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DETERMINATION OF VIABILITY AND GERMINATION CHARACTERISTICS OF LETTUCE (*LACTUCA SATIVA* L.) POLLEN UNDER DIFFERENT STORAGE CONDITIONS

ŞAHAN TEZCAN AND KENAN SÖNMEZ*

Department of Horticulture, Faculty of Agriculture, Eskişehir Osmangazi University, Eskişehir, Türkiye

*Corresponding author's email: ksonmez@ogu.edu.tr; ORCID: 0000-0003-4040-4555

Abstract

This study was conducted to determine the viability and germination characteristics of lettuce (*Lactuca sativa* L.) pollen under different storage conditions (refrigerator and room temperature), in different containers (Eppendorf tubes and watch glasses) and over different durations (0, 1, 3, 5, 7, 10, 15 and 30 days). To the best of our knowledge, this is the first study to comprehensively evaluate the combined effects of storage temperature, storage container, storage duration, and multiple germination media on lettuce pollen performance using both univariate and multivariate statistical approaches. Data were analysed using appropriate univariate and multivariate statistical methods to evaluate treatment effects and identify the major factors influencing pollen performance. Refrigerated storage and Eppendorf tubes significantly improved pollen germination compared with room temperature and watch glasses. Germination declined progressively with storage duration, while Medium 6 (45% sucrose supplemented with H_3BO_3 and $Ca(NO_3)_2$ without agar) produced the highest germination percentage. Multivariate analyses further identified storage duration and germination medium as the principal factors influencing pollen performance. These findings establish an optimized protocol for short-term lettuce pollen storage and provide valuable guidance for hybrid breeding, germplasm conservation, and future studies on lettuce reproductive biology.

Key words: *In vitro* germination; *Lactuca sativa*; Pollen storage; Sucrose; Temperature; Viability

Introduction

Lettuce (*Lactuca sativa* L.), a member of the Asteraceae family, ranks among the most widely consumed fresh vegetables globally. Due to its high water content, low caloric value and significant levels of vitamins A, C, K and folate, lettuce plays a vital role in human nutrition (Nicolle *et al.*, 2004). Annual global production is nearly 27 million tons, with intensive cultivation occurring in countries such as China, the USA and India (Anon., 2024). Recently, increasing attention has been directed toward the physiological and genetic characteristics of lettuce, particularly in relation to sustainable agriculture and climate change adaptation. New research continues to expand knowledge on high-temperature germination inhibition, seed quality and yield (Hartman *et al.*, 2014; Hassan *et al.*, 2021).

Lettuce exhibits a high self-pollination rate (up to 99%) and a brief flowering period, with flowers closing within 2–3 h (Eenink, 1983; Sarıçam *et al.*, 2017). Each flower head contains 10–20 individual florets, making emasculation a labour-intensive process (Hassan *et al.*, 2021). These reproductive traits complicate controlled hybridization and necessitate efficient pollen collection and storage. The global decline in pollinator populations further highlights the need for controlled pollination and effective pollen management (Thomann *et al.*, 2013). Pollen storage is essential for facilitating crosses between parental lines with asynchronous flowering, preserving genetic material for germplasm studies and enhancing the efficiency of breeding programmes (Barnabas & Kovacs, 1997; Sidhu, 2019; Volk *et al.*, 2026). Lettuce genetic resources are also tracked through online germplasm databases (Anon., 2023). Advances in molecular breeding strategies have increased interest in pollen biology, leading to new research on male-sterility mechanisms, quantitative trait locus (QTL) analyses and genomic resources (Hartman *et al.*, 2014; Seki, 2022).

Pollen viability is affected by multiple factors, including species, cultivar, harvest timing, storage temperature, humidity, container type and storage duration (Dafni & Firmage, 2000; Gill, 2014; Althiab-Almasaud *et al.*, 2024). Research consistently demonstrates that low-temperature and low-humidity conditions extend pollen viability (Barnabas & Kovacs, 1997; Maryam *et al.*, 2015; Aldahadha *et al.*, 2020; Kadri *et al.*, 2022). The type of pollen is also significant: binucleate pollen generally remains viable longer than trinucleate pollen, as the latter's higher respiration rate accelerates viability loss during storage (Sidhu, 2019; Carter *et al.*, 2025). Cryopreservation has been identified as the most effective method for long-term storage in *Luffa* species (Nagar *et al.*, 2023), while in apple (Čalić *et al.*, 2021) and pepper (Du *et al.*, 2025), pollen stored at −80°C retains viability for several months. For short-term storage (up to 30 days), refrigerator temperatures are considered effective (Nagar *et al.*, 2023; Dutta, 2025). Additionally, Du *et al.*, (2019) found that low-temperature storage significantly improved pollen viability in herbaceous peony species and that the choice of storage container was critical in minimizing moisture loss.

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The composition of the *İn vitro* pollen germination medium is a critical determinant of viability assessment outcomes. Standard formulations, such as the Brewbaker and Kwack (BK) medium, include boron, calcium and sucrose, which are essential for pollen tube elongation in many species (Tushabe & Rosbakh, 2021). Tushabe & Rosbakh (2021), using a database of more than 1,800 articles published between 1926 and 2019, developed species-optimized *In vitro* germination medium recipes and emphasized the need for methodological standardization in pollen research. Boron supports sugar transport via sugar–borate complexes, whereas calcium is involved in signal transduction, cytoskeletal organization and exocytosis during pollen tube growth (Zheng *et al.*, 2019). Sucrose acts as both an osmotic regulator and an energy source; its optimal concentration varies by species, ranging from 5–20% in fruit species to 10–45% in vegetable species (Shi *et al.*, 2023; Li *et al.*, 2025). For daylily (*Hemerocallis* spp.) pollen, Li *et al*., (2025) identified sucrose concentration as the key factor, with the optimal medium comprising 5% sucrose, H_3BO_3, KNO_3 and $Ca(NO_3)_2$. Shi *et al*., (2023) demonstrated, using an orthogonal experimental design, that sucrose, boric acid and calcium chloride levels influence germination and tube elongation in alfalfa pollen in a dose-dependent manner. *In vitro* pollen germination and viability assessment methods are increasingly applied to vegetable and fruit species, including eggplant (Ginoya *et al.*, 2022), apricot (Asma, 2008) and rose (Ercişli, 2007). General protocols have also been established for various species (Patel & Mankad, 2014). However, the inclusion of agar may negatively affect pollen tube elongation in certain species (Tushabe & Rosbakh, 2021). In addition, Lagera *et al*., (2017) found that the type and concentration of sugar significantly affect germination and tube growth in *Cassia alata* pollen.

