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Optimizing Wheat Pollen Preservation for Enhanced Viability and *In Vitro* Germination

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Abstract: Wheat pollen, which is characterized by its short lifespan, exhibits rapid germination after anthesis. The preservation of wheat pollen is contingent upon environmental factors including temperature, relative humidity, light, and wind. The aim is to explicate the process for efficiently storing wheat pollen, particularly with regard to breeding. The short longevity of wheat pollen grains renders it impractical to conduct tests for pollen viability and *in vitro* germination on a large scale. Herein, the impact of storage temperatures and duration was assessed on pollen viability and *in vitro* germination in order to optimize storage conditions for preserving pollen viability. Pollen grains from 50 diverse spring wheat genotypes, each with three replicates, were harvested and stored at temperatures of 22 °C, −20 °C, and 4 °C. Subsequently, pollen viability and *in vitro* germination rates were determined after storage for 1, 3, and 6 days. The results revealed that storage temperatures, durations, genotypes, and their interactions had a statistically significant impact on both pollen viability and *in vitro* germination. Notably, when pollen was kept at 22 °C, almost all genotypes exhibited a loss of pollen viability and *in vitro* germination after 1, 3, and 6 days of storage. Likewise, storage at −20 °C failed to extend pollen germination. However, at a storage temperature of 4 °C, the pollen of 36 wheat genotypes exhibited a range of 6–14% for *in vitro* pollen germination and even remained viable for 6 days. The ANOVA revealed a substantial variation in grain number per spike between wheat genotypes, thereby highlighting the significant influence of genetic variations on grain yield. Moreover, a slight positive association between the viability of wheat pollen and the number of grains was found in the current study, suggesting that a variety of factors affect the number of grains produced. Simple linear regression analysis further revealed a significant negative correlation between pollen viability, *in vitro* germination, and storage time and temperature. In conclusion, our findings underscore that 4 °C is the optimal temperature for preserving pollen viability and *in vitro* pollen germination in spring wheat for up to 6 days. The results of the present study suggests that the pollen viability of wheat is dependent on genotype, storage temperature, and storage duration. Thus, the 36 wheat genotypes identified during the present work could be efficiently maintained at 4 °C for short-term storage (6 days) and could be further used for genetic and breeding purposes.

Keywords: pollen viability; *in vitro* pollen germination; preservation; wheat breeding; heat tolerance

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1. Introduction

In flowering plants, the male gametophyte, or pollen grain, is released after the second pollen mitosis. When wheat pollen grains are mature, they are tricellular, possess high moisture content, and exhibit a short lifespan [1,2]. This limited viability is attributed to the respiratory activity in tricellular pollen necessitating successful pollination within a brief 30 to 40 min window post-pollen shedding for seed production [3]. The success of pollination

in crop plants hinges on the vigor and viability of pollen grains, with the collection stage playing a crucial role. Pollen from closed flowers, due to its immaturity and reduced susceptibility to contamination, is considered ideal for maintaining high viability [4].

Pollen viability is evaluated through stainability, germinability, and fertilization capability [5]. In genetic improvement programs, *in vitro* germination serves as a common viability assay, but specific protocols and culture media are required for each species [6,7]. Alternatively, cytological observations using vital fluorescent dyes offer indirect methods for assessing pollen viability [8]. Previous studies emphasize the importance of *in vitro* pollen germination and staining for assessing stored pollen, correlating well with fertile seed yields [9]. Numerous factors, including pollen grain vigor, age, growth stage, temperatures, flower physiological status, and the moisture content of the pollen during preservation, contribute to the duration of pollen viability, thereby leading to variability among crops and even genotypes within a species [10–12]. Storage conditions, particularly that of temperature, profoundly impact pollen viability [13]. Ultra-low temperature (cryo) preservation, which requires moderate dehydration, is effective in preventing the damage to cell membranes caused by ice crystal formation. However, achieving the right moisture level and thawing method is complex, making identification of the optimal preservation temperature essential [14–17].

In selective breeding projects, producing pollen grains with high viability that are appropriate for transportation as well as storage is crucial [18]. It speeds up the process of fertilization and allows for crosses between genotypes with varying flowering periods [6]. For breeders and conservators, identifying the viable pollen required for high seed sets is crucial [8]. The three main factors that determine a crop variety's capacity to reproduce in a certain environment are seed set, pollen viability, and pollen production [19].

Ineffective fertilization and seed set failure have been linked to a lack of viable pollen or a pollen's incapacity to germinate in the style and causes a decreased yield of grains [8,20]. It has been observed that pollen viability and seed set are highly correlated [21–24].

Pollen preservation is crucial for breeding and genetic research [8]. Limited data exist on wheat pollen storage at low temperatures, with one study reporting preservation at 5 °C for one day [25]. Pollen life spans differ significantly between plant species, genotypes, and cultivars [26]. For instance, *Agrostis stolonifera* L. pollen loses viability rapidly [27], while maize pollen becomes non-viable within two hours in field conditions [28]. Buckwheat, on the other hand, prefers low temperatures and high humidity for preserving pollen viability [29].

In breeding programs, understanding pollen viability and germination is crucial for controlled pollination. Preserving pollen viability is essential for overcoming barriers to hybridization, especially when dealing with plants or species with differing flowering times or which grow in distinct regions [30].

In wheat breeding programs, a significant challenge arises from the genetic variation in flowering time among elite parents. Storing pollen until the desired pollination time can help overcome these differences. Pollen preservation is a challenge despite the widespread belief that wheat pollen has a limited lifespan and becomes unviable in 30 to 40 min in the natural environment. While it is commonly believed that wheat pollen has a short lifespan and loses viability under natural conditions within 30 to 40 min [3] making pollen storage a problem, the different storage temperatures and optimal temperature for pollen germination depend on the species and also vary between cultivars [12].

