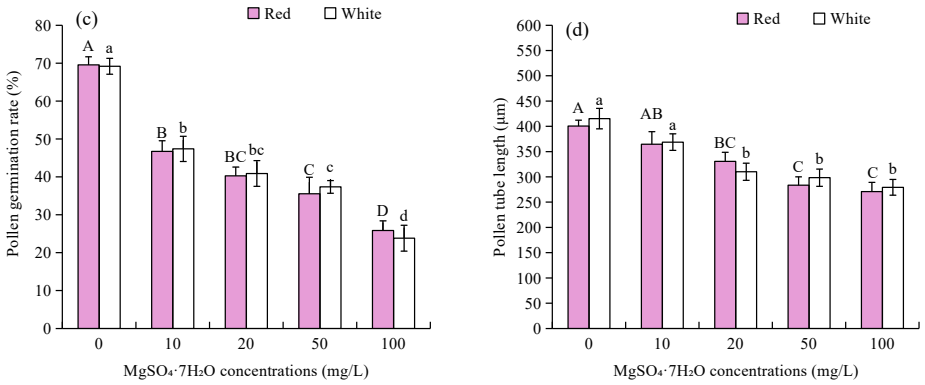

Figure 4: The effect of magnesium and potassium ion concentrations on pollen germination rate and pollen germination tube. (**a**,**c**) pollen germination rate; (**b**,**d**) pollen tube length. Uppercase letters represent significant differences among red pollen groups, and lowercase letters represent significant differences among white pollen groups; different uppercase or lowercase letters indicate significant differences at the (p = 0.05) level, whereas identical letters denote no significant difference.

3.2 Effect of Temperature on Pollen Germination and Pollen Tube Growth

Our data indicated that both the simple red and white *H. mutabilis* varieties exhibited a well-defined temperature optimum for pollen germination at 20°C. At this temperature, the pollen germination rates were 64.22 ± 2.18% for single petal red and 68.45 ± 1.96% for single petal white, respectively (Fig. 5a). Deviations from this optimal temperature (both higher and lower) resulted in a significant reduction in pollen germination. Under optimal conditions (20°C), pollen tubes reached lengths exceeding 400 μm after 3 h of incubation (Fig. 5b). In contrast, temperatures above and below the optimal range led to a significant reduction in pollen tube growth. These results suggest that *H. mutabilis* is particularly sensitive to temperature fluctuations, and both the germination rate and tube elongation are adversely affected by temperatures outside the optimal range. The comparison of the simple red and white varieties of *H. mutabilis* revealed similar responses to temperature, indicating that both varieties possess comparable temperature tolerance levels. Our results further support the observation that high temperatures (36–40°C) lead to a dramatic decrease in pollen viability.

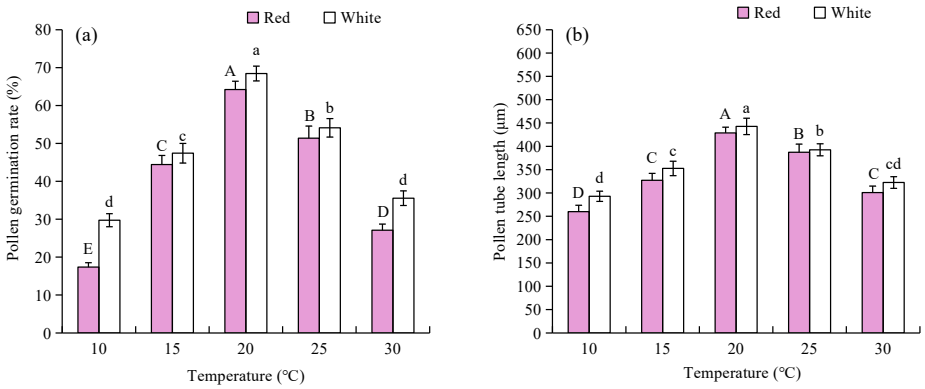

Figure 5: The effect of culture temperature on pollen germination rate and pollen germination tube. (**a**) pollen germination rate; (**b**) pollen tube length. Uppercase letters represent significant differences among red pollen groups, and lowercase letters represent significant differences among white pollen groups; different uppercase or lowercase letters indicate significant differences at the (p = 0.05) level, whereas identical letters denote no significant difference.

