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Pollen Viability and Stigma Receptivity in *Zephyranthes*: Implications for Controlled Pollination

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Abstract

Knowledge of pollen viability, pollen longevity, and stigma receptivity in *Zephyranthes* remains limited despite their importance for successful hybridization. Therefore, this study evaluated these reproductive traits in four *Zephyranthes* species (*Z. traubii*, *Z. candida*, *Z. grandiflora*, and *Z. citrina*) and one cultivar, 'Pim Chomphu', to provide baseline reproductive information for controlled pollination. Pollen viability was evaluated using tetrazolium staining at different collection times and storage conditions, whereas stigma receptivity was assessed using 6% hydrogen peroxide (H_2O_2), with reproductive outcomes following controlled self-pollination evaluated at the corresponding time points. Tetrazolium-based pollen viability exhibited taxon-specific temporal patterns; however, viability was generally high at 11:00, corresponding to 1 day after anthesis (1 DAA) in *Z. traubii* and the day of anthesis (0 DAA) in the remaining taxa. The combined protocol of desiccation followed by storage at −5 °C or −20 °C maintained tetrazolium-based pollen viability for up to 120 days. Although all taxa showed positive H_2O_2-based stigma responses across the evaluated time points, reproductive outcomes following self-pollination differed markedly among taxa. *Z. citrina* set fruit at all pollination times, whereas *Z. candida* failed to set fruit. The remaining taxa showed variable fruit set and seed germination depending on pollination timing. These results indicate that positive H_2O_2-based stigma responses should be interpreted as biochemical indicators of potential stigma receptivity and considered together with subsequent reproductive outcomes. These findings provide baseline reproductive information to support pollen management and controlled pollination in *Zephyranthes*.

Keywords: Amaryllidaceae; hydrogen peroxide test; long-term storage; self-pollination; tetrazolium test

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1. Introduction

Zephyranthes Herb. (Amaryllidaceae), commonly known as fairy lily or rain lily, is a bulbous ornamental genus native to tropical and subtropical regions of North America, the Caribbean, Mexico, and Argentina [1] and has become widely naturalized in regions such as Hawaii, Indonesia, Thailand, and South Africa [2]. In addition to their ornamental value, several species have attracted pharmacological interest due to diverse bioactivities, including anticancer, antifungal, antiviral, and antibacterial properties, as well as traditional use in diabetes management [2–4]. From a horticultural perspective, *Zephyranthes* is valued for its attractive, six-tepaled, funnel-shaped flowers, which display a wide range

of colors, including white, yellow, and pink. Individual flowers are short-lived, lasting only 1–2 days depending on environmental conditions; however, sequential flowering over consecutive days enhances ornamental value and marketability [2]. In commercial production, market competitiveness depends on the availability of diverse cultivars with variation in flower color, form, and other distinctive traits. Therefore, the development of new cultivars with novel floral colors and forms remains an important objective in ornamental breeding programs.

Conventional breeding in *Zephyranthes* relies primarily on hybridization, including interspecific and intergeneric crosses. Previous studies have demonstrated the feasibility of such crosses [1,5]. However, their efficiency is often limited by multiple reproductive barriers, including differences in floral morphology (e.g., floral tube length, spatial arrangement of reproductive organs, and pistil length), reduced pollen production, and reproductive incompatibility, such as self- and cross-incompatibility. In addition, temporal factors, including flowering synchrony and stigma receptivity, further influence fertilization success [1]. These constraints reduce the success rate of controlled crosses and hinder breeding progress. Among these factors, pollen viability, pollen longevity, and stigma receptivity are key reproductive traits that are closely associated with pollination and fertilization success and can be experimentally evaluated. Therefore, identifying taxon-specific patterns of pollen viability, pollen longevity, and stigma receptivity provides important baseline information for improving pollen management, pollination scheduling, and future controlled hybridization studies.

Pollen viability is an important reproductive trait that contributes to fertilization success and breeding efficiency, and has therefore been widely studied across diverse plant species [6–8]. It is influenced by multiple factors, including genotype, environmental conditions, and pollen age [9], as well as storage-related parameters, particularly temperature and moisture status, which play a key role in pollen longevity [10]. In *Zephyranthes*, however, studies on pollen viability and pollen storage remain limited, with available reports indicating variation among species and across collection times [11,12]. Pollen viability is commonly assessed using in vitro germination and staining-based assays [9]. In vitro pollen germination provides a direct assessment of pollen germination ability and the potential for pollen tube growth, although successful germination depends on species-specific culture media and often requires protocol optimization [13]. Staining methods provide an alternative approach by assessing different physiological characteristics of pollen. For example, the fluorochromatic reaction (FCR) test evaluates pollen viability based on membrane integrity and esterase activity [14], whereas the 2,3,5-triphenyl tetrazolium chloride (TTC) test estimates pollen metabolic viability based on dehydrogenase activity involved in cellular metabolism [6,9]. Each method has advantages and limitations depending on the study objectives. The FCR test provides sensitive detection of viable pollen grains but requires fluorescence microscopy and prompt evaluation because fluorescence gradually declines after staining [15]. TTC staining provides a rapid method for the comparative assessment of pollen metabolic viability across multiple samples. Although it reflects metabolic activity rather than directly assessing pollen germination [9], it has been widely applied in comparative studies of pollen viability.

In addition to pollen-related traits, temporal factors such as stigma receptivity influence pollination and fertilization success but remain insufficiently characterized in *Zephyranthes*. Stigma receptivity has been extensively investigated in other ornamental and crop species, including *Lilium* spp. [16], *Solanum lycopersicum* [17], and members of the Amaryllidaceae such as *Hippeastrum* and *Crinum* [18], where stigma receptivity has been shown to vary temporally during flower development. However, information on the timing of stigma receptivity and its relationship with reproductive outcomes in *Zephyran-*

thes has received little attention. Biochemical indicators associated with stigma receptivity, such as the H_2O_2 test, are commonly used to assess stigma-associated biochemical activity, although they do not always provide direct evidence of subsequent reproductive outcomes. Therefore, assessment of reproductive outcomes following controlled pollination provides additional context for interpreting the biological relevance of stigma receptivity. Despite their importance for breeding, information on pollen viability, pollen storability, and stigma receptivity remains limited across *Zephyranthes* taxa. We hypothesized that *Zephyranthes* taxa exhibit distinct patterns of pollen viability, pollen longevity, and H_2O_2-based stigma responses, together with variation in reproductive outcomes following controlled self-pollination. To test this hypothesis, the present study characterized these reproductive traits in four *Zephyranthes* species (*Z. traubii*, *Z. candida*, *Z. grandiflora*, and *Z. citrina*) and one cultivar, 'Pim Chomphu'. These taxa represent cultivated *Zephyranthes* germplasm with contrasting reproductive characteristics, including differences in anthesis timing, floral morphology, and reproductive performance. Such biological variation provides a suitable comparative framework for evaluating reproductive traits relevant to controlled pollination and ornamental breeding. The findings provide fundamental reproductive information to support the development of controlled pollination strategies in *Zephyranthes* breeding programs.