The viability and preservation of wheat pollen may be affected by exposing it to different temperatures [8,15]. At −20 °C, freezing could cause cell death [25], while a room temperature of 22 °C may cause metabolic modifications that impair the ability of pollen to germinate and fertilize [24]. However, storing wheat pollen in a refrigerator at 4 °C may slow down the degradation process and help maintain its viability [25].

There is not much information about ideal wheat storage conditions [3] as well as wheat storage at low storage temperatures (5 °C, and −20 °C) to develop or improve strategies for improving the pollen viability and germination of wheat in order to improve wheat

seed setting [25]. Examining appropriate storage conditions can address the difficulties in assessing pollen viability in multiple wheat lines, including genotypic variations, short post-shedding lifespans, and differences in flowering time [24]. To successfully advance breeding procedures, this study attempted to determine the ideal storage temperature and duration to maintain pollen's viability in the selected wheat lines. Therefore, we hypothesized that (i) the viability of wheat pollen may vary depending on several factors such as temperature, duration, and genotypes; (ii) exposure to temperatures of −20 °C and 22 °C may negatively impact the viability and preservation of wheat pollen; and (iii) storing wheat pollen at 4 °C in a refrigerator can preserve its viability for many days.

Taking into account the known susceptibility of pollen to climatic factors, the aforementioned hypotheses are based on the general principles of biological information preservation. Additional experimental research is necessary to confirm and measure the severity of negative effects.

The investigation results will provide valuable insights into the effects of temperature changes on wheat pollen, benefiting both agriculture and science.

2. Materials and Methods

Plant material and growth conditions: We collected pure seeds of 50 diverse spring wheat genotypes (Table S1) including landraces, pre-green revolution as well as post-green revolution varieties, recent cultivars, and advanced lines. The wheat genotypes were cultivated using a randomized complete block design (RCBD) with three replications at the National Institute for Genomics and Advanced Biotechnology (NIGAB) in Islamabad, Pakistan. The seeds were sown using a wheat planter in 1.2 m × 3 m plots, with each plot consisting of six rows spaced 20 cm apart. Standard agronomic practices were used throughout the experiment.

2.1. Pollen Collection and Storage

During the flowering stage, we collected spikes with yellow anthers to extract anthers for sampling. To extract the anthers, we carefully opened the glume and lemma using forceps. Subsequently, we stored the sampled anthers in tightly sealed plastic vials for future use. For each wheat genotype, anthers were stored at three different temperatures as follows: ambient temperature (22 °C), refrigeration (4 °C), and deep freezing (−20 °C). Pollen viability and *in vitro* pollen germination were evaluated after storage periods of 1, 3, and 6 days at each of these storage temperatures.

2.2. Pollen Viability

To determine the optimal conditions for preserving pollen viability, we tested three different storage temperatures (22 °C, 4 °C, and −20 °C) over four storage durations (0 days, 1st day, 3rd day, and 6th day). Pollen grains collected from 50 genotypes and stored under these conditions were placed on slides with one to two drops of ALEXANDER solution and covered with cover-slips [5]. A compound microscope (Olympus) with 5× magnification was used to assess the level of pollen staining with three microscopic fields of view. Pollen grains that stained fully and darkly (magenta-red or red) were classified as viable; those with light staining (magenta-red or red) were considered semi-viable; and those stained blue-green, blue, or not stained at all (lacking color) were non-viable (adapted from the study of [31]). Pollen viability was quantified as the percentage of stained pollen grains out of the total.

2.3. In Vitro Pollen Germination Test

To assess pollen germination across 50 wheat genotypes under various storage conditions and durations, an *in vitro* pollen germination test was conducted. A liquid pollen germination medium was prepared consisting of dissolved H_3BO_3 (0.05 g), $Ca(NO_3)_2 \cdot 4H_2O$ (0.03 g), BK Salts (including $MgSO_4 \cdot 7H_2O$ (0.2 g), KNO_3 (0.1 g), and 19% maltose), and polyethylene glycol (PEG6000, 13%) adjusted to a pH of 6, as described by the authors

of [32]. Using a light compound microscope (Olympus BX41 with DP12 camera), we counted pollen grains and germinated pollen grains from three microscopic fields of view to determine *in vitro* pollen germination. Pollen grains were considered germinated when the length of the pollen tube exceeded the diameter of the pollen grain, following the criteria of the study of [23].

2.4. Grain Number per Spike (GpS)

The number of grains per spike was counted from randomly selected spikes per replication from each line and then averaged out.

2.5. Statisticall Analysis

Descriptive statistics were performed using Excel and GraphPad Prism. Analysis of variance (ANOVA) was conducted using SPSS (v16.0) to assess variations among genotypes (G), storage time (ST), storage temperature (T), G × T, G × ST, and T × ST. Moreover, one-way ANOVA was conducted using R studio to assess variations among genotypes for GpS. Simple linear regression was performed using the R function of "jamovi" for all 50 spring wheat genotypes to investigate the relationship between pollen viability as well as *in vitro* pollen germination and storage days. Through this analysis, pollen viability and *in vitro* pollen germination were treated as dependent variables, while storage days were considered to be independent variables. Correlation analysis was performed using the R function of "jamovi" to find the relationship between pollen viability and grains per spike (GpS).