Seki (2022) elucidated the molecular basis of male sterility in lettuce using ddRAD-seq and resequencing analyses, and identified *LsACOS5* as a candidate gene. Hassan *et al*., (2021) provided a comprehensive review of lettuce genetic resources and molecular breeding strategies, summarizing the challenges posed by the plant's floral biology to breeding studies. Pollen storage methods have also been developed in other vegetable species; for example, effective storage protocols have been defined for pointed gourd (*Trichosanthes dioica* Roxb.) pollen (Hassan *et al.*, 2020). Hartman *et al.* (2014) compared abiotic-stress QTLs in lettuce crop–wild hybrids under greenhouse and field conditions and emphasized the importance of obtaining healthy pollen for the conservation and evaluation of genetic resources. Nevertheless, previous studies have primarily examined individual factors affecting pollen performance, such as storage temperature, storage duration, or germination media, in isolation. To date, no study has comprehensively evaluated the combined effects of storage temperature, storage container, storage duration, and multiple germination media on lettuce pollen using both conventional and multivariate statistical analyses. This knowledge gap limits the development of standardized protocols for lettuce breeding and germplasm conservation.

The present study was designed to bridge this gap by integrating multiple storage factors and germination media into a single experimental framework, thereby providing both biological insight and practical recommendations for lettuce breeding programmes.

In addition, multivariate statistical analyses were employed to identify the relative importance of storage-related factors and to provide a more comprehensive interpretation of pollen performance than could be achieved using univariate analyses alone.

Accordingly, the present study aimed to determine (i) the effects of different storage temperatures, container types and durations on pollen viability; (ii) a comparative evaluation of 12 different *İn vitro* germination media; and (iii) practical recommendations for the short-term storage of lettuce pollen.

We hypothesised that refrigerated storage in sealed containers, combined with sucrose-enriched germination media containing boric acid and calcium nitrate, would maximize pollen germination and viability during short-term storage.

Materials and Methods

Plant material and pollen collection: The research was conducted at the United Genetics Turkey Seed and Seedling Co. Ltd. R&D centre from November 2020 to July 2021. The LW16 lettuce variety, developed by United Genetics Turkey Seed and Seedling Co. Ltd., served as the pollen donor. This genotype was selected because of its stable flowering behaviour, reliable pollen production, and its routine use in the company's lettuce breeding programme, making it an appropriate experimental material for pollen storage studies. This variety is distinguished by its curly, glossy green leaves and a medium to late bolting tendency. Pollen was obtained from lettuce (*Lactuca sativa* L.) plants and used in this study. Pollen was collected during the full-flowering period in the early morning hours from flowers with dehisced anthers, using a sterile scalpel and a brush. The collected pollen was dried in an oven at 45°C for 24 h to remove moisture before processing, and then divided for use in the experiments. The drying procedure was adopted to minimise moisture content before storage while maintaining pollen integrity, following practices commonly recommended for short-term pollen preservation.

Storage conditions and durations: Two storage conditions were applied in the experiment: (1) refrigerator (4 ± 1°C) and (2) room conditions (20–22°C). Under both conditions, pollen was kept in two different storage containers: (a) 1.5 mL capped Eppendorf tubes and (b) watch glasses. Pollen was sampled on days 0, 1, 3, 5, 7, 10, 15 and 30 and subjected to *İn vitro* germination tests. Each treatment combination consisted of three independent biological replicates, and each replicate was evaluated separately during statistical analysis. The experiment was conducted using a completely randomized factorial design consisting of storage temperature, storage container, storage duration, and germination medium as the main experimental factors.

Germination media: Pollen germination experiments were carried out in 12 different media prepared on the basis of culture media recommended in the literature (Table 1). The media consisted of combinations with or without H_3BO_3 (100 ppm) and $Ca(NO_3)_2 \cdot 4H_2O$ (100 ppm), with

0%, 40% or 45% sucrose, and with or without 1% agar. For the germination tests, pollen was transferred onto the prepared media and incubated in a humidity chamber at 25 ± 1°C for 24 h. At the end of incubation, at least 100 pollen grains were counted across 10 microscope fields of view for each replicate and classified as germinated, non-germinated or underdeveloped (pollen tube shorter than half the pollen diameter). Pollen grains were considered germinated when the pollen tube length exceeded the diameter of the pollen grain.

Table 1. Composition of the 12 *In vitro* pollen germination media used in the experiment.

Medium No.	$H_3BO_3 + Ca(NO_3)_2$ (100 ppm)	Sucrose (%)	Agar (%)
1.	Present	0 (none)	1
2.	Present	40	1
3.	Present	45	1
4.	Present	0 (none)	0 (none)
5.	Present	40	0 (none)
6.	Present	45	0 (none)
7.	Absent (pure water)	0 (none)	1
8.	Absent (pure water)	40	1
9.	Absent (pure water)	45	1
10.	Absent (pure water)	0 (none)	0 (none)
11.	Absent (pure water)	40	0 (none)
12.	Absent (pure water)	45	0 (none)

All media were prepared with pure water; boric acid (H_3BO_3) and calcium nitrate ($Ca(NO_3)_2 \cdot 4H_2O$) were added at 100 ppm and agar at 1% (w/v)

Traits evaluated: The following ratios were calculated for each sample: germination rate (%) = (germinated pollen / total pollen) × 100; viability rate (%) = [(germinated + underdeveloped) / total pollen] × 100.

Statistical analysis

Data were analysed using SPSS (version 26.0; IBM Corp., Armonk, NY, USA). One-way analysis of variance (ANOVA) was applied separately for the factors of storage condition, storage container, storage duration and germination medium; statistically significant differences between means were determined by Duncan's multiple range test (at the 5% probability level). In addition to the means, ± standard deviation and coefficient of variation (CV%) values are presented. Pearson correlation analysis was applied to assess the linear relationships among the factors. Principal component analysis (PCA) was performed to explore the multivariate structure, and canonical discriminant analysis (CDA) was conducted to evaluate group separation. Prior to analysis, data were examined for compliance with the assumptions of normality and homogeneity of variance.