3.3 Influence of Environmental Factors (Light, Humidity, and pH) on Pollen Germination and Pollen Tube Growth

The impact of light intensity on pollen germination and pollen tube growth was also assessed. Pollen germination was found to be most successful in the dark, with the germination rate decreasing progressively as light intensity increased. Interestingly, pollen tubes grew longer under light conditions within the range of 6000–12,000 lux, but exposure to excessively high light intensities led to a decline in both germination rate and tube length. This suggests that moderate light conditions may enhance pollen tube growth, but excessive light intensity may inhibit germination and elongation, possibly due to the adverse effects of light stress. The humidity experiment revealed that both pollen germination rate and pollen tube growth were optimal at higher humidity levels. This suggests that *in vitro* culture conditions for *H. mutabilis* require elevated humidity for maximal pollen germination and tube elongation, although further optimization of specific humidity levels is needed to fine-tune these conditions (Fig. 6).

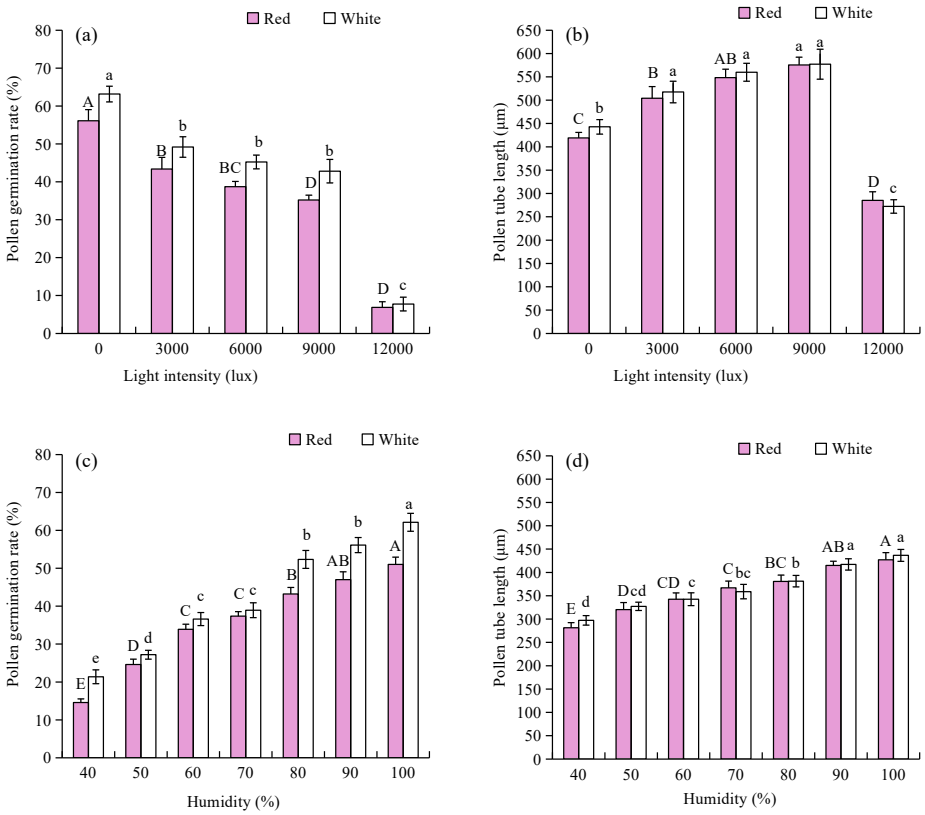

Figure 6: The effects of light intensity and humidity on pollen germination rate and pollen germination tube. (**a**,**c**) pollen germination rate; (**b**,**d**) pollen tube length. Uppercase letters represent significant differences among red pollen groups, and lowercase letters represent significant differences among white pollen groups; different uppercase or lowercase letters indicate significant differences at the (p = 0.05) level, whereas identical letters denote no significant difference.

In addition, the effect of pH on pollen germination was evaluated. The germination rate reached above 70% at a pH value of 6.1, which also corresponded to the longest pollen tube length (Fig. 7a). Both varieties of H. mutabilis exhibited similar responses to pH variation, with a decrease in germination rate and tube length at pH values higher or lower than the optimal 6.1 (Fig. 7b). These results suggest that *H. mutabilis*

exhibits a narrow pH tolerance range, with optimal germination and growth occurring at slightly acidic conditions (pH 6.1).