3. Results

3.1. Assessment of Pollen Viability and Germination across Genotypes

The assessment of pollen viability and *in vitro* pollen germination revealed substantial variability among different genotypes with different a storage time (ST) and temperature conditions, as depicted in Figures 1 and 2. Histograms illustrating pollen viability exhibited pronounced variation regarding storage duration (Figure 3). Freshly collected pollen (with no storage, 0 days) exhibited the highest pollen viability percentages across all 50 spring wheat genotypes. The pollen viability percentages ranged from 71% to 100% for these fresh samples (Figure 1A−D and Table S3). Similarly, *in vitro* pollen germination rates ranged from 63% to 98.6% for the same fresh pollen samples (Figure 2A,B and Table S3). Notably, for all genotypes and under all storage temperature conditions, both *in vitro* pollen germination and pollen viability exhibited significant decreases as the storage time elapsed (Figure 3; Tables S2 and S3).

Specifically, at 0 days of storage (fresh pollen), the pollen viability was high at 90.03% and was accompanied by an *in vitro* pollen germination rate of 83.40%. However, after the first day of storage, these values dropped substantially to 59.75% for pollen viability and 55.65% for *in vitro* pollen germination. Furthermore, after three days of storage, pollen viability decreased to 45.65%, while *in vitro* pollen germination plummeted to 30.93%. Finally, after six days of storage, both pollen viability and *in vitro* pollen germination reached their lowest levels at 35.87% and 3.10%, respectively (Tables S1 and S2; Figure 3). These findings underscore that, at each storage duration, *in vitro* pollen germination was consistently lower than pollen viability.

To assess variations among genotypes, storage time (ST), and storage temperature (T) as well as the interactions between genotypes (G) and ST, G and T, and T ×S T, an analysis of variation (ANOVA) was conducted. The analysis revealed statistically significant differences ($p < 0.001$) in pollen viability and *in vitro* pollen germination among genotypes, pollen storage temperatures, and storage duration (Table 1). Importantly, the ANOVA also indicated significant impacts ($p < 0.001$) of genotypes on both pollen viability and *in vitro* pollen germination rates as well as significant interactions with storage time (ST) and temperature (T) (Table 1).

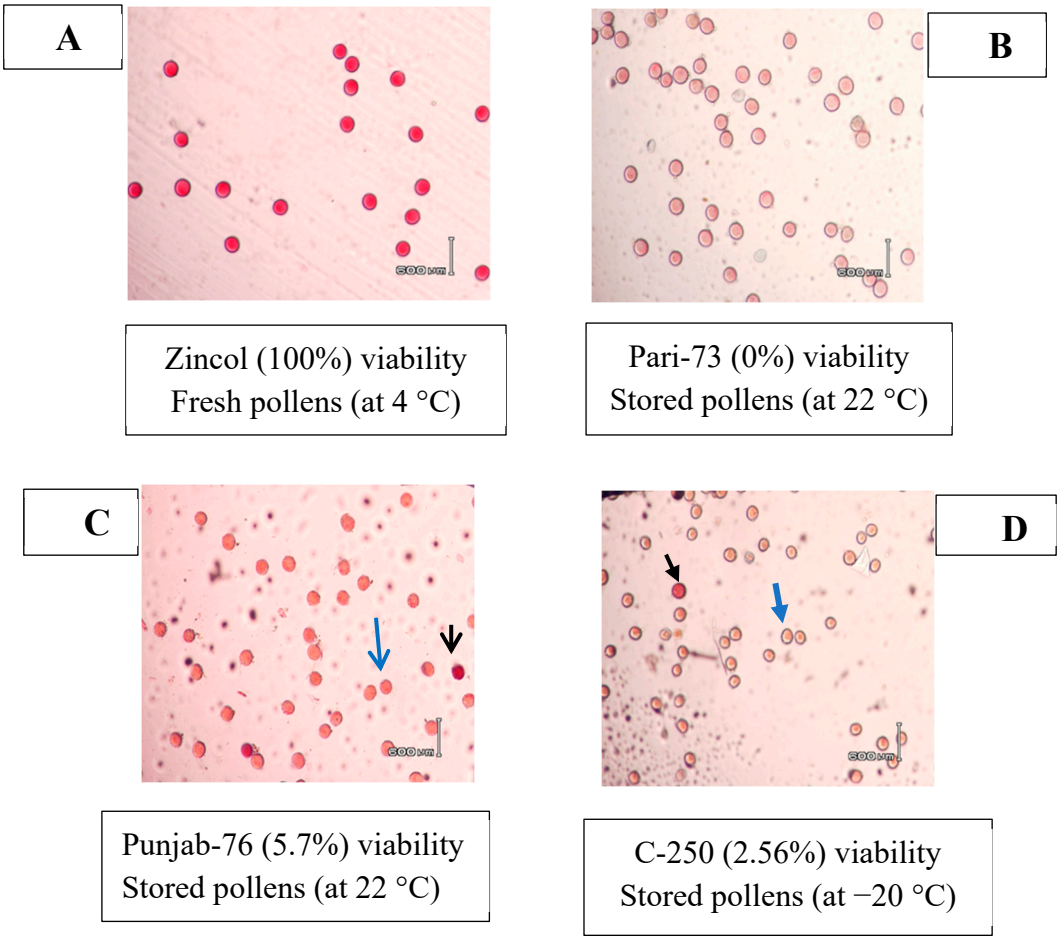

Figure 1. Pollen grains of spring wheat lines shown as follows: (**A**) indicates 100% viability at 4 °C for fresh pollens; (**B**) represents all non-viable pollens after storage for 6 days at 22 °C; (**C**) indicates 5.7% viability after storage for 6 days at 22 °C, with the blue arrow indicating semi-viability and the black arrow indicating viability; (**D**) shows 2.56% viability after storage for 6 days at −20 °C, with the black arrow specifying viability and the blue arrow showing non-viability. All images were taken under a light compound microscope (OLYMPUS) at 5× magnification.

