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ARTICLE

Evaluation of pollen vivability in some spray Chrysanthemum varieties on storage period

Avaliação da viabilidade do pólen em algumas variedades de crisântemo em spray no período de armazenamento

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Abstract: In producing chrysanthemum hybrids, more seed sets per fruit are preferred. Only successful pollination and fertilization allow seeds to develop. High pollen viability and pollen germination rate are intimately correlated with successful fertilization. Chrysanthemum pollen and their storage duration have only been the subject of a relatively few investigations. The study aimed to determine pollen's viability and germination during the 4 days, which were kept at 24 ºC. In the present study, two different Chrysanthemum species (*Chrysanthemum coronarium* L. and *Chrysanthemum segetum* L.) and two commercial Chrysanthemum varieties (Chic and Haydar) that belong to *Chrysanthemum morifolium* Ramat. species as spray chrysanthemum pollens were used for experimental material. The grains pollen were stored in an incubator which was 24 ºC and 60% humidity. The pollen viability was tested with TTC (2,3,5 Triphenyl Tetrazolium Chloride) staining test and pollen germination was evaluated hanging drop method with modified ME_{3-m} medium, daily for 4 days including day 0. The results showed that the viability and germination of all pollens used in this study decreased day by day. Depending on the species/varieties, the viability rates ranged from 12.83% to 32.04% on the first day and between 0.57-2.33% on the last day. Pollen germination rates differed between 16.76% - 3.45% on the 0th day and 0.0-0.17% on the 4th day.
Keywords: Chrysanthemum, clocreto de 2,3,5 trifenil tetrazólio, duration of pollen, pollen germinability.

Resumo: Na produção de híbridos de crisântemo, prefere-semais conjuntos de sementes por fruto. Somente a polinização e a fertilização bem sucedidas permitem o desenvolvimento das sementes. A alta viabilidade do pólen e a taxa de germinação do pólen estão intimamente correlacionadas com o sucesso da fertilização. O pólen do crisântemo e sua duração e armazenamento foram objeto de relativamente poucas investigações. O estudo teve como objetivo determinar a viabilidade e germinação do pólen durante 4 dias, os quais foram mantidos a 24 ºC. No presente estudo, duas espécies diferentes de crisântemo (*Chrysanthemum coronarium* L. e *Chrysanthemum segetum* L.) e duas variedades comerciais de crisântemo (Chic e Haydar) que pertencem à espécie*Chrysanthemum morifolium* Ramat. foram usadas como pólen de crisântemo em spray como material experimental. Os grãos de pólen foram armazenados em incubadora a 24 ºC e 60% de umidade. A viabilidade do pólen foi testada com teste de coloração TTC (Cloreto de 2,3,5 trifenil tetrazólio) e a germinação do pólen foi avaliada pelo método de gota suspensa com meio ME3-m modificado, diariamente durante 4 dias incluindo o dia 0. Os resultados mostraram que a viabilidade e a germinação de todos os pólens utilizados neste estudo diminuiram dia a dia. Dependendo da espécie/variedade, as taxas de viabilidade variaram entre 12,83% e 32,04% no primeiro dia e entre 0,57-2,33% no último dia. As taxas de germinação de pólen diferiram entre 16,76% - 3,45% no 0º dia e 0,0-0,17% no 4º dia.
Palavras-chave: clocreto de 2,3,5 trifenil tetrazólio, Crisântemo, duração do pólen, germinabilidade do pólen, viabilidade do pólen.

Introduction

Chrysanthemums, which are native to the northern hemisphere, especially to Europe and Asia, are one of the most important floriculture products in the cut flower, flowering pot plants, and herbaceous perennial markets globally (Wang et al., 2018; Yan et al., 2019). There are more than 200 species of chrysanthemums in the world and new chrysanthemum varieties in different shapes, types, and colors that meet consumer demands are developed and marketed every year. In the international trade of cut flowers, chrysanthemums are second after roses and are worth approximately 147 million Euro and 399 444 thousand pieces of chrysanthemum were sold worldwide according to Royal FloraHolland (2020) (AIPH/Union Fleurs, 2021). In 2021, the combined share of the Netherlands and Colombia in chrysanthemum exports was 92%.