2. Materials and Methods

2.1. Plant Materials and Growth Conditions

Four *Zephyranthes* species (*Z. traubii*, *Z. candida*, *Z. grandiflora*, and *Z. citrina*) and one *Zephyranthes* cultivar ('Pim Chomphu') were used in this study (Table 1). The taxa were identified by Assistant Prof. Dr. Chamaiporn Anuwong, Department of Plant Production Technology, Faculty of Agricultural Technology, King Mongkut's Institute of Technology Ladkrabang, Bangkok, Thailand. Approximately 150 bulbs of each taxon were grown as stock plants to provide flowers for subsequent experiments, including pollen viability assessment, pollen storage, stigma receptivity, and controlled pollination. All bulbs were mature and capable of flowering during the experimental period.

Table 1. Bulb and floral characteristics of the *Zephyranthes* taxa used in this study. Values are mean ± SD (n = 10).

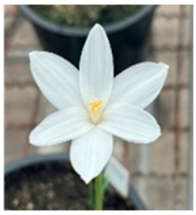
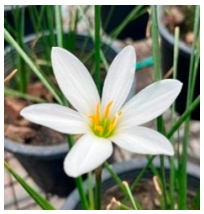
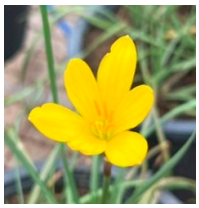
Zephyranthes Taxa		Bulb Diameter (cm)	Flower Color	Flower Diameter (cm)	Style Length (cm)	Flowering Period	Anthesis Time	Flower Longevity (DAA)
	*Z. traubii*	1.82 ± 0.10	White	3.92 ± 0.33	13.68 ± 0.09	Jan. to Dec.	Evening	3
	*Z. candida*	1.73 ± 0.02	White, green throat	4.49 ± 0.65	2.06 ± 0.10	Jul. to Dec.	Morning	2

Table 1. *Cont.*

Zephyranthes Taxa		Bulb Diameter (cm)	Flower Color	Flower Diameter (cm)	Style Length (cm)	Flowering Period	Anthesis Time	Flower Longevity (DAA)
	*Z. grandiflora*	2.23 ± 0.08	Purplish pink	5.59 ± 0.56	2.95 ± 0.35	May to Dec.	Morning	2
	*Z. citrina*	1.93 ± 0.03	Yellow	2.83 ± 0.24	2.09 ± 0.09	Apr. to Nov.	Morning	2
	'Pim Chomphu'	1.16 ± 0.12	Light pink	4.53 ± 0.12	3.11 ± 0.18	May to Oct.	Morning	2

Bulbs were planted in 8-inch-diameter (20.32 cm) pots containing a growing medium consisting of soil, coarse sand, manure, and chopped coconut husk at a volumetric ratio of 2:2:1:1 (*v*/*v*), with three to five bulbs per pot depending on bulb size. Plants were maintained in a greenhouse at the Department of Agricultural Technology, Thammasat University, Rangsit Campus, Pathum Thani, Thailand (14°04′27″ N 100°36′08″ E), under natural environmental conditions, with temperatures ranging from 16.4 to 40.8 °C and relative humidity ranging from 20.4 to 96.7% during the experimental period. The flowering periods of the five *Zephyranthes* taxa are summarized in Table 1. The experiments were conducted from January 2024 to October 2025. Irrigation was provided once or twice daily depending on weather conditions, and a granular compound fertilizer (16–16–16 N–P_2O_5–K_2O) was applied at 0.2 g per pot one week after planting and every two weeks thereafter. Insect pests were managed using insecticides as required to maintain plant health throughout the experimental period.

2.2. Assessment of Pollen Viability

2.2.1. Pollen Viability at Different Pollen Collection Times

Preliminary experiments using both in vitro pollen germination and TTC staining were conducted to evaluate methods for pollen viability assessment and to identify the appropriate floral stage for pollen collection. However, in vitro pollen germination was consistently very low or absent across the evaluated taxa, limiting its applicability for comparative analysis [19]. Therefore, the TTC staining method, which estimates pollen metabolic viability based on dehydrogenase activity [6], was selected for pollen viability assessment in the present study. The preliminary TTC assessment (Table S1) indicated that pollen viability was higher at 1 day after anthesis (DAA) in *Z. traubii*, whereas the remaining taxa generally exhibited higher or comparable viability at 0 DAA. Therefore, subsequent pollen sampling was conducted at the floral stages exhibiting relatively high tetrazolium-

based pollen viability. Based on these differences in flowering stage and anthesis pattern, pollen collection was performed at species-specific time points. For *Z. traubii*, pollen was collected at 18:00 and 20:00 on 0 DAA, and at 09:00, 11:00, 13:00, and 15:00 on 1 DAA. For *Z. candida*, *Z. grandiflora*, *Z. citrina*, and the 'Pim Chomphu' cultivar, pollen was collected at 09:00, 11:00, 13:00, and 15:00 on 0 DAA (Figure S1).

To assess pollen viability, pollen grains were collected from at least three flowers of each taxon at each sampling time. Three independent biological replicates were prepared, each consisting of pollen pooled from a different set of flowers. For each replicate, a sample of pollen was placed on a glass slide, and a drop of 0.4% (*w*/*v*) TTC solution prepared in 10% (*w*/*v*) sucrose was added before covering with a coverslip. The slides were placed in glass Petri dishes lined with moistened tissue paper, covered, and incubated in the dark at 25 °C for 1 h following Jirakiattikul [20]. After incubation, pollen grains were examined under a compound light microscope (Alphaphot-YS2-T, Nikon, Tokyo, Japan) at 100× magnification. Pollen grains showing clear red staining were counted as TTC-positive pollen grains and considered metabolically active, whereas unstained or weakly stained pollen grains were counted as TTC-negative pollen grains. A total of at least 300 pollen grains were randomly counted for each replicate. The experiment was arranged in a completely randomized design (CRD) with three replicates. Pollen viability was calculated as follows:

$$\text{Pollen viability}(\%) = \frac{\text{Number of stained pollen grains}}{\text{Total number of pollen grains counted}} \times 100$$

2.2.2. Pollen Viability During Storage

Pollen of *Z. traubii*, *Z. grandiflora*, *Z. citrina*, and the cultivar 'Pim Chomphu' was selected for the storage experiment due to sufficient pollen availability. Pollen grains were collected at the time point that exhibited the highest pollen viability in Section 2.2.1, and divided into three independent replicates, each consisting of pooled pollen from multiple flowers. Pollen samples were evaluated under four storage conditions: 25 °C, 4 °C, −5 °C, and −20 °C. Prior to storage, pollen samples intended for storage at −5 °C and −20 °C were desiccated over silica gel at 25 °C for 24 h to reduce moisture content and minimize freezing injury during low-temperature storage, whereas desiccation was not applied to samples stored at 25 °C and 4 °C, where freezing damage is not a concern.

Pollen viability was assessed after 0, 3, 6, 9, 12, 15, 30, 45, 60, 90, and 120 days of storage using the TTC staining procedure described in Section 2.2.1. Before assessment, pollen samples stored at −5 °C and −20 °C were equilibrated in a water bath at 35 °C for 1 h for rehydration and recovery, following the method of Sukhvibul and Considine [21]. After equilibration, pollen viability was assessed using the TTC staining procedure described in Section 2.2.1. The experiment was arranged in a CRD with four storage conditions and three replicates.