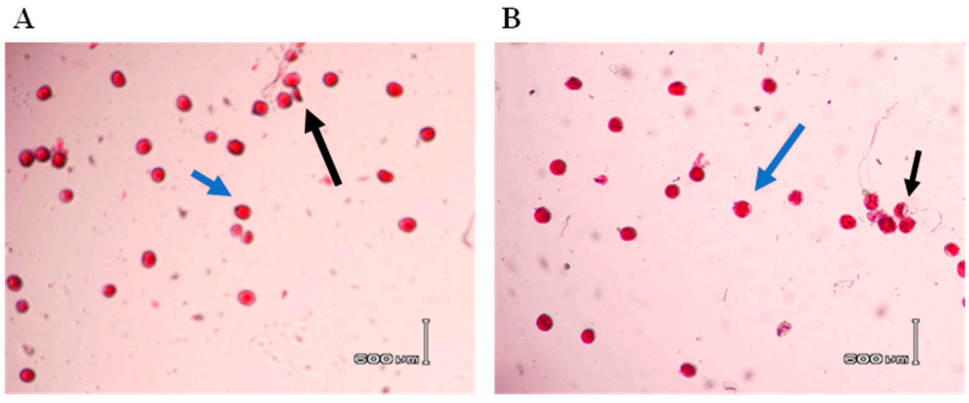

Figure 2. *In vitro* pollen germination after 6 days at 4 °C. (**A**) Akber-19 and (**B**) WL711. The blue arrow indicates no germination and the black arrow indicates germination having a pollen tube (5× under a light compound microscope).

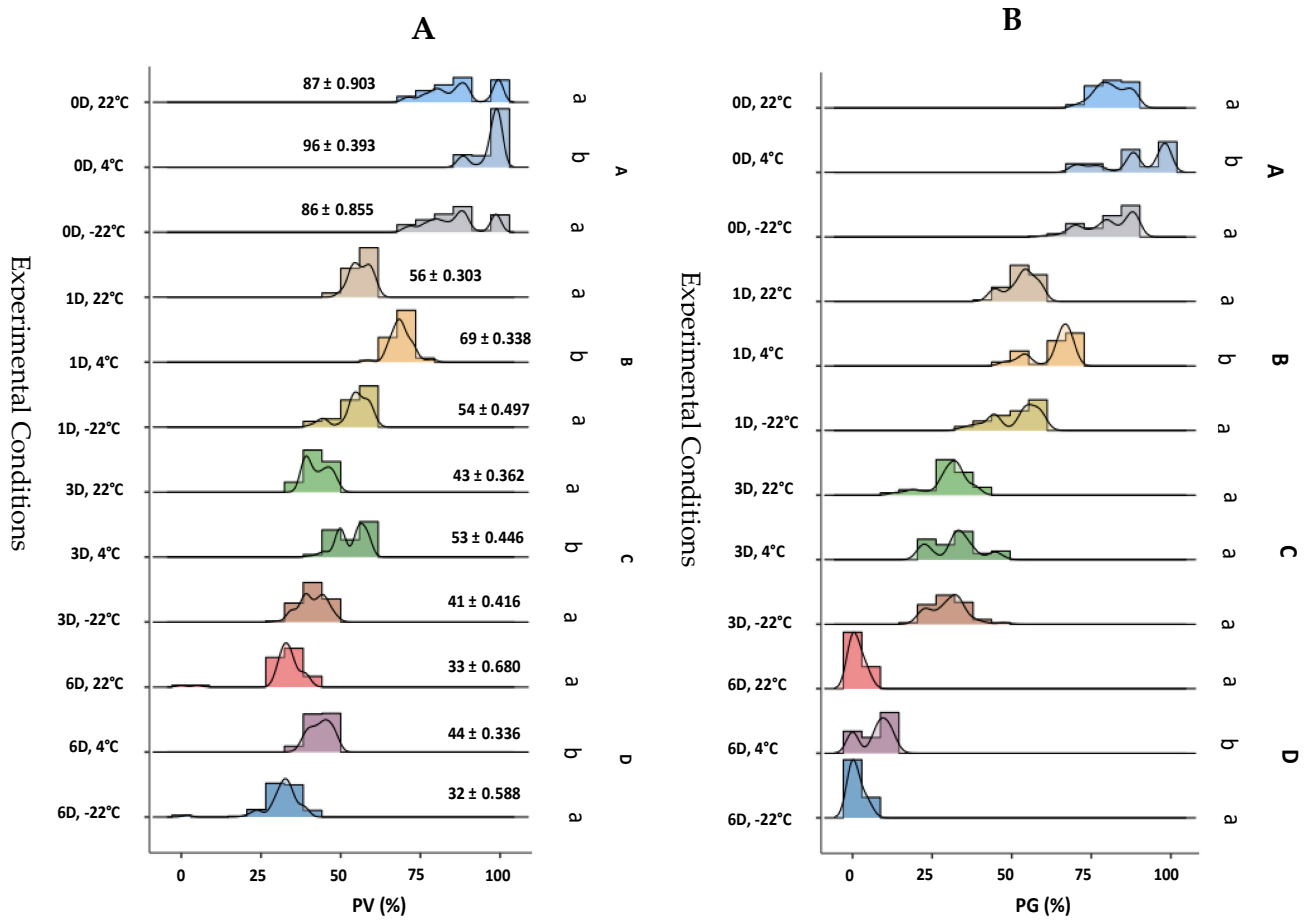

Figure 3. Histogram showing the effect of experimental conditions on (**A**) pollen viability (PV %) and (**B**) *in vitro* pollen germination. The capital letters represent the least significance difference (LSD) at varying storage time levels. The small letters represent the least significance difference (LSD) at varying storage temperature levels within each storage time. Mean ± SE valves are given for each experimental condition.