Classical and modern breeding methods such as hybridization and mutation breeding are used in the development of new chrysanthemum varieties (Kumari et. al., 2019). Among the main breeding targets in the breeding of cut flower chrysanthemums; are different colors, shapes, and types of flowers, earliness, high yield, and quality, resistance to stress conditions, long vase life, and resistance to transportation conditions (Anderson, 2006; Wang et al., 2014a; Zhao et al., 2022; Eisa et al., 2022). Mutation breeding enables new cultivars to be obtained quickly due to the high heterozygosity of chrysanthemums, increasing the mutation rate (Miler and Kulus, 2018; Wang et al., 2014b). However, in mutation breeding, mutations occur suddenly in an unpredictable way and cause only one change, making it very difficult to develop varieties with many desired characteristics. Therefore, conventional cross-breeding between parental cultivars with contrasting target traits remains the most effective approach for breeding new cultivars due to the genetic heterozygosity of chrysanthemum cultivars (Zhang et al., 2018). In breeding studies, knowing the fertility of the parents in the determination of hybrid combinations greatly affects the success of the breeder (Zlesak, 2007). Successful pollination is dependent on pollen viability, which is also crucial for flowering plant fertilization, fruit set, embryo development, seed quality, and breeding productivit. Because of its significance, pollen has been the subject of several studies on pollen viability detection and germination of pollen (Khan et al., 2021; Kılıç, 2023). From theory to reality, all of the aforementioned studies are essential for plant cross breeding. Especially considering that the fertility of the parents is the most important factor in the formation of fruit and seed sets. The breeder must know the pollen viability and germination rate of the species/variety that can be used as the parents, and the duration of their viability (Spaargaren and Van Geest, 2018). Only a small number of studies have examined its reproductive processes, such as the observed pollen morphology

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and observation of flowering characteristics. Contrarily, pollen-related research on chrysanthemums is dispersed and little understood, which is not helpful for the plant's acceptance and use in society.

Therefore, the determination of pollen viability, optimized *in vitro* germination, and determining the effects of different storage times (0, 1, 2, 3, and 4 days) on pollen viability have been analyzed in this study, and the results based on the above studies are supposed to provide a reference for the research of the hybridization and breeding.

Material and Method

Plant Material

The study was carried out in September 2021 in the Post-Harvest Physiology Laboratory of the Department of Horticulture, Faculty of Agriculture, Ankara University. In the present study, two different Chrysanthemum species (*Chrysanthemum coronarium* L. and *Chrysanthemum segetum* L.) and two commercial Chrysanthemum varieties (Chic and Haydar) that belong to *Chrysanthemum morifolium* Ramat. species as spray chrysanthemum (Table 1, Fig. 1) pollens were used for experimental material. Pollens were obtained from the chrysanthemum cultivars/species grown in the chrysanthemum breeding greenhouse of Ankara University, Faculty of Agriculture, Department of Horticulture (39°57′40.2′′N 32°51′51.7′′E). The pollen of the plants was taken in the greenhouse with 20-25 °C and 65-70% humidity The EC of the nutrient solution was 1.5-1.7 mS cm^{-1} at the beginning of the development period of the plants, 1,7-2,0 mS cm^{-1} during the flowering period, and the pH of the nutrient solution was 6.5.

Table 1. Some characteristics of the spray chrysanthemum species and cultivars used in the study (Anonymous, 2023).

Variety/Species	Color	Flower type	Diameter of flower (mm)
Chrysanthemum coronarium	Yellow	Single	45-50
Chrysanthemum segetum L.	Yellow	Single	45-50
Chic	White	Single	65
Haydar	Bicolor (purple-white)	Single	65

The pollen of the chrysanthemum cultivars used in the study; when the disk flowers opened 4-5 rows from the outside to the inside pollens were placed in glass petri dishes with a brush. For some of the pollen brought to the laboratory, pollen viability and germination tests were determined immediately (0 day, fresh pollen), while other pollen was transferred to 4 different glass bottles for pollen viability and germination tests at the same time every day and kept in the climate cabinet (24 °C and 65% humidity) to be used every day until the 4th. day.