2.3. Assessment of Stigma Receptivity

2.3.1. Hydrogen Peroxide (H_2O_2) Test

Stigma-associated biochemical activity related to receptivity was evaluated using an H_2O_2 assay based on bubble formation on the stigma surface, which provides a qualitative indication of this activity [22]. Stigma receptivity was assessed throughout the floral lifespan of each taxon, with sampling times adjusted according to differences in flowering behavior.

In *Z. traubii*, which exhibits evening anthesis and extended floral longevity, stigma receptivity was assessed over 0, 1, and 2 DAA. Sampling was conducted at 17:00–19:00 and 19:00–21:00 on 0 DAA; at 08:00–10:00, 10:00–12:00, 12:00–14:00, 14:00–16:00, 17:00–19:00,

and 19:00–21:00 on 1 DAA; and at 08:00–10:00, 10:00–12:00, 12:00–14:00, and 14:00–16:00 on 2 DAA. In contrast, *Z. candida*, *Z. grandiflora*, *Z. citrina*, and the cultivar 'Pim Chomphu', which exhibit daytime anthesis, were sampled at 0 and 1 DAA during 08:00–10:00, 10:00–12:00, 12:00–14:00, and 14:00–16:00.

For each sample, two drops of 6% (*v*/*v*) H_2O_2 solution, prepared by diluting commercial 30% H_2O_2 with distilled water, were applied to the stigma surface, following the method of Dafni and Maués [22], and the reaction was immediately observed under a stereomicroscope (Olympus SZ30, Olympus Corporation, Tokyo, Japan). Observation magnification was adjusted according to stigma size: 80× for *Z. traubii*, *Z. candida*, and *Z. citrina*; 60× for the cultivar 'Pim Chomphu'; and 40× for *Z. grandiflora*. The formation of oxygen bubbles was used as a qualitative biochemical indicator of stigma receptivity. The experiment consisted of ten replicates, with one flower representing one replicate. Stigma receptivity was visually scored according to the extent of bubble formation on the stigma surface following H_2O_2 application: + = low or few small bubbles; ++ = moderate or moderate bubble formation; and +++ = high or abundant bubble formation covering most of the stigma surface (Figure 1). Representative levels of bubble formation were established during preliminary observations, and the same observer applied the predefined scoring criteria consistently throughout the experiment.

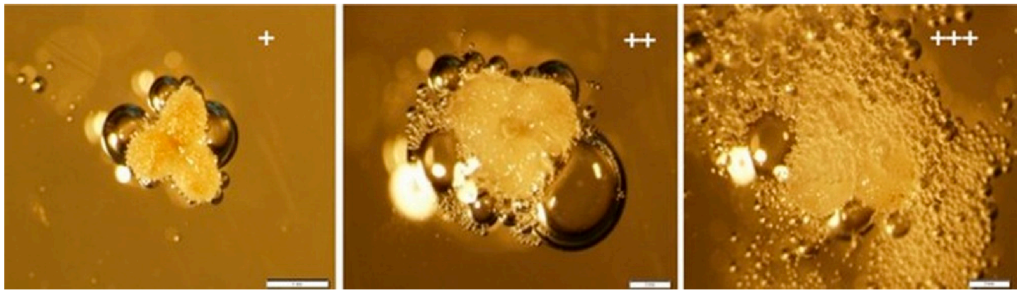


Figure 1. Bubble formation on the stigma surface following hydrogen peroxide (H_2O_2) application. Bubble intensity was visually scored as + (low or few small bubbles), ++ (moderate or moderate bubble formation), and +++ (high or abundant bubble formation covering most of the stigma surface). Scale bar = 1 mm.

2.3.2. Controlled Self-Pollination and Reproductive Outcomes

Controlled self-pollination was conducted at the same time points used in the H_2O_2 test to allow comparison between H_2O_2-based stigma responses and subsequent reproductive outcomes at the same floral developmental stages. Anthers were removed from selected flowers one day before anthesis, and the flowers were covered with plastic tubes to prevent unintended pollination. Pollination was performed at the time points described in Section 2.3.1 using freshly collected pollen obtained from flowers of the same taxon on the day of anthesis. For controlled self-pollination, the anthers were detached using forceps and gently brought into contact with the stigma until the entire stigmatic surface was visibly covered with pollen. All pollinations were performed by the same operator using the same pollination procedure across taxa and pollination times. Depending on flower availability, 15–40 flowers per taxon were pollinated at each pollination time point. Fruit set (%), days to fruit maturity, and number of seeds per fruit were recorded. Fruit set (%) was calculated as the proportion of fruits formed relative to the total number of pollinated flowers at each pollination time point.

Seeds were subjected to a top-of-paper germination test and incubated at 25 ± 2 °C. Distilled water was added every two days to maintain moisture, and germination was monitored for 14 days. Seeds from each fruit were treated as a single experimental unit, and all seeds within that fruit were included in the germination test. A seed was considered

germinated when the radicle reached at least 2 mm in length. Germination percentage (%) and mean emergence time (MET) were recorded. Germination percentage (%) was calculated as the proportion of germinated seeds relative to the total number of seeds per fruit. MET was calculated as follows:

$$MET = \Sigma(T_i N_i)/\Sigma N_i$$

where N_i is the number of seeds germinated at time T_i, and T_i is the number of days from the start of the germination test [23].

2.4. Statistical Analysis

Data were analyzed using analysis of variance (ANOVA) in a CRD. Mean comparisons among treatments were performed using Tukey's honestly significant difference (HSD) test at $p < 0.05$. All values are presented as mean ± standard deviation (SD). Statistical analyses were performed using SPSS version 21.0 (IBM Corp., Armonk, NY, USA). Fruit set data were presented descriptively and were not subjected to statistical analysis because fruit set (%) was calculated from the total number of fruits formed relative to the number of pollinated flowers at each pollination time point, which varied among treatments depending on flower availability.

3. Results

3.1. Effect of Collection Time on Pollen Viability

Tetrazolium-based pollen viability varied among collection times and exhibited distinct taxon-specific temporal patterns across the *Zephyranthes* taxa examined. In *Z. traubii*, pollen viability increased after anthesis and reached its maximum on 1 DAA, peaking at 13:00 (90.11 ± 1.92%), which was not significantly different from that at 11:00 (86.78 ± 0.19%). In contrast, pollen collected shortly after flower opening on 0 DAA (18:00) exhibited the lowest viability (65.22 ± 2.01%) (Figure 2a).

In *Z. candida*, pollen viability was relatively low compared with the other taxa but increased from morning to midday, reaching a maximum at 13:00 (47.17 ± 6.83%), although no significant differences were detected among 11:00, 13:00, and 15:00. The lowest viability was observed at 09:00 (9.67 ± 0.67%) (Figure 2b).

In *Z. grandiflora* and 'Pim Chomphu', pollen viability was highest in the morning (09:00–11:00) and declined significantly thereafter. No significant differences were observed between 09:00 and 11:00 in either taxon, but both time points showed significantly higher viability than those at 13:00 and 15:00 (Figure 2c,e).