Results

Effect of storage condition and container: The results of the analysis of variance are summarized in Table 2. The effect of storage condition on germination rate was statistically significant ($F = 37.025$, $p<0.01$, CV = 129.66%). The germination rate of pollen stored in the refrigerator (13.74 ± 16.57 a) was statistically higher than that of pollen stored under room conditions (8.62 ± 11.53 b). In contrast, no significant difference in viability rate was observed between refrigerator (39.66 ± 21.94) and room conditions (39.33 ± 16.86) ($F = 0.086$, $p = 0.769$, NS).

The storage-container factor had a statistically significant effect on both germination and viability rates. Pollen stored in Eppendorf tubes was markedly superior to that stored on watch glasses in terms of both germination rate (14.34 ± 16.12 a vs. 8.02 ± 11.86 b; $F = 57.598$, $p<0.01$) and viability rate (41.14 ± 21.55 a vs. 37.85 ± 17.21 b; $F = 8.196$, $p<0.01$).

Effect of storage duration: Storage duration had a statistically significanteffect on germination rate ($F = 47.173$, $p<0.01$; Table 3). The highest germination rate was obtained on day 0 (22.36 ± 17.36%, a); this rate declined markedly with increasing storage duration, falling to 1.75 ± 5.44% (e) by day 30. According to Duncan's test, day 0 alone formed group "a"; days 1 and 3 formed group "b"; days 5, 7 and 10 formed group "c"; day 15 formed group "d"; and day 30 formed group "e". These findings indicate that lettuce pollen rapidly loses viability and has a short shelf life for practical breeding purposes. In terms of viability rate, the differences among durations were not statistically significant ($F = 1.705$, $p = 0.104$, NS). Overall, germination decreased by more than 90% during the 30-day storage period, indicating a substantial reduction in pollen performance over time (Fig. 1).

Table 3. Germination and viability rates by storage duration.

Duration (days)	Germination rate (%)	Viability rate (%)
0	22.36 ± 17.36 a	39.87 ± 22.65
1	18.43 ± 15.33 b	37.60 ± 22.93
3	16.64 ± 14.82 b	40.32 ± 21.78
5	10.54 ± 12.57 c	37.52 ± 19.00
7	8.45 ± 11.11 c	36.30 ± 19.58
10	7.92 ± 13.67 c	41.91 ± 18.25
15	3.34 ± 7.68 d	40.40 ± 15.19
30	1.75 ± 5.44 e	42.04 ± 15.06
F	**47.173**	**1.705**
p	**0.0000**	**0.1038**
Significance	**	**NS**
CV (%)	**129.66**	**49.53**

Different letters within a column indicate statistical differences (Duncan, $p<0.05$). **: $p<0.01$; NS: not significant. No letters are given for viability rate because the F test was not significant

Table 2. Analysis of variance results for the effect of storage condition and storage container.

Source	Variable	Mean ± SD	F value	p value	Sig.
Storage condition	Germination rate (%)	Refrigerator: 13.74 ± 16.57 a; Room: 8.62 ± 11.53 b	37.025	0.0000	**
	Viability rate (%)	Refrigerator: 39.66 ± 21.94; Room: 39.33 ± 16.86	0.086	0.7691	NS
Storage container	Germination rate (%)	Eppendorf: 14.34 ± 16.12 a; Watch glass: 8.02 ± 11.86 b	57.598	0.0000	**
	Viability rate (%)	Eppendorf: 41.14 ± 21.55 a; Watch glass: 37.85 ± 17.21 b	8.196	0.0043	**

**: Significant at $p<0.01$; NS: Not significant; SD: Standard deviation. Means followed by the same letter within a factor do not differ statistically (Duncan, $p<0.05$)