Fig. 1. Chrysanthemum species/varieties used in the study, (A) *Chrysanthemum coronarium* L. (B); *Chrysanthemum segetum* L. (C) Chic; (D) Haydar.

Pollen viability

In the study, TTC (2,3,5 Triphenyl Tetrazolium Chloride) staining test was used to determine pollen viability rates. To prepare 10 ml of TTC: 100 mg of TTC (Merck, CAS: 298-96-4) in 1 mL of distilled water and 5.4 grams of sucrose in 9 mL of distilled water were dissolved separately then both solutions were mixed with each other. A drop of the solution was placed on microscope slides and pollen grains of each type were spread on the stain with a brush. Pollen grains were determined using a light microscope (x 100). They were counted after waiting for 120 minutes in the darkness. The viability of pollen was determined according to staining level: pollen with red or dark pink color as viable, with light red or pink color as semi-viable and with yellow-green color or colorless as non-viable (Eti, 1990). The pollen viability rate was calculated by using the following formula:

$$\text{Pollen viability (\%)} = \frac{\text{Viable pollen} + \left(\frac{\text{Semi viable pollen}}{2}\right)}{\text{Total pollen}} \text{ x } 100$$

$$\text{Pollen germination (\%)} = \frac{\text{Germinated pollen x100}}{\text{Total pollen}}$$

Pollen germination

In the study, the hanging drop method was used to determine pollen germination rate, and pollen grains germinated *in vitro* on modified Monnier Culture medium $ME_{3\text{-}m}$ added to 200 g L^{-1} PEG 4000 (Zhao et al., 2005; Zhao et al., 2008; Shivanna, 2019). The nutrients and their concentrations in the modified Monnier culture medium were given in Table 2. Then pollen was sprinkled onto the slide with culture mediım with a brush. The slides were placed into Petri dishes with wet filter papers at the bottom instantly and the dishes were incubated at 60±5% humidity and 24 °C for at least 24 h.

Pollen grains were screened on each slide under 40x and 100x microscope magnification (Leica DM1000) and pollen tubes longer than 1.5 times the pollen diameter (Fig. 2) were evaluated germinated (Chen et al., 2009). The pollen germinability was calculated by using the following formula:

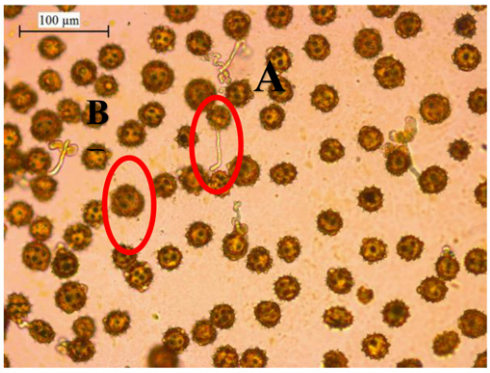

Fig. 2. Pollen germination and pollen tube (A) germinated pollen, (B) ungerminated pollen.

Table 2. The nutrients and their concentrations in the modified Monnier culture medium (Zhao et al.2005; Dursun, 2023)

Macro Elements	Concentration (mg L^{-1})
KNO_3	950
$MgSO_4.7H_2O$	370
KH_2PO_4	85
$CaCl_2$ $2H_2O$	880
NH_4NO_3	412.5
Micro Elements	**Concentration (mg L^{-1})**
$MnSO_4.H_2O$	16.80
$ZnSO_4.H_2O$	10.50
H_3BO_3	50
KI	0.83
$Na_2MoO_4.2H_2O$	0.25
Na_2EDTA	7.45
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
$FeSO_4.7H_2O$	5.55
KCl	175
Vitamins	**Concentration**
B_6	1.0
B_1	1.0
Other	**Concentration** (g L^{-1})
PEG 4000	200
pH	**5.8**

Statistical Analysis

Experiments were conducted on eight replicates and randomized four fields and roughly 500 pollen grains were counted in each area. Analysis of the pollen viability and pollen germinability data was conducted using the IBM SPSS Statistics 26 software. Pairwise comparisons between all means were subjected to the Duncan's Multiple Range (DMR) test and differences were determined using analysis of variance (ANOVA).