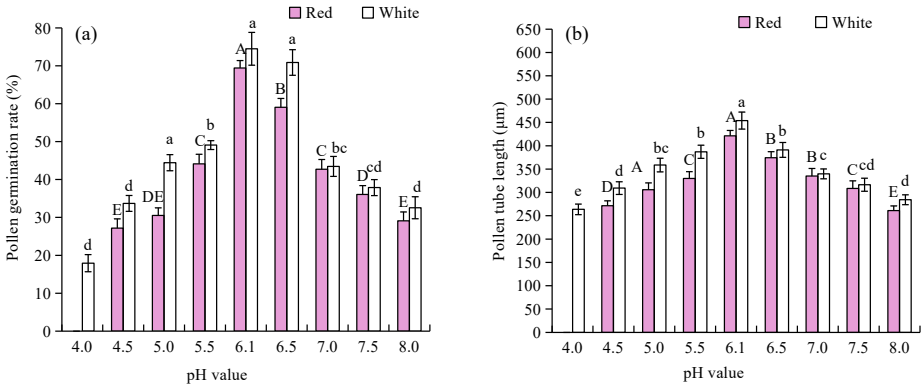

Figure 7: The effect of pH on pollen germination rate and pollen germination tube. (**a**) pollen germination rate; (**b**) pollen tube length. Uppercase letters represent significant differences among red pollen groups, and lowercase letters represent significant differences among white pollen groups; different uppercase or lowercase letters indicate significant differences at the (p = 0.05) level, whereas identical letters denote no significant difference.

4 Discussion

Pollen germination and pollen tube growth are critical determinants of fertilization success in flowering plants, and they are highly sensitive to both nutrient composition and physical environment [25,29]. In this study, we systematically optimized the culture medium and key environmental conditions for *in vitro* pollen germination of *H. mutabilis*, a species with high ornamental and ecological value but lacking detailed reproductive data. Our results establish, for the first time, a robust protocol for this species and reveal several features that are distinct from many other angiosperms, while sharing commonalities with other Malvaceae members.

Sucrose is indispensable for pollen germination, serving both as an energy source and as an osmotic regulator [9,10,14]. In *H. mutabilis*, no germination occurred in the absence of sucrose, and the optimal concentration was 300 g/L—markedly higher than the typical 10–20% (100–200 g/L) reported for many angiosperms such as *Impatiens cordata* (10–15%) [30] and *Torreya grandis* (15%) [5]. This high optimum is consistent with observations in other Malvaceae species. For example, *Hibiscus rosa-sinensis* exhibits optimal germination at 250–300 g/L sucrose [26], and *Gossypium hirsutum* (cotton) requires 250–350 g/L [14]. Microscopic examination in our study revealed that sucrose concentrations ≤200 g/L caused rapid pollen bursting due to excessive water uptake, indicating that a high external osmotic pressure is essential to prevent cytoplasmic leakage in *H. mutabilis*. Moreover, the rapid tube elongation (reaching >467 μm within 3 h) demands substantial carbohydrate energy, a function efficiently provided by high sucrose availability [28]. Taken together, a high sucrose requirement appears to be a conserved trait within Malvaceae, likely reflecting a shared adaptation of pollen wall structure and osmoregulation.

Boric acid (H_3BO_3) and calcium (Ca^{2+}) are well-known promoters of pollen germination and tube growth [12,30]. In *H. mutabilis*, the absence of either component completely abolished germination, confirming their absolute requirement. The optimal concentrations (50 mg/L H_3BO_3, 150 mg/L $CaCl_2$) are similar to those reported for *Hibiscus rosa-sinensis* (50 and 100–150 mg/L, respectively) [6] and for *Dioscorea* spp. [12]. Boron is thought to stabilize the cell wall by cross-linking rhamnogalacturonan-II, while Ca^{2+} regulates actin dynamics and vesicle fusion at the elongating tip [12,30]. In contrast, the addition of KNO_2

or $MgSO_4 \cdot 7H_2O$—even at low concentrations (10–20 mg/L)—significantly reduced both the germination rate and tube length in *H. mutabilis*. This inhibitory effect has also been observed in other species; for instance, high K^+ concentrations impair pollen viability in *Gossypium* [14], and excess Mg^{2+} can interfere with Ca^{2+}-dependent signaling [8]. Therefore, the standard BK medium, which contains K^+ and Mg^{2+}, is suboptimal for *H. mutabilis*, and we recommend omitting these ions entirely for *in vitro* germination assays of this species.