Table 1. Analysis of variance (ANOVA) for the pollen viability and *in vitro* pollen germination of 50 spring wheat genotypes, three storage temperatures, and three storage times.

Traits	SOV	DF	Sum Sq	Mean Sq	F-Value	Pr (>F)
Pollen viability	Genotypes (G)	49	7048	144	5.878	$<2 \times 10^{-16}$ ***
	Storage temperature (T)	2	53,909	26,954	1101.57	$<2 \times 10^{-16}$ ***
	Storage time (ST)	3	752,099	250,700	10,245.6	$<2 \times 10^{-16}$ ***
	G × T	98	6774	69	2.825	$<2 \times 10^{-16}$ ***
	G × ST	147	14,811	101	4.99	$<2 \times 10^{-16}$ ***
	T × ST	6	891	149	5.592	9.29×10^{-6} ***
In Vitro pollen germination	Genotypes (G)	49	6406	131	3.411	6.34×10^{-14} ***
	Storage temperature (T)	2	13,419	6710	175.072	$<2 \times 10^{-16}$ ***
	Storage time (ST)	3	1,536,105	512,035	13,360.3	$<2 \times 10^{-16}$ ***
	G × T	98	10,303	105	2.743	4.40×10^{-16} ***
	G × ST	147	18,398	125	3.635	$<2 \times 10^{-16}$ ***
	T × ST	6	7700	1283	33.955	$<2 \times 10^{-16}$ ***

SOV = source of variation, DF = degree of freedom, Sum Sq = sum of square, Mean Sq = mean of square. Significance codes 0 "***" 0.001.

3.2. Influence of Pollen Viability on Mean Number of Grains Per Spike

The ANOVA showed significant differences among 50 spring genotypes for GpS (Table 2). However, a weak positive correlation was observed between PV and GpS (Table 3). Genotypes WL 711 and Ihsan-16 had larger grain numbers per spike (GpS 60), while genotype Pasina-17 had the lowest (GpS 29) (Table S3).

Table 2. One-way ANOVA for GpS of 50 spring wheat genotypes.

Source of Variation	Df	Sum Sq	Mean Sq	F Value	Pr(>F)
Genotypes	49	10,027	204.6	38.61	$<2 \times 10^{-16}$ ***
Residuals	100	530	5.3		

Signif. codes: 0 '***', 0.001.

Table 3. Correlation between pollen viability and grain number per spike.

		Pv	GpS
Pv	Pearson's r	—	
	df	—	
	p-value	—	
GpS	Pearson's r	0.148	—
	df	48	—
	p-value	0.303	—

3.3. Influence of Storage Duration and Temperature on Pollen Viability and Germination

The impact of storage duration and temperature on both pollen viability (PV) and *in vitro* pollen germination (PG) was conspicuous, with more pronounced declines observed at 22 °C and −20 °C. At an ambient temperature (22 °C), one day of storage resulted in approximately 56.46% pollen viability and 53.19% *in vitro* pollen germination. Similarly, at −20 °C, after one day of storage, pollen exhibited viability of approximately 53.94% and *in vitro* pollen germination of 50.67%. Subsequently, after three and six days of storage, pollen viability dropped to 41.04% and 31.59%, and *in vitro* pollen germination declined to 30.16% and 1.07%, respectively (Tables S1 and S2).

In contrast, when pollen was stored at 4 °C compared with storage at 22 °C and −20 °C, it significantly preserved both PV and PG across all storage durations (1D, 3D, and 6D). After 6 days of storage at 4 °C, pollen from 36 out of 50 spring wheat genotypes still maintained germination rates ranging from 6% to 14% (Table S2) (Figure 2). Conversely, when stored at 22 °C and −20 °C, only 16 and 12 genotypes retained germination rates of about 3–5% and 2–5%, respectively (Table S2). Least significant difference (LSD) analysis conducted among different storage temperatures for each storage duration of both pollen viability and germination indicated that the 4 °C storage temperature exhibited significant variation compared with temperatures of 22 °C and −20 °C (Figure 3). Pollen viability and *in vitro* pollen germination from all 50 spring wheat genotype samples stored at room temperature and at −20 °C showed nearly identical trends (Tables S1 and S2).

3.4. Correlation of Pollen Viability and Germination with Storage Duration

Linear regression analysis revealed significant linear regressions between pollen viability and the storage day at various storage temperatures, namely 22 °C, −20 °C, and 4 °C (Figure 4A–C). Simple linear regression analysis demonstrated notably strong correlations, with R2 values of 0.821 ($p < 0.001$) at 22 °C, 0.764 ($p < 0.001$) at −20 °C, and 0.877 ($p < 0.001$) at 4 °C. Negative regression results indicated that pollen viability declined with an increase in storage time. The maximum pollen viability on the first day of storage was demonstrated to be 55%, 52%, and 66% at 22 °C, −20 °C, and 4 °C, respectively. After 3 days of storage, pollen germination decreased to 47%, 44%, and 58% at 22 °C, −20 °C, and 4 °C, respectively. Subsequently, on the 6th day of storage, germination rates reduced to 31.5%, 31%, and 43% at 22 °C, −20 °C, and 4 °C, respectively. These results underscore that pollen germination

was notably higher at the 4 °C storage temperature compared with the temperatures of 22 °C and −20 °C across the different storage durations.