Results

In this study the quality characteristics of pollen viability and germination belonging to chrysanthemum species and variety at different holding times were examined. The effect of species/variety x residence time interaction on pollen viability and pollen germination rate was found to be statistically significant ($p \leq 0.05$).

In the species/variety x holding time interaction, the highest pollen viability rate was determined in *Chrysanthemum coronarium* (32.04% and 26.07% respectively) on the 0th and 1st day. The lowest percentage of viability pollen rate was detected in Haydar cultivar (0.57%) on the 4th day. The viable pollen rate in all species/varieties decreased below 50% as of the 3rd day (Table 3 and Fig. 3). Pollen viability rates from day 0 to 4th day; It decreased by 90.68% in Chic cultivar, 95.55% in Haydar cultivars, 97.40% in *Chrysanthemum segetum* L. and 97.44% in *Chrysanthemum coronarium* L. (Fig. 4).

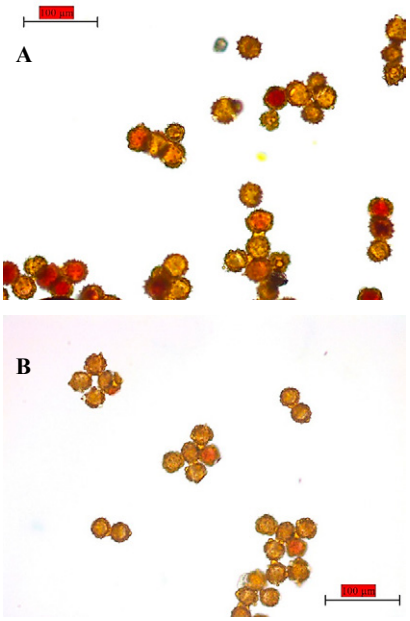

Fig. 3. In vitro pollen viability under 40x microscopic magnification for Chrysanthemum coronarium L. (A) = 0th day, (B) 4th day.

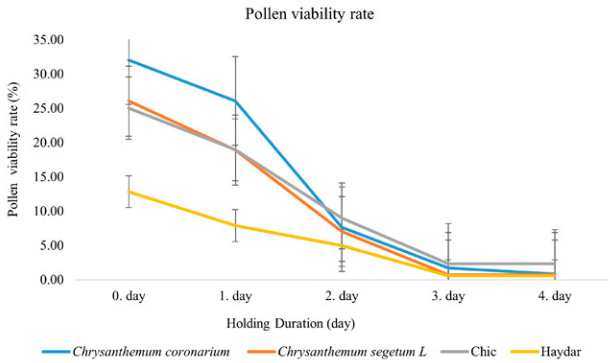

Fig. 4. *In vitro* pollen viability under 40x microscopic magnification for *Chrysanthemum coronarium* L. (A) = 0th. day, (B) 4th day day.

Table 3. Pollen viability rates (%) in chrysanthemum species/varieties according to different holding times.

Species/varieties	Holding Times					
	0th day	1st day	2nd day	3rd day	4th day	Average
Chrysanthemum coronarium	32.04 a	26.07 a	7.65 ab	1.70 b	0.83 b	13.66 A
Chrysanthemum segetum	26.04 b	18.89 b	7.02 ab	0.70 b	0.70 b	10.89 A
Chic	25.01 b	18.94 b	8.99 a	2.33 a	2.33 a	12.27 A
Haydar	12.83 c	7.89 c	4.99 b	0.57 b	0.57 b	5.65 B
Average	23.98 A	17.95 B	7.16 C	1.325 D	1.105 E	