Similarly, *Z. citrina* maintained consistently high pollen viability from 09:00 to 13:00 (86.33–91.45%), followed by a significant decline at 15:00, when viability reached its lowest level (53.67 ± 2.31%) (Figure 2d).

Across the *Zephyranthes* taxa examined, 11:00 represented a practical common sampling time for obtaining relatively high tetrazolium-based pollen viability, as viability at this time was either maximal or not significantly different from the highest recorded value within each taxon.

3.2. Pollen Viability Under Different Storage Protocols

Tetrazolium-based pollen viability declined over time in all four *Zephyranthes* taxa included in the storage experiment, although the rate of decline varied markedly among the storage protocols evaluated (Figure 3).

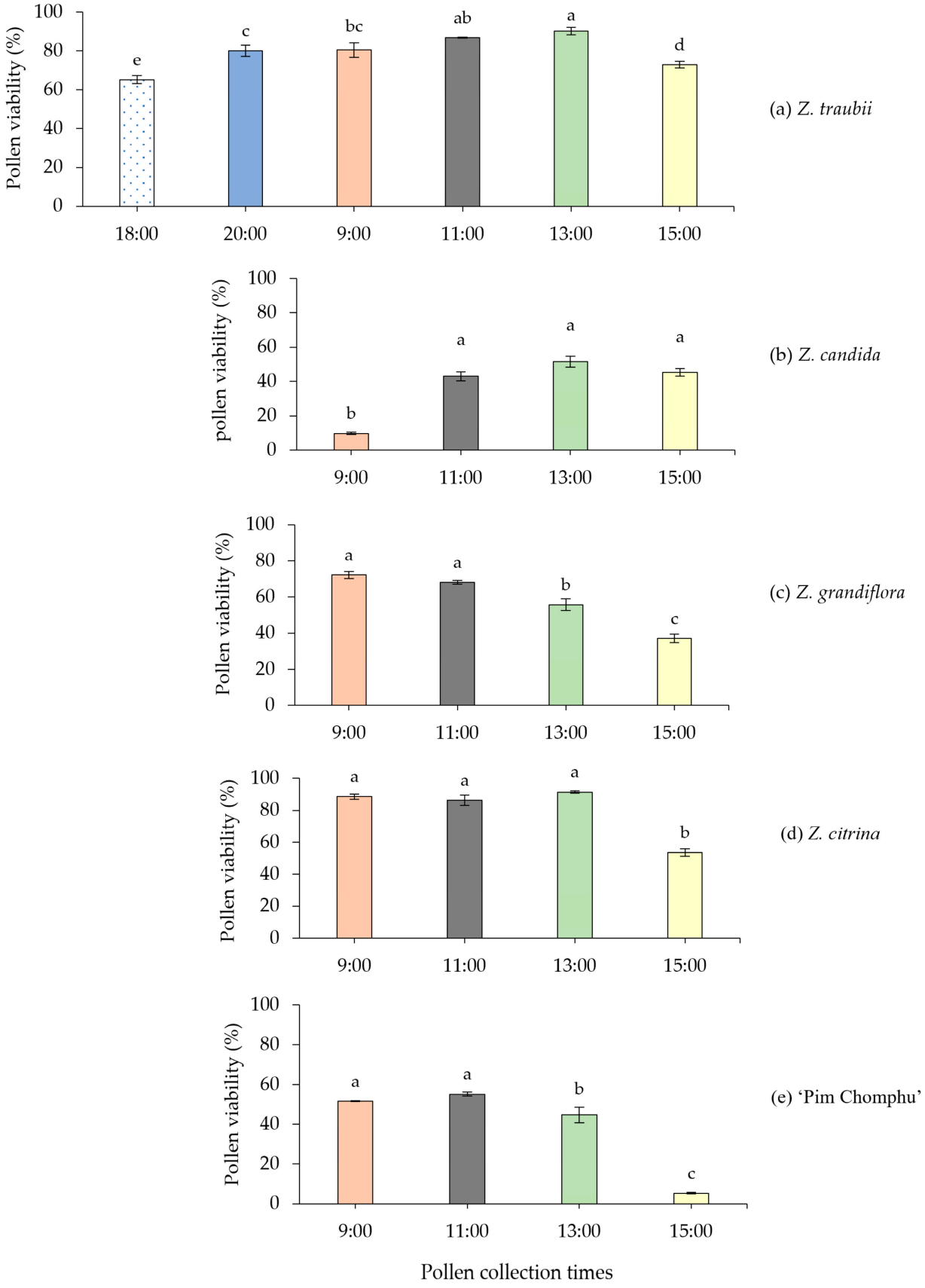


Figure 2. Tetrazolium-based pollen viability of (**a**) *Z. traubii*, (**b**) *Z. candida*, (**c**) *Z. grandiflora*, (**d**) *Z. citrina*, and (**e**) 'Pim Chomphu' at different collection times. Values represent the mean ± SD of three replicates. Different letters indicate significant differences among pollen collection times within each panel according to Tukey's HSD test at $p < 0.05$.