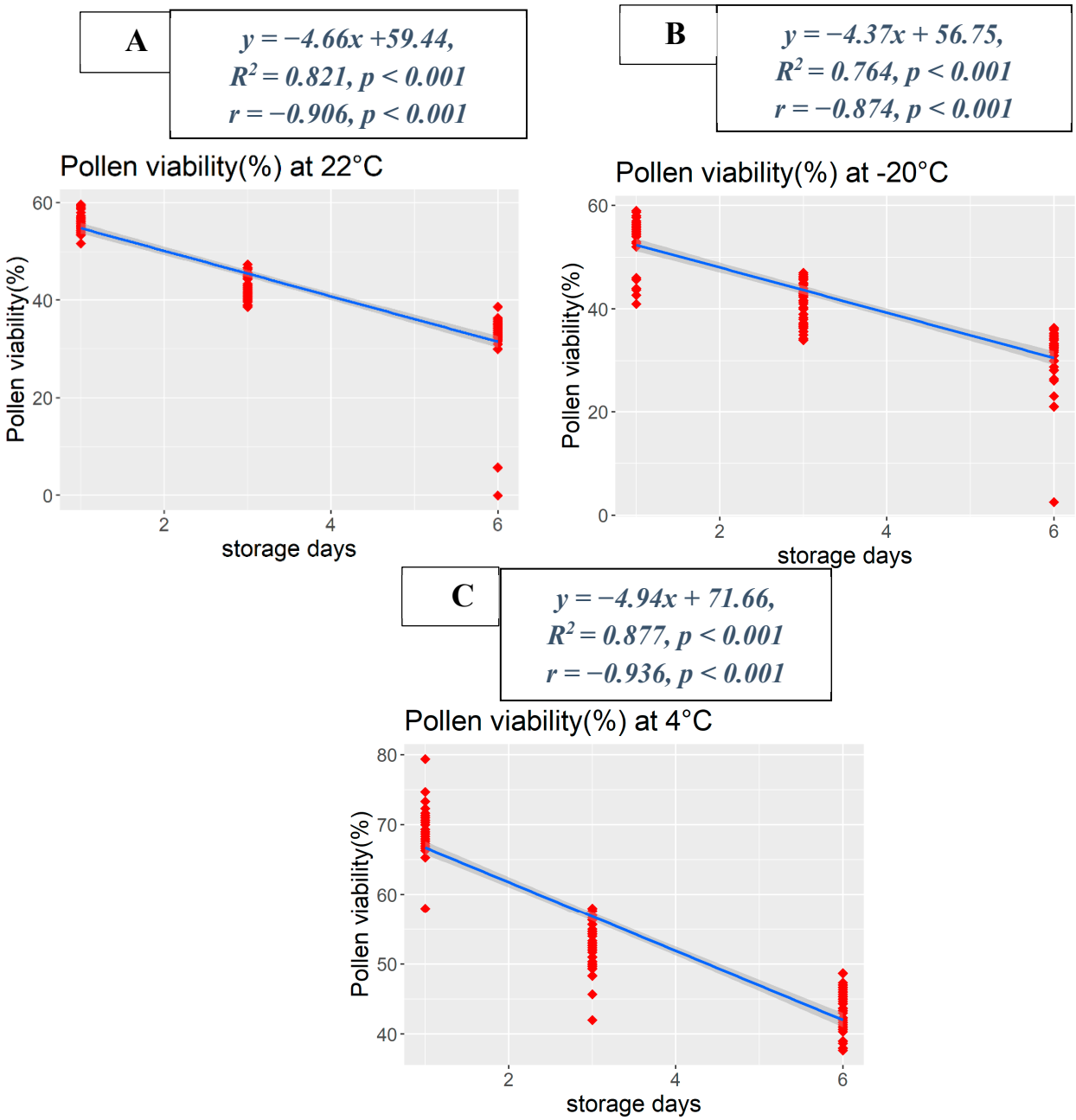

Figure 4. Correlation and simple linear regression between pollen viability and storage days in 50 spring wheat genotypes. (**A**) (22 °C), (**B**) (−20 °C), and (**C**) (4 °C). The red dots represent data points in these plots, providing insights into the correlation between variables.

Likewise, linear regression analysis demonstrated significant linear regressions between pollen germination and storage days at 22 °C, −20 °C, and 4 °C (Figure 5A–C). Simple linear regression analysis revealed strong correlations, with R2 values of 0.958 ($p < 0.001$) at 22 °C, 0.937 ($p < 0.001$) at −20 °C, and 0.909 ($p < 0.001$) at 4 °C. Negative regression results indicated that pollen germination declined with an increase in storage time. The maximum pollen germination on the first day of storage was shown to be 52%, 50%, and 60% at 22 °C, −20 °C, and 4 °C, respectively. After 3 days of storage, pollen germination decreased to 32%, 31%, and 38% at 22 °C, −20 °C, and 4 °C, respectively. Sub-

sequently, on the 6th day of storage, germination rates plummeted to 1%, 1.3%, and 5.2% at 22 °C, −20 °C, and 4 °C, respectively. These findings emphasize that pollen germination exhibited significantly higher rates when stored at 4 °C compared with 22 °C and −20 °C across various storage durations.