Temperature is a critical factor because it affects membrane fluidity, enzyme activity, and metabolic rates [15,16]. *H. mutabilis* pollen exhibited a clear optimum at 20°C, with sharp declines at higher or lower temperatures. This optimum is lower than that of tropical *Hibiscus* species (e.g., *H. rosa-sinensis* often tested at 25–30°C) [26] but matches the typical spring/autumn temperatures in its native subtropical China. Similar temperature optima have been reported for other temperate or subtropical plants, such as *Cocos nucifera* (coconut) grown at lower altitudes [15] and *Paeonia lactiflora* [20]. Interestingly, the two color varieties (red and white) showed nearly identical temperature responses, suggesting a conserved physiological basis for temperature sensitivity. The sharp decline above 25°C may be due to heat-induced oxidative damage to membranes and proteins, as shown in *Arabidopsis thaliana* [31] and apple [16]. These results imply that controlled crossing blocks for *H. mutabilis* should be maintained at 18–22°C to maximize pollination success.

Light intensity had a dual effect. Pollen germination was highest in complete darkness, consistent with many reports that light—especially high intensity—induces oxidative stress and inhibits germination [13,17]. However, moderate light (6000–12,000 lux) promoted longer pollen tubes, suggesting that once germination is initiated, low to moderate light may enhance energy production through photosynthetic pigments present in the pollen wall, or may reduce inhibitory dark-factors [18]. Excessive light (>12,000 lux) inhibited both germination and tube growth, likely due to reactive oxygen species damage [18]. Humidity was also critical: 100% relative humidity yielded the best germination and tube elongation. This is expected because pollen must hydrate to become metabolically active; low humidity causes desiccation and irreversible loss of viability [19,20]. In practice, maintaining near-saturation humidity in incubation chambers is essential. Finally, pH had a narrow optimum at 6.1 (slightly acidic), with rapid declines on either side. This sensitivity to pH is common among many species, because H^+ concentration affects cell wall expansibility and ion transport across the plasma membrane [5,21]. For example, *Salix viminalis* also shows optimal pollen germination at pH 6.0–6.5 [21]. The strict pH requirement of *H. mutabilis* suggests that standard media should be carefully buffered to pH 6.1 when used for breeding or conservation purposes.

The optimized protocol established here has several practical applications. First, the defined culture medium (300 g/L sucrose, 150 mg/L $CaCl_2$, 50 mg/L H_3BO_3) can be used to screen high-viability pollen donors for controlled crosses, potentially increasing hybridization success rates [31,32]. Second, the temperature sensitivity identified (20°C optimum) suggests that controlled-environment crossing blocks should be maintained within 18–22°C during flowering [33]. Third, the negative effect of KNO_2 and $MgSO_4 \cdot 7H_2O$ indicates that standard BK medium requires modification for *H. mutabilis*. Finally, the species' narrow pH tolerance (optimal 6.1) and high humidity requirement (100%) should guide *in vitro* conservation efforts for pollen storage and germplasm preservation [34].

5 Conclusions

This study provides a comprehensive analysis of the factors influencing pollen germination and tube growth in *H. mutabilis*. By identifying the optimal culture medium composition (300 g/L sucrose, 150 mg/L $CaCl_2$, and 50 mg/L H_3BO_3, without KNO_2 or $MgSO_4 \cdot 7H_2O$) and environmental conditions (20°C,

100% relative humidity, pH 6.1, and dark for germination), we have established a robust framework for enhancing pollen viability and supporting interspecific breeding efforts. Comparisons with other Malvaceae species confirm a family-specific high sucrose requirement and the inhibitory effect of K^+/Mg^{2+}, while the moderate temperature optimum reflects the subtropical origin of *H. mutabilis*. These findings contribute to the understanding of *H. mutabilis* reproductive biology and offer practical insights for the conservation and utilization of its genetic resources. Future research should explore the molecular mechanisms underlying pollen sensitivity to environmental factors, particularly the high sucrose requirement and the inhibition by K^+/Mg^{2+}, and investigate the potential for genetic improvement through targeted breeding strategies.

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