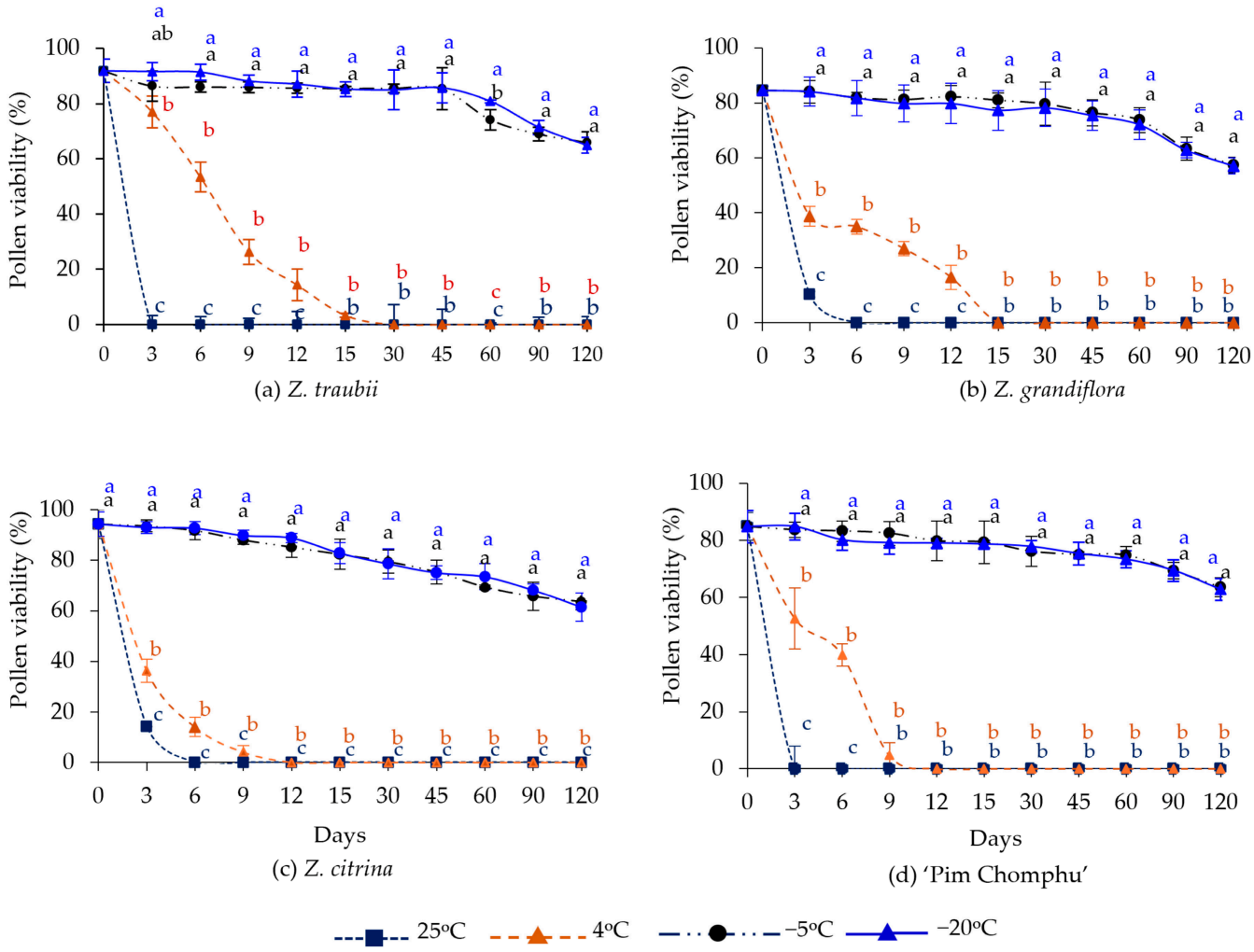


Figure 3. Tetrazolium-based pollen viability of (**a**) *Z. traubii*, (**b**) *Z. grandiflora*, (**c**) *Z. citrina*, and (**d**) 'Pim Chomphu' during storage at 25 °C, 4 °C, −5 °C, and −20 °C for up to 120 days. Values present as the mean ± SD of three replicates. Different letters within each panel indicate significant differences among treatment means according to Tukey's HSD test at $p < 0.05$.

At 25 °C, viability decreased rapidly in all taxa, although initial viability was relatively high (84.56 ± 1.54–94.44 ± 4.86%). In *Z. traubii* and 'Pim Chomphu', no viable pollen was detected by day 3 of storage. Similarly, in *Z. grandiflora* and *Z. citrina*, viability declined sharply within 3–6 days and no viable pollen was detected thereafter.

Storage at 4 °C moderately extended pollen longevity; however, viability gradually decreased over time in all taxa. Viability declined to low levels (3.33 ± 0.67–16.44 ± 4.40%) within two weeks of storage, depending on the taxon, and became undetectable after 12–30 days.

In contrast, the combined protocols of desiccation followed by storage at −5 °C or −20 °C maintained tetrazolium-based pollen viability throughout the 120-day storage period in all taxa. After 120 days, tetrazolium-based viability remained relatively high, ranging from 57.00 ± 2.85% to 66.00 ± 3.93%, with no significant differences observed between the two desiccation–low-temperature storage conditions. These values represented a reduction of approximately 21–33% relative to the initial viability of each taxon. In all taxa, tetrazolium-based pollen viability was maintained substantially longer under the combined protocol of pre-desiccation followed by storage at −5 °C or −20 °C than under the non-desiccated storage protocols at 25 °C or 4 °C.

3.3. H_2O_2-Based Stigma Response and Reproductive Outcomes After Self-Pollination

3.3.1. H_2O_2-Based Stigma Response

All *Zephyranthes* taxa exhibited positive H_2O_2-based stigma responses at all evaluated time points during their respective flowering periods, as indicated by bubble formation following H_2O_2 application (Table 2). Positive H_2O_2 responses were observed throughout the floral lifespan of each taxon, with *Z. traubii* showing responses from 0 to 2 DAA, whereas *Z. candida*, *Z. grandiflora*, *Z. citrina*, and 'Pim Chomphu' exhibited responses from 0 to 1 DAA. The intensity of the H_2O_2 response, based on the extent of bubble formation, varied among taxa. In *Z. traubii*, *Z. candida*, and 'Pim Chomphu', H_2O_2 response intensity ranged from low (+) to high (+++). *Z. grandiflora* exhibited lower response intensity overall, with bubble formation ranging from low (+) to moderate (++), whereas *Z. citrina* exhibited stronger responses, ranging from moderate (++) to high (+++).

Table 2. H_2O_2-based stigma response of *Zephyranthes* taxa at different time points during flowering, as assessed by bubble formation following hydrogen peroxide (H_2O_2) application.

Zephyranthes Taxa	DAA	Stigma Receptivity Based on Bubble Formation Intensity					
		8:00–10:00	10:00–12:00	12:00–14:00	14:00–16:00	17:00–19:00	19:00–21:00
Z. traubii	0 [1]	-	-	-	-	+ [2]	++
	1	++	++	++	+++	+++	+++
	2	+++	+++	+++	++	-	-
Z. candida	0	+	+++	++	++	-	-
	1	++	+	+	+	-	-
Z. grandiflora	0	+	+	+	++	-	-
	1	++	+	+	+	-	-
Z. citrina	0	++	++	+++	++	-	-
	1	++	+++	+++	++	-	-
'Pim Chomphu'	0	++	++	++	+++	-	-
	1	+++	+++	+++	+	-	-

[1] Evaluations were conducted at 0, 1, and 2 DAA for Z. *traubii*, and at 0 and 1 DAA for *Z. candida*, *Z. grandiflora*, *Z. citrina*, and 'Pim Chomphu'. [2] Bubble formation intensity following H_2O_2 application was scored as + (low or few small bubbles), ++ (moderate or moderate bubble formation), and +++ (high or abundant bubble formation covering most of the stigma surface). - indicates not evaluated.

3.3.2. Reproductive Outcomes After Controlled Self-Pollination

Controlled self-pollination resulted in substantial variation in reproductive outcomes among *Zephyranthes* taxa, although positive H_2O_2-based stigma responses were observed at all evaluated pollination times (Figure 4). Fruit set, seed production, and seed germination differed markedly among taxa and pollination times.

Among the taxa, *Z. citrina* exhibited consistently high fruit set after self-pollination with 100% fruit set at all pollination times on both 0 and 1 DAA. Fruits matured within 14.76 ± 1.15–15.76 ± 2.68 days after pollination (DAP). Germination percentages remained relatively stable across pollination times, ranging from 61.09 ± 6.12% to 65.44 ± 7.70% on 0 DAA and from 46.93 ± 9.84% to 66.36 ± 6.36% on 1 DAA. MET varied between 4.39 ± 0.76 and 5.37 ± 1.38 days on 0 DAA and was generally lower on 1 DAA (3.63 ± 0.56–4.52 ± 0.73 days).