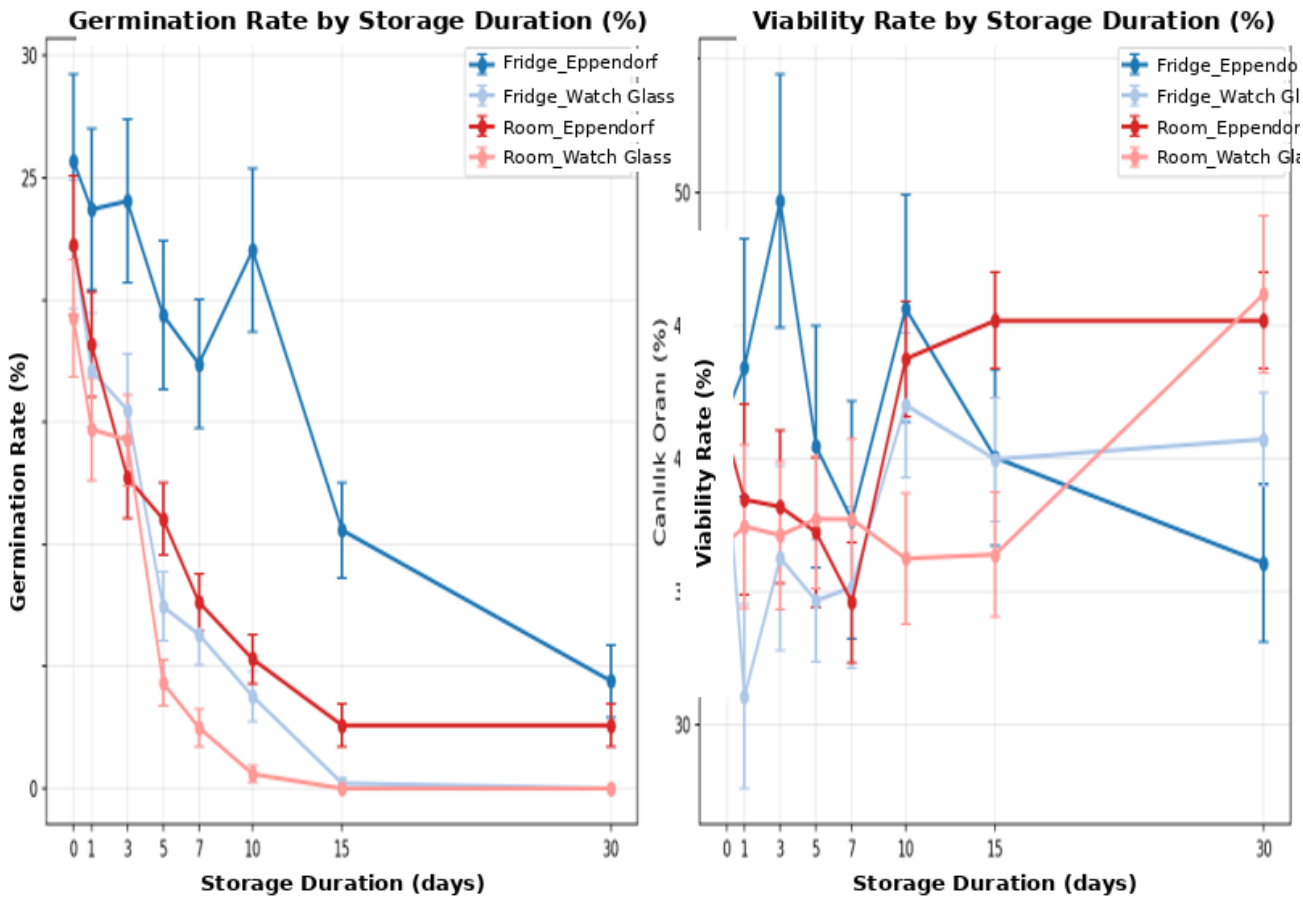

Fig. 1. Change in germination and viability rate depending on storage duration (by storage condition and storage container).

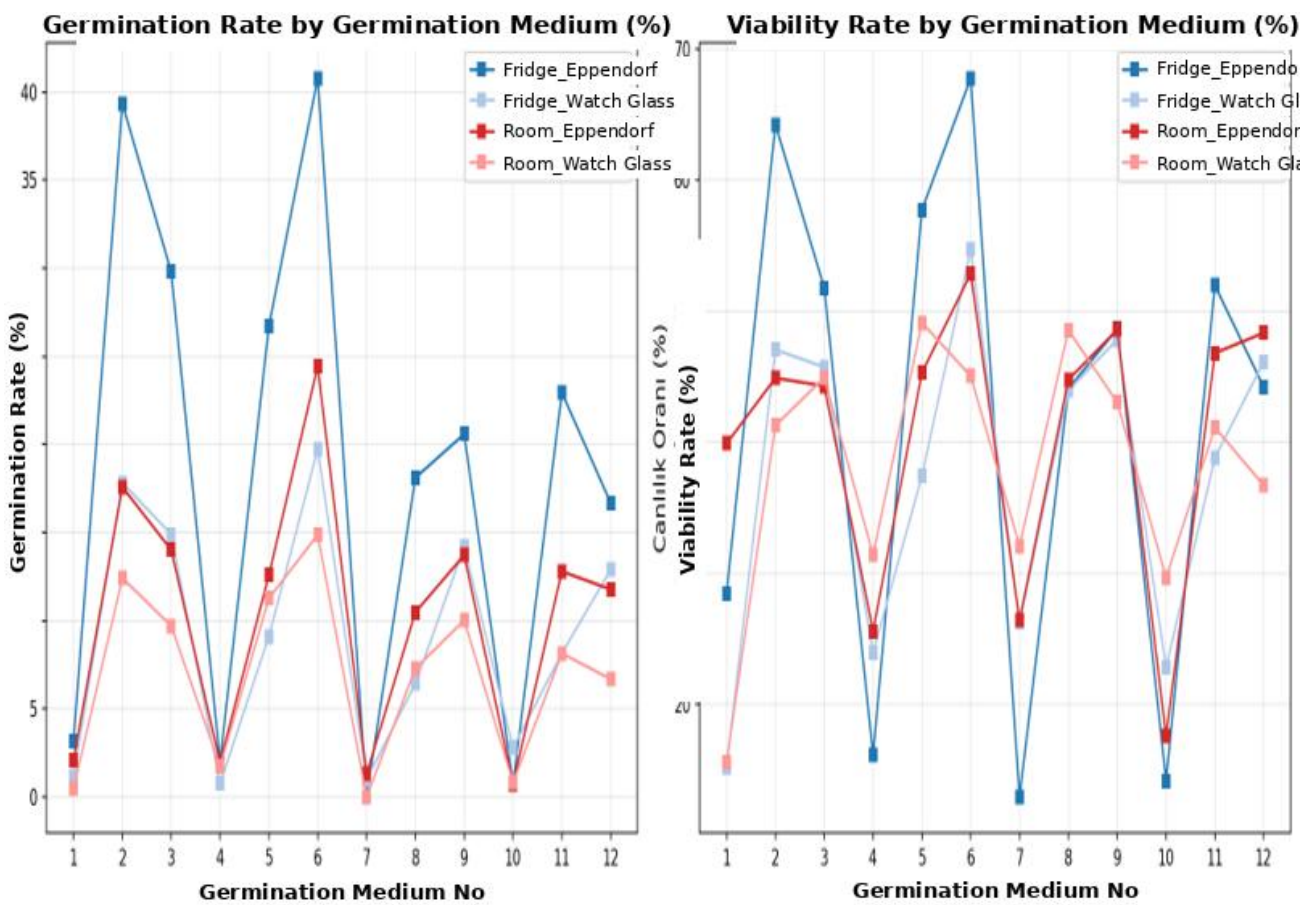

Fig. 2. Mean germination and viability rates by germination medium.