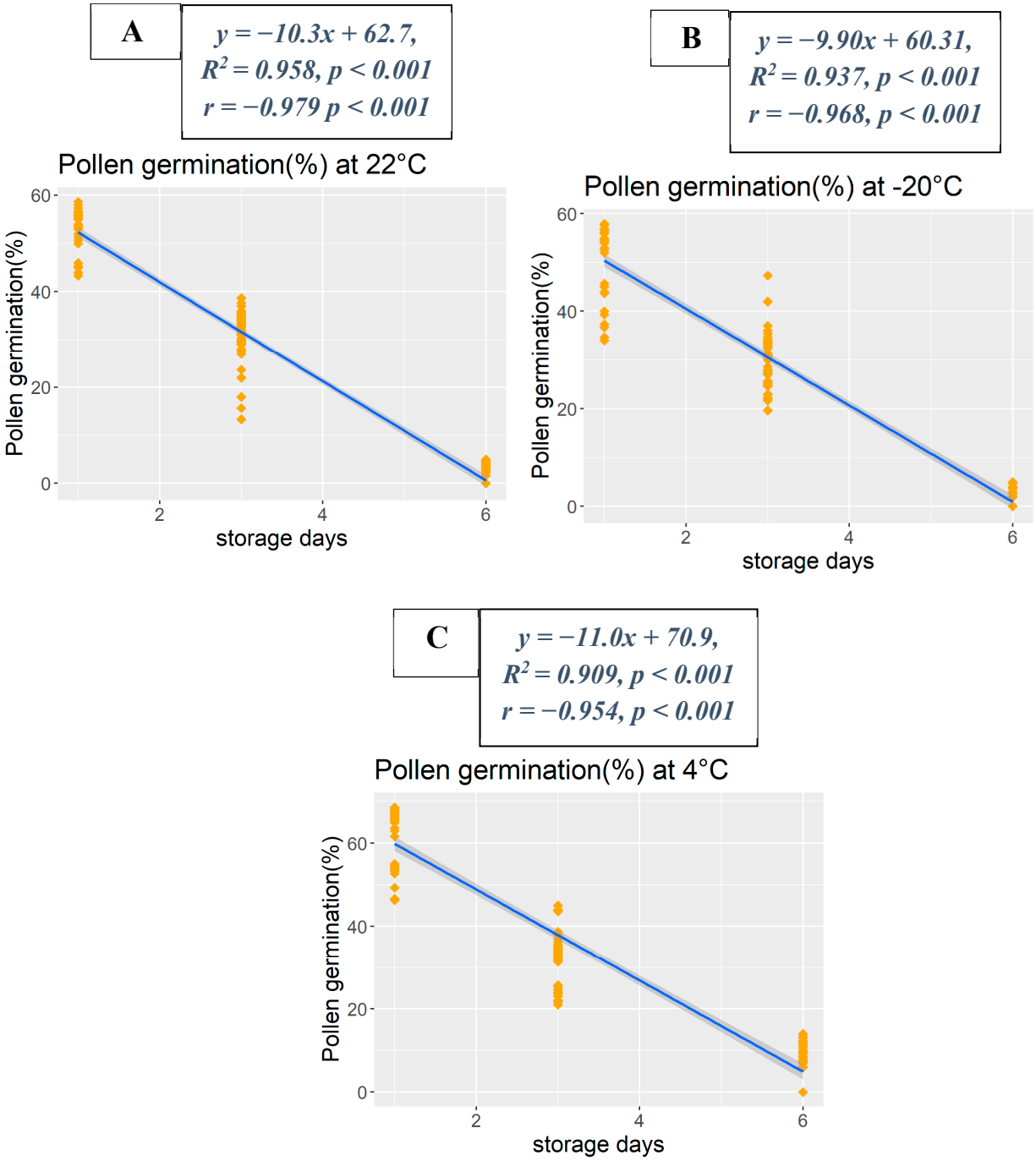

Figure 5. Correlation and simple linear regression between *in vitro* pollen germination and storage days in 50 spring wheat genotypes. (**A**) (22 °C), (**B**) (−20 °C) and (**C**) (4 °C). The yellow dots represent data points in these plots, providing insights into the correlation between variables.

4. Discussion

The purpose of this report was to provide information on the optimal storage conditions for wheat pollen to be used in the future to increase the fertilization potential of specific wheat genotypes, as there have been no reports of a 4 °C temperature influence on

the pollen longevity of wheat undergoing six days storage conditions. Wheat is recognized as a self-pollinating crop [33,34] and is distinguished through its relatively high moisture content and limited shelf life [2]. Therefore, it is imperative to conduct pollination within a tight timeframe of 30–40 min following pollen shedding to ensure a successful seed set [3].

Pollen can cause double successful fertilization through germination and the release of pollen tubes when it reaches the stigma of a flower [5]. Apart from germination, the process through which a pollen tube develops, pollen viability indicates the presence of various germination enzymes [35,36]. Pollen viability plays a pivotal role in fertilization [37], embryonic development [38], and seed quality [39]. The longevity and viability of plant pollen vary significantly among species and are influenced by environmental factors [5]. The capacity of pollen to maintain viability over time and under different storage conditions hinges on both its genetic characteristics and environmental factors [40–42]. Optimal storage conditions for pollen also differ across species and cultivars [43]. In our current investigation, we observed substantial variations in the longevity of wheat pollen among different genotypes, storage temperatures, and storage durations. These findings are consistent with earlier research [5,17,28,44–49].

However, studies focusing on the short-term storage of spring wheat pollen at various temperatures are relatively scarce [25]. Proper storage temperatures are crucial for preserving pollen viability [50]. Hence, the primary objective of our study was to establish the optimal temperature range for storing spring wheat pollen as well as to determine the duration for which wheat pollen can be stored under diverse conditions without compromising viability. The longevity of pollen in rice, wheat, and maize can vary from mere minutes to several hours [28]. Our study highlighted genotype-specific variations in pollen viability and longevity among all the observed genotypes, which align with findings from previous research [40–42,51]. Genetic diversity may be the cause of the changes in the genotypes for the study's pollen germination and viability [52]. Moreover, variations in the pollen's susceptibility to desiccation have been connected to variations in pollen longevity from genotype to genotype [53]. Furthermore, numerous factors can influence pollen viability including pollen handling during collection, the maturity stage of flowering, and environmental conditions such as air temperature and moisture content [54,55]. The current study found that fresh pollen at zero days of storage had the highest percentages of both in pollen germination and the viability of all genotypes, while the percentages of preserved pollen viability and germination drastically decreased throughout storage. The findings showed that pollens stored in refrigerators (−20 °C) did not increase the lifespan of wheat, and there were no appreciable variations in the percentages of viablity and germination among pollens stored at temperatures of (22 °C and −20 °C). However, the decline in both pollen viability and germinability was observed to occur much more quickly at room temperature (22 °C) than at 4 °C. Lower temperatures are typically utilized for long-term pollen preservation due to reduced pollen respiration and the decreased consumption of soluble sugars and organic acids [56,57]. The findings showed that pollen kept at 4 °C for six days maintained a greater germination percentage than that observed at 22 °C and −20 °C, which is consistent with earlier findings [48,57]. The decline in pollen germination at 22 °C in our current study may be attributed to the inactivation of crucial germination enzymes and substrates as well as the reduced ability of pollen grains to germinate when stored at room temperature. [58]. However, the decline at −20 °C may be attributed to the freezing and thawing of the pollen grains [59].