In *Z. traubii*, fruit set ranged from 2.50% to 36.67%, with the highest value recorded at 12:00–14:00 on 1 DAA. No fruit set was observed at 14:00–16:00 on 1 DAA or at any pollination time on 2 DAA. Fruit maturation time was generally consistent but was slightly reduced when pollination was performed at 10:00–12:00. Germination varied markedly among pollination times, with the highest values recorded at 12:00–14:00. MET ranged from 3.19 ± 0.36 to 5.10 ± 0.47 days.

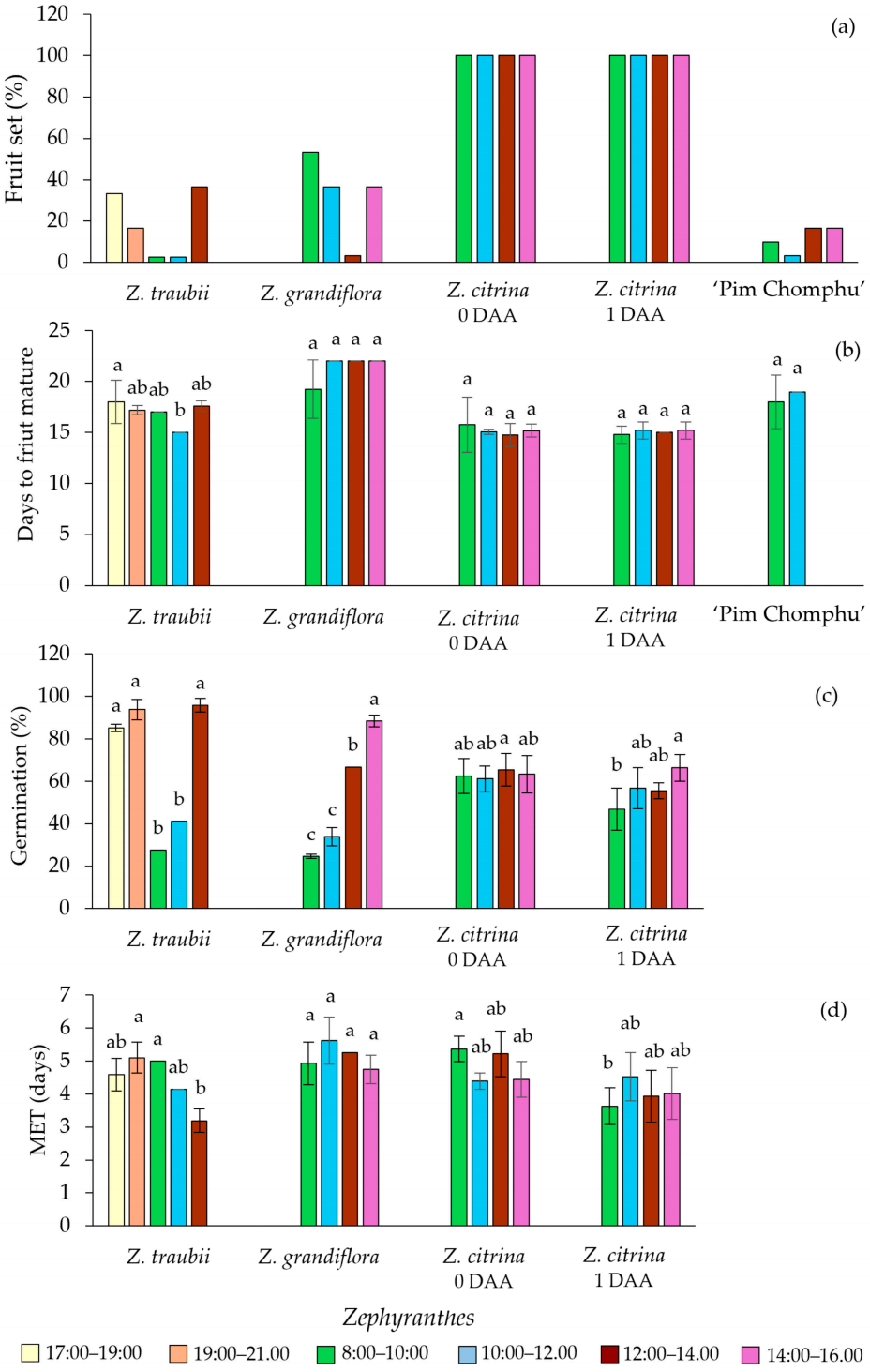


Figure 4. (**a**) Fruit set (%), (**b**) days to fruit maturity, (**c**) germination (%), and (**d**) mean emergence time (MET; days) following self-pollination at different time points after anthesis in *Z. traubii*, *Z. grandiflora*, *Z. citrina*, and 'Pim Chomphu'. Different lowercase letters above the columns indicate significant differences among collection times within each taxon ($p < 0.05$), and error bars represent ± SD. No statistical analysis was performed for fruit set (%). Note: Only pollination time points that resulted in successful fruit set are presented. In *Z. traubii*, data were obtained at 0 DAA (17:00–19:00 and 19:00–21:00) and 1 DAA (08:00–10:00, 10:00–12:00, and 12:00–14:00). In *Z. grandiflora* and 'Pim Chomphu', data were obtained only on 0 DAA, whereas in *Z. citrina*, data were obtained at both 0 and 1 DAA. *Z. candida* did not produce fruit following self-pollination and was therefore excluded from the figure.

Z. grandiflora and 'Pim Chomphu' exhibited limited fruit set after self-pollination, with fruit set restricted to 0 DAA. In *Z. grandiflora*, fruit set ranged from 3.33% to 53.33%. The highest fruit set occurred at 08:00–10:00. Fruit maturation time showed only slight variation across pollination times. Germination percentage increased with later pollination, reaching a maximum of 88.50 ± 5.26% at 14:00–16:00. MET ranged from 4.74 ± 0.43 to 5.62 ± 0.71 days and varied only slightly among pollination times.

'Pim Chomphu' showed low fruit set (3.33–16.67%). Although fruits developed following pollination at selected time points, none of the resulting seeds germinated. *Z. candida* failed to produce fruits following self-pollination at any pollination time, whereas positive H_2O_2-based stigma responses were detected throughout the flowering period.

4. Discussion

This study characterized temporal variation in tetrazolium-based pollen viability, H_2O_2-based stigma responses, and reproductive outcomes following controlled self-pollination, demonstrating variation among *Zephyranthes* taxa in these reproductive traits. It also evaluated pollen storage responses under different storage conditions. By examining these reproductive traits together, the study provides baseline reproductive information to support future controlled pollination studies and breeding efforts in *Zephyranthes*.