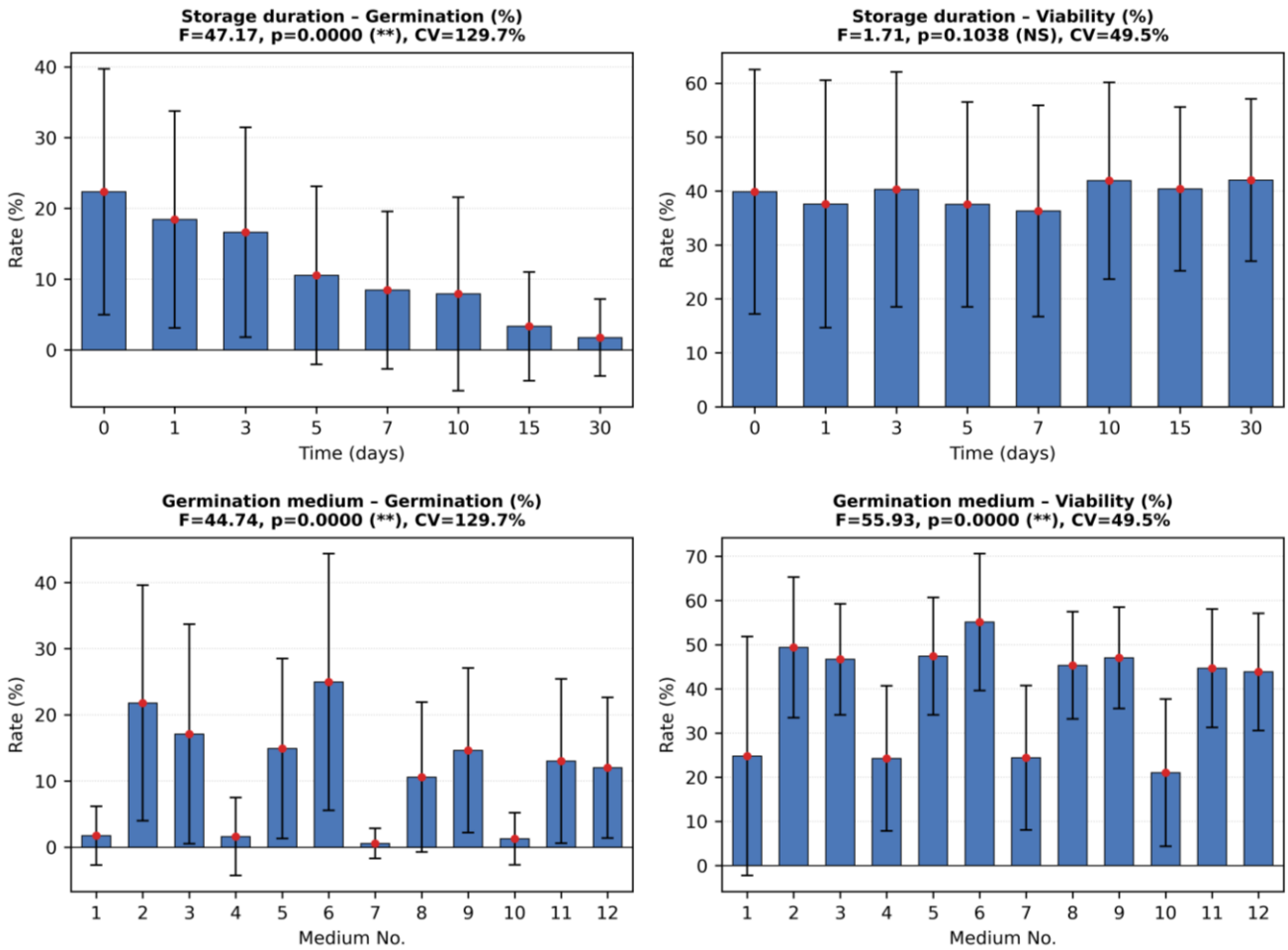

Fig. 3. Duncan groups for germination and viability rate across storage-duration and germination-medium factors (mean ± standard deviation).

The germination medium had a statistically significant effect on both germination rate ($F = 44.736$, $p<0.01$) and viability rate ($F = 55.932$, $p<0.01$) (Table 4). In terms of germination rate, Medium 6 (24.96 ± 19.38%, a) and Medium 2 (21.79 ± 17.80%, a) were in the same group and exhibited the highest performance. These were followed by Medium 3 (17.10%, b), Medium 5 (14.91%, b), Medium 9 (14.63%, b) and Medium 11 (13.01%, b). The pure-water, sucrose-free Medium 7 gave the lowest germination rate (0.57 ± 2.27%, e) (Fig. 2).

In terms of viability rate, the best result was likewise obtained from Medium 6 (55.10 ± 15.49%, a) (Fig. 3).

Correlation analysis: The results of the Pearson correlation analysis are given in Table 5. A significant negative correlation was found between storage duration and germination rate ($r = -0.433$, $p<0.01$). This finding statistically confirms that pollen loses viability as storage duration increases. The strong positive correlation between germination rate and viability rate ($r = 0.644$, $p<0.01$) indicates, as expected, a close relationship between the two variables. No statistically significant relationship was observed between storage duration and viability rate ($r = 0.070$, $p = 0.173$, NS). These findings indicate that storage duration is one of the principal factors affecting pollen germination under the evaluated storage conditions (Fig. 4).

Principal component analysis (PCA): According to the PCA results, seven components were obtained, and the first two explained 70.41% of the total variance (PC1: 42.85%; PC2: 27.56%). On the PC1 axis, germination rate and viability rate showed high positive loadings, whereas storage duration loaded negatively. On the PC2 axis, germination medium number and container type emerged as the determining variables. The biplot confirmed that PC1 and PC2 adequately represented the essential information in the dataset (Fig. 5). These results reveal that storage duration and germination medium composition constitute the two principal axes in determining lettuce pollen viability. Biologically, these findings suggest that storage duration and germination medium exert the greatest influence on maintaining pollen functionality during storage.

Canonical discriminant analysis (CDA): Canonical discriminant analysis was applied to assess which variables separated the groups corresponding to the different storage durations (0, 1, 3, 5, 7, 10, 15 and 30 days). Three canonical functions were obtained in total, and the overall classification accuracy of the model was 47.4%. This moderate performance is consistent with the nature of the data set, particularly given the wide distribution of germination rates, as indicated by its high coefficient of variation (129.66%). Examination of the canonical plot (Figs. 6 and 7) showed that the early-period groups (0–3 days) were clearly separated from the late-period groups (15–30 days). In this separation, storage duration and germination medium acted as the main discriminant variables.

Table 4. Germination and viability rates by germination medium.