In our study, *in vitro* pollen germination percentages consistently lagged behind pollen viability test results for all 50 spring wheat genotypes examined. This discrepancy may be attributed to various uncontrollable variables including pollen density, the choice of the most suitable growth medium, and the specific environmental requirements of each genotype [60]. Consistent with our findings, Cheng and McComb [61] also reported low and variable germination rates among wheat pollen grains under *in vitro* conditions, with a maximum germination rate of 6.8%. Devrnja et al. [62] also noted that trinucleate pollen germinates more rapidly but has a shorter lifespan than binucleate pollen. Furthermore,

some species with trinucleate pollen may encounter difficulties in developing pollen tubes *in vitro*, as indicated by the study of [63].

A slight positive association between the viability of wheat pollen and the number of grains was found in the current study, thereby suggesting that a variety of factors affect the number of grains produced [64]. While more pollen viability usually corresponds to more grains, other factors like genetics, environmental conditions, or associations with additional variables can reduce the association [8,24].

The present study's one-way ANOVA revealed significant variations in grain per spike between wheat genotypes, thus indicating that genetic variations likely have a large impact on the grain yield of various genotypes of wheat. By creating more resilient and productive wheat cultivars, breeders can enhance crop efficiency, adaptability to various environments, and food safety worldwide by strategically combining high-yielding wheat types. The results of this research concur with those of earlier studies [65–67].

Our study highlighted that pollen viability and *in vitro* pollen germination were notably higher at 4 °C than at −20 °C and 22 °C across all selected spring wheat genotypes. In a prior study, spring wheat genotypes stored at 5 °C exhibited approximately 1.64% pollen germination after 24 h of storage but then experienced a complete loss of viability, with 0.00% germination after 48h and 72 h [25]. In contrast, our study found that spring wheat genotypes maintained 6–14% germination at 4 °C after six days of storage. The data analysis emphasized the significant impact of genotypes on pollen viability and germination rates as well as their interactions with storage temperatures and durations, corroborating earlier findings [25].

Our results demonstrated a significant negative correlation and linear regression between both pollen viability and *in vitro* pollen germination with storage duration (days) at 22 °C, −20 °C, and 4 °C. These findings illustrate that an increase in storage duration led to a reduction in the viability and longevity of wheat pollen. This decrease in pollen viability and longevity with prolonged storage days has been documented in previous studies [28,41,44,45,54,55].

When comparing staining techniques to *in vitro* germination, viability is frequently overestimated [68]. Pollen germination is a measure of viability; a decline does not signify pollen death but rather unfavorable germination circumstances [69]. Moreover, Sunilkumar et al. [70] observed that the rate of pollen germination for *in vivo* germination is a more reliable predictor of viability than that determined through *in vitro* pollen germination.

There are limits to using *in vitro* germination assays to evaluate pollen viability because such tests only indicate the possibility of germination; they do not guarantee the subsequent production of a pollen tube, its passage to an ovule, and effective fertilization [71]. However, the use of Alexander's staining [72] in the present investigation has demonstrated protoplasm as a reliable measure of pollen grain vitality. This addition confirms that protoplasm is the most accurate measure to determine pollen viability.

Numerous techniques have been used in the published literature to overcome these drawbacks, such as measuring the actual growth of pollen tubes and using fluorescent labeling for detailed tracking, to improve the accuracy of viability estimates by combining field research with on-site observations and the use of molecular markers [73–75].

5. Conclusions

The results of this study indicate that wheat pollen viability and germination are influenced by factors such as variety, storage duration, and temperature. The study revealed that pollen from different spring wheat genotypes displayed varying levels of viability and germination capacity. Notably, even after six days of storage at a temperature of 4 °C, 36 spring wheat genotypes still exhibited germination rates ranging from 6% to 14%, thereby indicating that they remained viable.

In light of these findings, it is advisable to limit the storage of pollen to a maximum of six days at a temperature of 4 °C. Furthermore, the study suggests a correlation between storage duration (in days) and variations in both pollen viability and *in vitro* pollen germi-

nation. This insight could prove valuable in the development of a standardized protocol for pollen storage in breeding programs.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy14010201/s1, Table S1: List of 50 spring wheat genotypes used in this study; Table S2: Mean pollen viability (%) at 0D, 1D, 3D, and 6D; Table S3: Mean *in vitro* pollen germination (%) at 0D, 1D, 3D, and 6D; Table S4; Mean grains per spike (GpS).

Author Contributions: I.K. performed the experiment, analyzed data, and wrote the first draft; M.S. conceptualized the study and wrote the first draft; A.S. conceptualized the study; M.S. and M.K.N. supervised the experiments; A.S. and M.K.N. improved the manuscript; Z.Z. validated analysis; and J.C. provided resources and funding. All authors have read and agreed to the published version of the manuscript.

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