Tetrazolium-based pollen viability, used in the present study as an indicator of pollen metabolic viability rather than as a measure of pollen germination and pollen tube growth, exhibited clear temporal patterns that varied among *Zephyranthes* taxa, with peak viability occurring at different time points depending on the species. In taxa with daytime anthesis, including *Z. candida*, *Z. grandiflora*, *Z. citrina*, and 'Pim Chomphu', peak viability was observed at specific times between morning and early afternoon, although the exact timing differed among taxa. Peak viability occurred later in *Z. candida* than in *Z. grandiflora*, *Z. citrina* and 'Pim Chomphu', likely reflecting variation in flower opening and progression to full anthesis among taxa. Unlike the other taxa, *Z. traubii* reached maximum pollen viability on the day following anthesis. The decline in pollen viability at later sampling times may reflect progressive pollen aging and exposure to environmental conditions known to affect pollen longevity, including temperature and humidity [24,25]. From a practical breeding perspective, pollen collection at 11:00 represented a broadly suitable sampling time across the taxa examined, as viability at this stage was high or statistically comparable to the maximum value in each taxon. These results are consistent with those of Anuwong et al. [11], who reported that pollen of the *Zephyranthes* hybrid 'POS' exhibited maximum viability at 09:00–11:00 and declined thereafter. These findings reinforce the concept that peak pollen viability is taxon-specific and temporally variable. Similar temporal patterns have been documented in other species. For example, pollen viability in *Zea mays* cultivars was highest during the early morning hours, typically around 08:00–10:00 on the second day after anthesis [26]. Similarly, in *Hemerocallis* spp., pollen viability was highest during the morning hours, generally before midday, on the day of anthesis [6]. In *Macrotyloma uniflorum*, pollen viability was highest in freshly collected pollen at or just before anthesis and declined rapidly under ambient conditions [27]. These results indicate that pollen collection time can vary substantially among taxa, even within the same genus. Therefore, taxon-specific pollen collection schedules are important for improving pollen management in *Zephyranthes*.

Pollen storage is an important strategy in plant breeding, as it enables flexibility in pollination when flowering is not synchronized. However, its effectiveness depends largely on storage conditions, particularly temperature and pollen moisture status, which play a critical role in maintaining pollen viability and longevity [28–30]. In the present study, tetrazolium-based pollen longevity differed markedly among the storage conditions.

Rapid viability loss at 25 °C indicated sensitivity to non-desiccated room-temperature storage. By comparison, storage at 4 °C moderately prolonged pollen viability but was insufficient for maintaining viability over extended storage periods. These findings are consistent with those of Calic et al. [31], who reported that high temperatures accelerate the decline in pollen viability during storage, a process often accompanied by increased metabolic activity and oxidative stress [32]. Similarly, Anuwong et al. [11] reported that *Zephyranthes* hybrid pollen could be stored for up to 12 days at 5 °C. In practical terms, short-term storage at 4 °C may be suitable for bridging brief asynchrony in flowering (e.g., 2–3 days). Because pollen stored at 4 °C in the present study was not desiccated prior to storage, the relative contributions of storage temperature and pollen moisture content could not be distinguished. Pre-desiccation may further improve pollen longevity, but this requires experimental verification. In contrast, the combined protocol of pollen desiccation followed by storage at −5 °C or −20 °C effectively maintained pollen viability for up to 120 days. Therefore, the improved pollen longevity observed under these storage conditions should be interpreted as the combined effect of pre-desiccation content and sub-zero storage. The combined protocol evaluated in the present study may provide a practical approach for pollen preservation in *Zephyranthes* breeding programs, particularly when parental taxa do not flower synchronously or when pollen must be transported or stored for controlled pollination. The retention of tetrazolium-based pollen viability following the combined protocol of pre-desiccation and storage at −5 °C or −20 °C suggests that this combined protocol may be a useful approach for maintaining pollen metabolic viability under the tested conditions in *Zephyranthes*. The improved pollen longevity observed under this combined protocol is consistent with previous reports demonstrating that reducing pollen moisture before low-temperature storage supports long-term pollen preservation [33]. However, the relative contribution of each factor could not be determined in the present study. Similar storage-related responses have been reported in other species. Almeida et al. [34] reported that *Amaryllis* pollen could be maintained for several weeks to approximately 75 days at 8 °C and −20 °C. Likewise, Fayos et al. [35] showed that *Allium cepa* pollen could be stored for up to 2 years at −20 °C and −80 °C. Together, these findings support the potential application of the combined pre-desiccation and sub-zero storage protocol for preserving tetrazolium-based pollen viability in *Zephyranthes*.

The stigmas examined in the present study exhibited minimal secretion, consistent with the dry-type stigma previously reported in *Zephyranthes* [36,37]. Consequently, assessments based on stigmatic exudates were considered less informative and were not investigated further. Instead, stigma receptivity was evaluated using an H_2O_2 assay, which detects stigma-associated enzymatic activity and serves as a biochemical indicator of stigma receptivity [22]. In this study, the H_2O_2 assay revealed positive stigma responses throughout the evaluated flowering period in all taxa, with taxon-specific variation in duration and intensity. The assay reflects oxidative enzyme activity at the stigmatic surface, particularly peroxidase activity [38]. Previous studies have shown that stigma-associated enzyme activities, including peroxidases and esterases, can serve as biochemical indicators of stigma receptivity [39]. Such stigma-associated enzymatic activities have been implicated in pollen–stigma interactions, including processes related to pollen germination, pollen tube growth, and compatibility responses during pollination [40,41]. Variation in bubble intensity among taxa suggests differences in the biochemical activity detected by the H_2O_2 assay, although the specific enzymatic mechanisms were not investigated in the present study. These findings are consistent with previous reports in other plant species. In 18 Bromeliaceae species, stigma receptivity assessed using H_2O_2 staining was highest between 08:00 and 12:00 and declined thereafter, with species-specific differences in the timing of minimum receptivity [42]. Similarly, stigma receptivity in *Garcinia imberti* was

observed only after flower opening [43]. For a more comprehensive evaluation of stigma receptivity, the H_2O_2 assay may be complemented by other techniques, including histochemical staining, fluorescence-based methods, or direct measurements of stigma enzyme activity. However, H_2O_2 bubbling reflects stigma-associated enzymatic activity and should therefore be interpreted as a biochemical indicator of potential stigma receptivity, with reproductive significance requiring confirmation through subsequent pollination outcomes.

Differences between H_2O_2-based stigma responses and subsequent reproductive outcomes were evident among the *Zephyranthes* taxa evaluated in the present study. Although all taxa exhibited positive H_2O_2-based stigma responses throughout the flowering period, they differed markedly in fruit set, seed production, and seed germination following controlled self-pollination. These findings indicate that H_2O_2-based stigma responses alone were insufficient to predict reproductive outcomes following controlled self-pollination. Self-pollination performance in *Zephyranthes* appears to be highly taxon-dependent. Therefore, practical pollination periods in *Zephyranthes* should be defined by considering reproductive outcomes together with biochemical assessments of stigma receptivity. Among the evaluated taxa, *Z. citrina* exhibited consistently high self-pollination performance, achieving 100% fruit set across all pollination times on both 0 and 1 DAA. This stable fruit set indicates a broad period during which self-pollination can result in fruit formation under the tested conditions. This observation is consistent with previous reports describing *Z. citrina* as self-compatible [37], and the floral morphology of *Z. citrina* may facilitate self-pollen deposition. Its consistently high fruit set and reliable seed production following self-pollination suggest that *Z. citrina* may serve as a useful taxon for controlled pollination studies and comparative investigations of reproductive traits among *Zephyranthes* taxa.