No.	Medium composition	Germination rate (%)	Viability rate (%)
1	Water + H_3BO_3 + $Ca(NO_3)_2$ (1% agar, no sucrose)	1.74 ± 4.45 d	24.79 ± 27.05 d
2	Water + H_3BO_3 + $Ca(NO_3)_2$ (1% agar, 40% sucrose)	21.79 ± 17.80 a	49.37 ± 15.93 b
3	Water + H_3BO_3 + $Ca(NO_3)_2$ (1% agar, 45% sucrose)	17.10 ± 16.58 b	46.68 ± 12.58 b
4	Water + H_3BO_3 + $Ca(NO_3)_2$ (no agar, no sucrose)	1.61 ± 5.90 d	24.26 ± 16.44 d
5	Water + H_3BO_3 + $Ca(NO_3)_2$ (no agar, 40% sucrose)	14.91 ± 13.60 b	47.40 ± 13.28 b
6	Water + H_3BO_3 + $Ca(NO_3)_2$ (no agar, 45% sucrose)	24.96 ± 19.38 a	55.10 ± 15.49 a
7	Pure water (1% agar, no sucrose)	0.57 ± 2.27 e	24.41 ± 16.36 d
8	Pure water (1% agar, 40% sucrose)	10.58 ± 11.33 c	45.33 ± 12.14 c
9	Pure water (1% agar, 45% sucrose)	14.63 ± 12.43 b	47.03 ± 11.48 b
10	Pure water (no agar, no sucrose)	1.26 ± 3.94 d	21.04 ± 16.67 d
11	Pure water (no agar, 40% sucrose)	13.01 ± 12.41 b	44.67 ± 13.39 c
12	Pure water (no agar, 45% sucrose)	12.01 ± 10.64 c	43.85 ± 13.28 c
F		**44.736**	**55.932**
p		**0.0000**	**0.0000**
Significance		**	**
CV (%)		**129.66**	**49.53**

Different letters within a column indicate statistical differences (Duncan, $p<0.05$). **: $p<0.01$

Table 5. Pearson correlation coefficients among the main variables.

Variable	Duration	Medium No.	Germination rate	Viability rate
Storage duration	1.00	−0.00 NS	−0.433 **	0.070 NS
Germination medium No.	−0.00 NS	1.00	−0.044 NS	0.049 NS
Germination rate	−0.433 **	−0.044 NS	1.00	0.644 **
Viability rate	0.070 NS	0.049 NS	0.644 **	1.00

**: Significant at $p<0.01$; NS: Not significant ($p>0.05$)

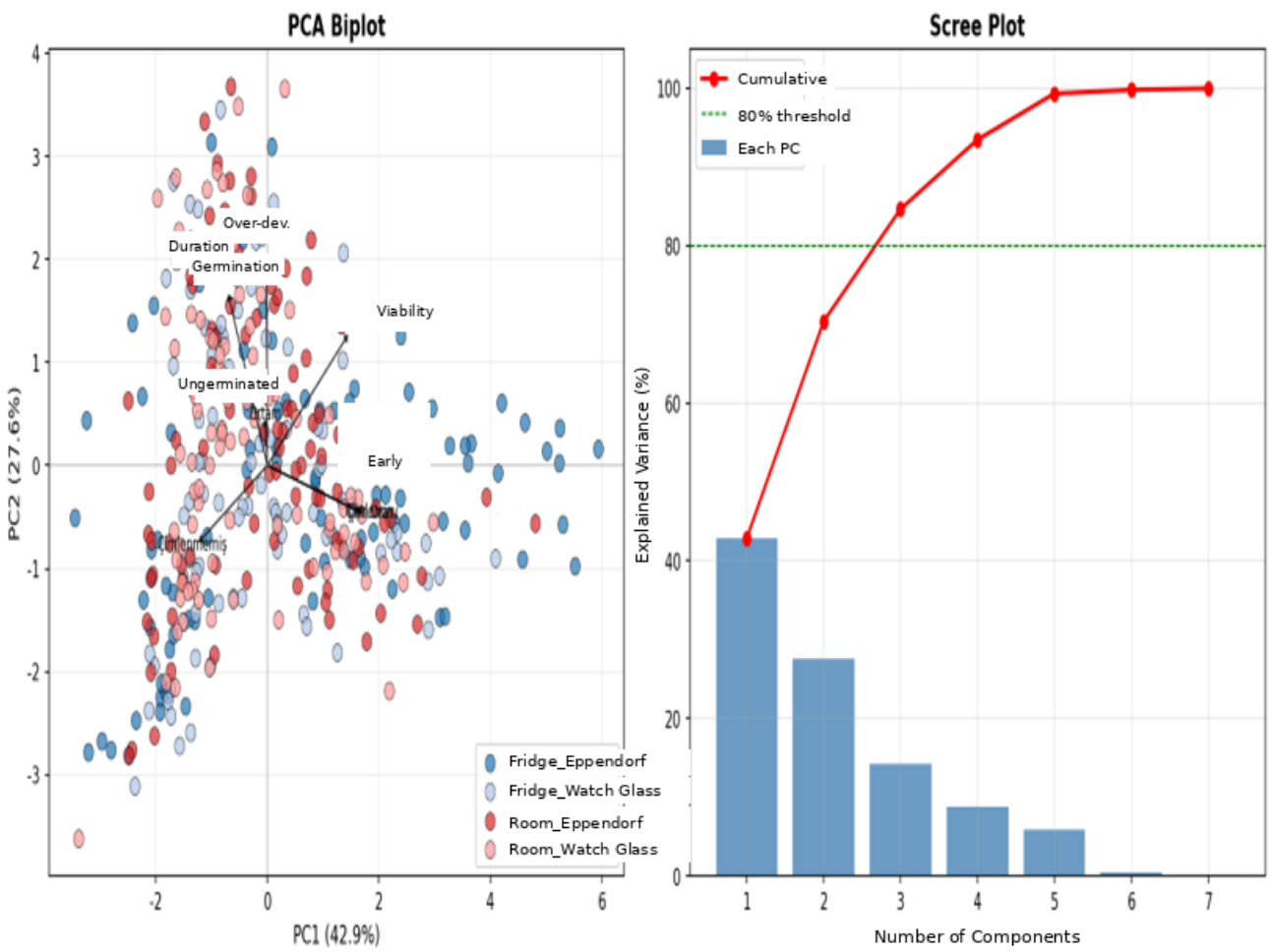

Fig. 5. PCA biplot (PC1 = 42.85%, PC2 = 27.56% of variance). Colours and symbols indicate storage condition and container type.

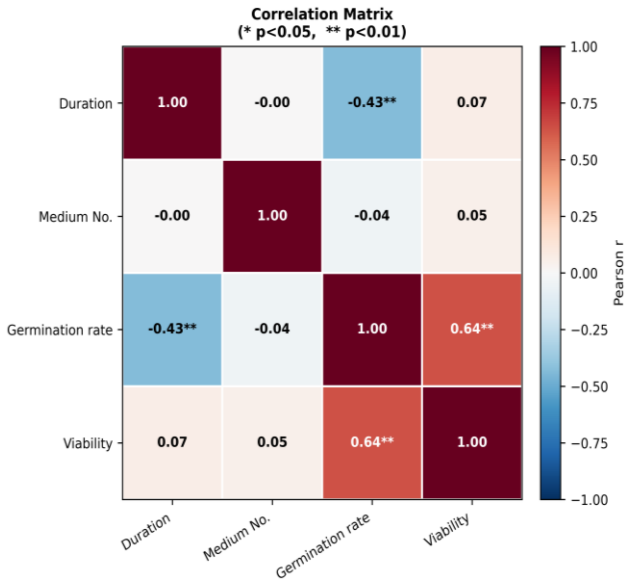

Fig. 4. Heat map of the Pearson correlation matrix.

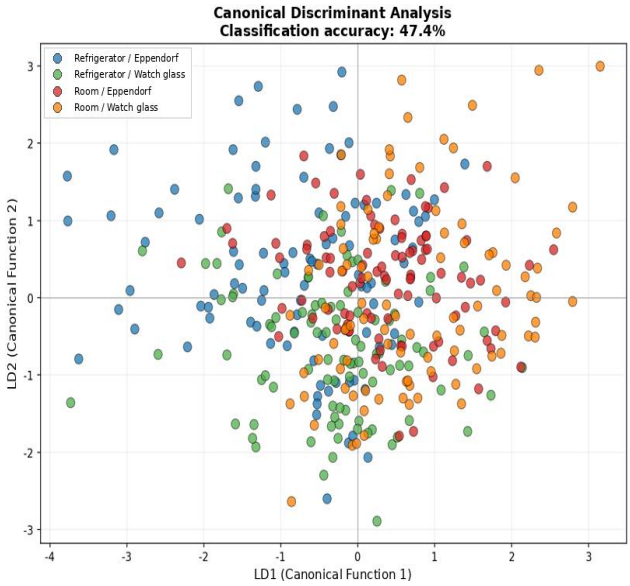

Fig. 6. Canonical discriminant analysis (CDA) biplot. Different symbols represent storage-duration groups.

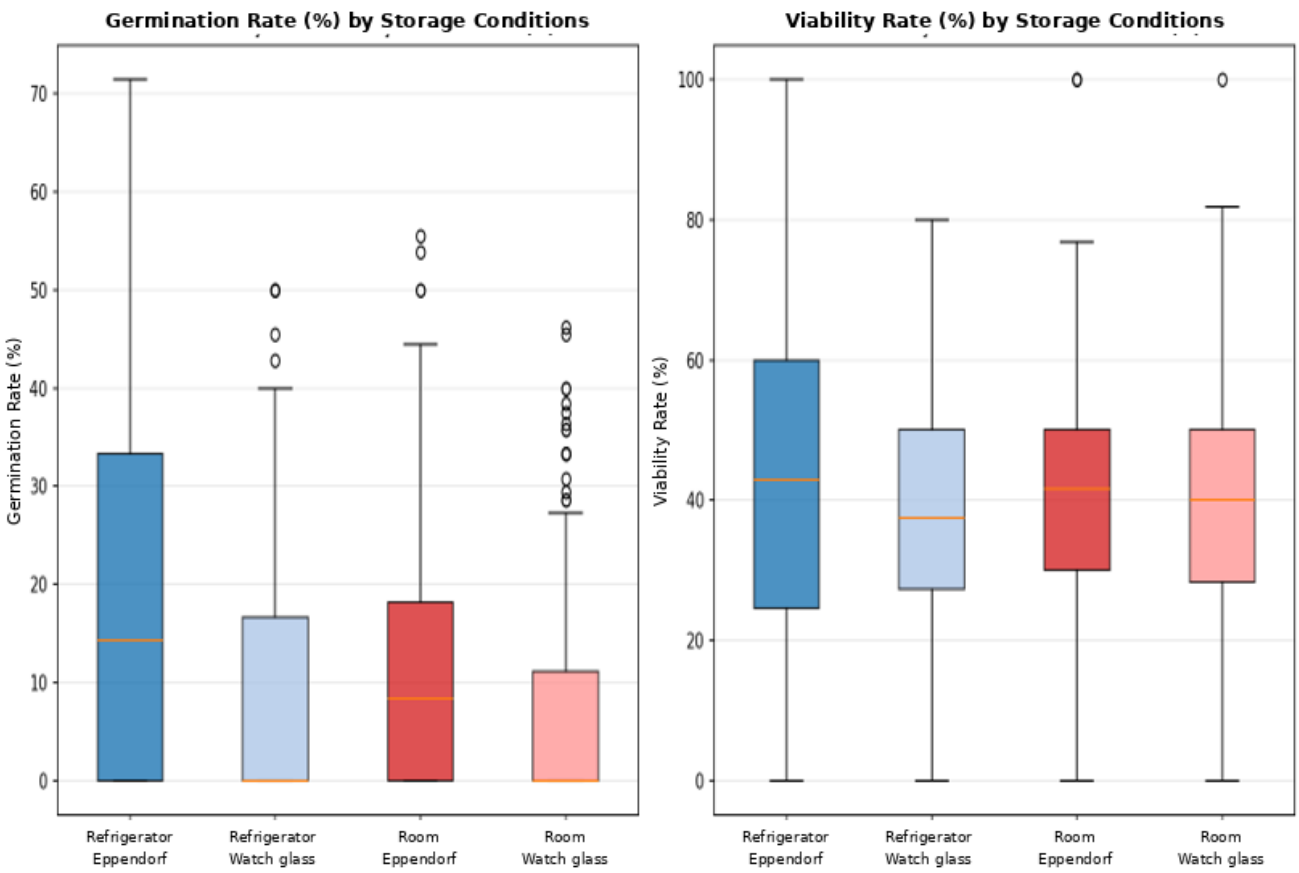

Fig. 7. Box plots of germination and viability rate for all storage condition × container groups.