In contrast to *Z. citrina*, *Z. traubii* exhibited a markedly narrower period during which controlled self-pollination resulted in fruit set, indicating that fruit formation in this species may depend on a more limited pollination window. Fruit set occurred only when pollination was performed from the early stages of anthesis (17:00–19:00) until 12:00–14:00 on the following day, whereas no fruit set was observed thereafter. Although the present results suggest that fruit set varied according to pollination timing, the possible contribution of other biological factors cannot be completely excluded. Therefore, further studies are required to determine the mechanisms underlying this rapid decline in fruit set. Similar temporal limitations have been reported in other flowering plants, where flower age influences stigma receptivity, pollen performance, and reproductive success [44–46]. However, overall fruit set remained low (2.50–36.67%), suggesting that factors other than pollination timing also contribute to reduced fruit production. One possible explanation is the exceptionally long style of *Z. traubii* (13.68 ± 0.09 cm), which may increase the distance and time required for pollen tubes to reach the ovules. Because pollen tube growth is a time-dependent process, longer stylar pathways may reduce fertilization efficiency, particularly when the period available for successful pollen tube growth is limited [47,48]. Nevertheless, pollen tube growth was not directly examined in the present study; therefore, this explanation should be considered a plausible hypothesis rather than a confirmed mechanism. Thus, fruit set following controlled self-pollination in *Z. traubii* varied with pollination timing and may also be influenced by floral morphology.

In *Z. grandiflora*, fruit set occurred at all pollination periods on 0 DAA, indicating that controlled self-pollination resulted in fruit formation across the daytime anthesis period under the tested conditions. However, overall fruit set varied widely (3.33–53.33%), suggesting that self-pollination performance differed among pollination times. The highest fruit set was observed during 08:00–10:00, whereas seed germination percentage increased at later pollination times, reaching a maximum at 14:00–16:00. These results suggest that both fruit set and subsequent seed germination varied among pollination times. Although

fruit set was relatively low at later pollination times, particularly at 14:00–16:00, seeds from the fruits produced during these periods exhibited high germination capacity. The contrasting temporal patterns of fruit set and seed germination indicate that both traits should be considered when evaluating reproductive outcomes in *Z. grandiflora.* This interpretation is consistent with previous studies showing that pollination outcomes vary according to pollination timing and floral developmental stage [7,44,49].

'Pim Chomphu' exhibited low fruit set following self-pollination, and seeds obtained from the few developing fruits failed to germinate. This pattern suggests that reproductive failure may have occurred after pollination, although the stage at which failure occurred was not directly examined. Similar post-pollination reproductive failure, including embryo abortion and reduced seed viability following self-pollination, has been reported in species such as *Cassia fasciculata* [50] and *Pseudowintera axillaris* [51]. Further investigation of pollen tube growth using aniline blue staining, together with observations of embryo development, would help identify the stage at which reproductive failure occurs in this cultivar.

The absence of fruit set in *Z. candida* despite positive H_2O_2-based stigma responses throughout the flowering period suggests that self-pollination was unsuccessful under the tested conditions. This result may reflect reproductive barriers, including possible self-incompatibility or other post-pollination limitations, but the underlying mechanism cannot be confirmed without direct observation of pollen tube growth and embryo development. This interpretation is consistent with the findings of Ghosh and Shivanna [37], who reported that pollen tube growth in *Z. candida* was inhibited at the stigma–style junction, which was interpreted as evidence of a possible pre-fertilization barrier in their study. Although similar pollen tube inhibition has previously been reported in *Z. candida*, whether the same reproductive barrier occurred in the material examined in the present study remains unknown. Direct observation using aniline blue staining would be necessary to determine whether similar pollen tube inhibition occurs in the material examined in the present study.

This study was conducted under natural greenhouse conditions, with most experiments performed during the rainy season (May–October). Because flowering times differed among taxa, some observations were made under slightly different environmental conditions. Consequently, slight differences in environmental conditions among observation periods may have contributed to some of the observed differences in reproductive traits. From a practical perspective, pollination performed at 12:00–14:00 on 1 DAA may represent a suitable reference period for controlled pollination in *Z. traubii*, as this interval resulted in relatively high fruit set and seed germination under self-pollination conditions and may be more practical than pollination during anthesis at 19:00–21:00. In *Z. grandiflora*, pollination during 14:00–16:00 on 0 DAA corresponded to high seed germination, whereas *Z. citrina* exhibited consistently high fruit set and seed germination across all pollination periods, indicating a broad self-pollination window under the tested conditions. For *Z. candida* and 'Pim Chomphu', the period between 10:00 and 12:00 on 0 DAA may provide a practical starting point for future cross-pollination studies because flowers were fully open during this period. However, its suitability for controlled crossing remains to be verified using compatible pollen donors through assessment of pollen tube growth and subsequent reproductive outcomes.

Overall, these findings indicate substantial taxon-dependent variation in pollination timing responses among the evaluated *Zephyranthes* taxa and that biochemical assessments of stigma receptivity should be interpreted together with downstream reproductive outcomes. The observed differences among taxa suggest that additional biological factors beyond tetrazolium-based pollen viability and H_2O_2-based stigma responses contribute to subsequent reproductive outcomes following controlled self-pollination. Thus, controlled

pollination strategies in *Zephyranthes* may be informed by an integrated assessment of reproductive traits rather than relying on a single indicator of pollen or stigma function.

5. Conclusions

This study revealed substantial variation in tetrazolium-based pollen viability, pollen longevity under different storage protocols, H_2O_2-based stigma responses, and reproductive outcomes following controlled self-pollination among *Zephyranthes* taxa. Tetrazolium-based pollen viability showed taxon-specific temporal patterns, while the combined protocols of pollen desiccation followed by storage at −5 °C and −20 °C maintained tetrazolium-based pollen viability for up to 120 days. Although all taxa showed positive H_2O_2-based stigma responses, reproductive outcomes following self-pollination differed markedly among taxa, indicating that H_2O_2-based responses alone were insufficient to predict subsequent reproductive outcomes under the tested conditions. Across the evaluated taxa, *Z. citrina* showed consistently high fruit set, with relatively stable seed germination after self-pollination, whereas *Z. traubii* and *Z. grandiflora* showed variation in fruit set and seed germination depending on pollination timing, 'Pim Chomphu' showed low fruit set with non-germinating seeds, and *Z. candida* produced no fruit following self-pollination under the tested conditions. The present findings provide baseline reproductive information to support pollen handling, pollination scheduling, and future controlled crossing studies in *Zephyranthes*.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijpb17080068/s1, Table S1. Preliminary assessment of pollen viability in *Z. traubii* at 1 and 2 days after anthesis (1 and 2 DAA), and in *Z. candida*, *Z. grandiflora*, *Z. citrina*, and 'Pim Chomphu' at anthesis and 1 day after anthesis (0 and 1 DAA), as determined by the tetrazolium test. Figure S1: Floral characteristic at the time of pollen collection: (a) *Z. traubii*; (b) *Z. candida*; (c) *Z. grandiflora*; (d) *Z. citrina*; and (e) 'Pim Chomphu'.

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Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

ANOVA	Analysis of variance
CRD	Completely randomized design
DAA	Days after anthesis
DAP	Days after pollination
MET	Mean emergence time

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