Discussion

The present study demonstrated that storage temperature, storage container, storage duration, and germination medium collectively influenced lettuce pollen viability and germination. Refrigerated storage in Eppendorf tubes consistently produced superior pollen performance, while germination progressively declined with increasing storage duration. Among the evaluated media, Medium 6 provided the highest germination percentage, and multivariate analyses confirmed storage duration and germination medium as the dominant factors affecting pollen performance.

Our findings demonstrate that refrigerator conditions offer a clear advantage over room temperature for storing lettuce pollen. Cold storage is known to slow metabolic processes in pollen—particularly cellular respiration—thereby reducing oxidative damage and moisture loss

(Macovei *et al.*, 2016; Carter *et al.*, 2025). Similar results have been reported in many other species: cold-storage treatments were found to be markedly superior in pistachio (Aldahadha *et al.*, 2020) and date palm (Maryam *et al.*, 2015; Mesnoua *et al.*, 2018; Kadri *et al.*, 2022). In kiwifruit, pollen stored at room temperature was reported to lose viability completely within 42 days, whereas low temperatures markedly preserved it (Dutta, 2025). However, lettuce pollen has a much shorter shelf life than most fruit species, consistent with our finding that the germination rate declined from 22.36% to 1.75% over 30 days of storage.

The superior results obtained with Eppendorf tubes compared with watch glasses can be explained by the moisture control provided by their hermetic caps and by their protection of pollen from external environmental effects. The hermetic sealing and small internal volume of Eppendorf tubes may also have reduced exposure to moisture fluctuations and external environmental variation during storage, thereby contributing to improved pollen preservation. Lower temperatures are likely to reduce metabolic activity, membrane deterioration, and oxidative stress within pollen grains, thereby slowing the loss of viability during storage. This physiological response provides a biological explanation for the improved germination observed under refrigerated conditions. Indeed, Aslantaş & Pırlak (2001) also showed that strawberry pollen was preserved more effectively in closed containers. Similarly, in maize pollen, the combination of low temperature and adequate gas exchange in closed containers has been identified as the main factor preserving viability (Carter *et al.*, 2025). The moisture level of pollen in the container directly affects oxidative stress and enzymatic degradation (Du *et al.*, 2019).

With respect to germination media, it is noteworthy that the agar-free Medium 6—containing H_3BO_3 and $Ca(NO_3)_2$ at a 45% sucrose concentration—exhibited the highest performance. The superior performance of this medium is likely attributable to the complementary roles of its individual components. Boron promotes pollen tube wall formation, whereas calcium is essential for membrane stability and pollen tube elongation. Together with the high sucrose concentration, these components create favourable conditions for successful pollen germination and tube development. Boron is known to play an indispensable role in pollen tube growth and in maintaining cell-wall integrity, a view also supported by Zheng *et al*., (2019) and Muengkaew *et al*., (2017). The high sucrose concentration provides osmotic adjustment and supplies the energy required for germination (Lagera *et al.*, 2017). The success of agar-free treatments in certain media indicates that lettuce pollen tends to germinate in liquid media.

The PCA and CDA findings are mutually supportive: both analyses revealed that storage duration and germination medium were the most decisive factors. Similar multivariate approaches have been applied in other fruit and vegetable species and have been found to provide more comprehensive interpretations than univariate methods (Xiong *et al.*, 2020). The moderate (47.4%) classification accuracy of the CDA model reflects the high variability of the dataset and numerous uncontrollable micro-environmental factors; it is not a deficiency in the research design but rather a reflection of the biological nature of lettuce pollen. These findings indicate that maintaining pollen viability depends more strongly on storage duration and the composition of the germination medium than on individual storage variables alone, highlighting the importance of integrating multiple factors when developing pollen preservation protocols.

When evaluated in the context of lettuce breeding programmes, it is recommended that pollen be used within 1–3 days, or that refrigerator storage, combined with Eppendorf tubes, be used during the hybridisation process. Tushabe and Rosbakh (2021) emphasised the decisive role of germination medium selection across many species, and our findings confirm this for lettuce. The absence of any systematic evaluation over the four decades since the pioneering study by Eenink (1983) on lettuce pollen underscores the originality and scientific contribution of the present study.

From a practical perspective, the optimized storage protocol proposed in this study may facilitate controlled hybridization programmes, improve pollen exchange between breeding locations, and contribute to more efficient germplasm conservation in lettuce breeding. Overall, the findings provide both mechanistic insight and practical guidance for optimizing lettuce pollen storage, thereby establishing a strong foundation for the recommendations presented in the following conclusion.

A limitation of the present study is that only one lettuce genotype was evaluated under short-term storage conditions. Future studies should investigate additional genotypes and longer storage periods to determine the broader applicability of the proposed storage protocol.

Conclusion

This study establishes an integrated protocol for optimizing short-term lettuce pollen storage by simultaneously evaluating storage temperature, storage container, storage duration, and germination medium within a single experimental framework.

The results demonstrated that refrigerated storage in closed Eppendorf tubes effectively preserved lettuce pollen viability during short-term storage, whereas pollen quality declined progressively with increasing storage duration. Among the evaluated germination media, the agar-free medium containing H_3BO_3, $Ca(NO_3)_2$ and 45% sucrose consistently produced the highest germination and viability. Multivariate analyses further confirmed that storage duration and germination medium were the dominant factors influencing pollen performance.

From a practical standpoint, pollen should be used fresh whenever possible in lettuce breeding programmes and, when storage is unavoidable, kept in closed Eppendorf tubes at 4°C. Beyond lettuce, the experimental framework developed in this study may provide a useful reference for optimizing pollen storage protocols in other economically important vegetable crops. Future research testing freezing and cryopreservation conditions is of great importance for extending the storage life of lettuce pollen (Volk *et al.*, 2026). Implementation of this protocol may improve synchronization of flowering between parental lines, facilitate pollen transport between breeding locations, and enhance the efficiency of controlled hybridization programmes.

Author's Contribution: ŞT conducted the experiments, collected and analyzed the data, and prepared the initial draft of the manuscript. KS conceived and supervised the study, designed the experimental methodology, interpreted the results, critically revised the manuscript, and approved the final version for publication. Both authors read and approved the final manuscript.

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