$p \leq 0.05$

In the study, as in the pollen viability rates in all cultivars and species, the highest pollen germination rates were obtained from non-stored (fresh) pollen (day 0), and pollen germination rates decreased with the prolongation of the storage period. The highest germination rate was found in Chic cultivars with a rate of 16.76% on day 0, while the lowest germination rate was determined in Haydar cultivars with 3.45%. On the 4th day, the highest germination rate was found in *Chrysanthemum coronarium* with a rate of 17%, while pollen germination was not observed in Haydar cultivars. From the 0th to the 4th day, germination rates decreased by 98.44% in *Chrysanthemum coronarium* (Fig. 5), 99.82% in Chic cultivars, 99.83% in *Chrysanthemum segetum*, and 100% in Haydar cultivars (Table 4, Fig. 6).

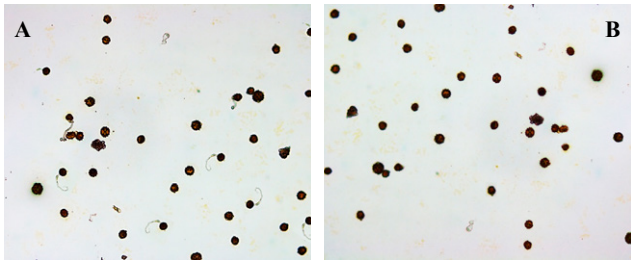

Fig. 5. *In vitro* pollen germination under 10x microscopic magnification for *Chrysanthemum coronarium* (A) = 0th. day, (B) 4th day.

Table 4. Pollen germination rates (%) in chrysanthemum species/varieties according to different holding times.

Species/varieties	Holding Times					Average
	0th day	1st day	2nd day	3rd day	4th day	
Chrysanthemum coronarium	10.93 b	7.88 a	6.22 a	1.55 a	0.17 a	5.35 A
Chrysanthemum segetum	11.81 b	6.81 b	4.74 b	0.02 a	0.02 b	4.90 A
Chic	16.76 a	6.65 b	3.58 c	0.03 a	0.03 b	5.62 A
Haydar	3.45 c	1.15 c	0.54 d	0.01 b	0.00 b	1.07 B
Average	10.74 A	5.62 B	3.77 C	0.40 D	0.055 E	

$p \leq 0.05$

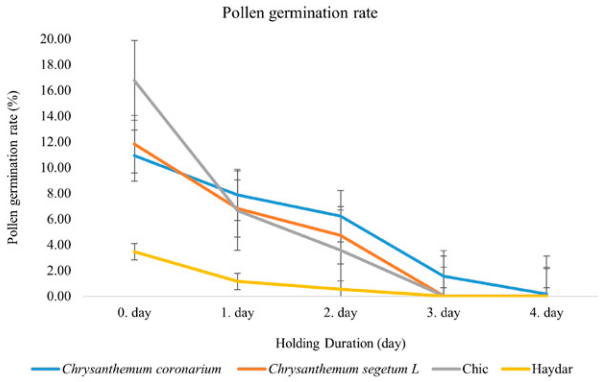

Fig. 6. Pollen germination rates (%) in chrysanthemum species/varieties according to different holding times.

Data Availability

Experiments were conducted on eight replicates and randomized four fields and roughly 500 pollen grains were counted in each area. Analysis of the pollen viability and pollen germinability data was conducted using the IBM SPSS Statistics 26 software. Pairwise comparisons between all means were subjected to the Duncan's Multiple Range (DMR) test and differences were determined using analysis of variance (ANOVA).

Discussion

The most important factor affecting the success of chrysanthemum breeding is the fertility of the genotypes used as parents. High pollen viability and germination rates are preferred in the male parents, whereas high rates of fertilization, fruit set, and seed formation are anticipated in the female parents. Knowing the pollen viability is crucial for successful pollination. When it comes to plant breeding and production, stored pollen is extremely valuable as a tool for overcoming challenges brought on by variations in flowering time, season, location, and partner availability, provided it recovers with appropriate pollination competence. Since it typically reflects the true ability to fertilize, in vitro pollen germination testing is a trustworthy method for determining pollen viability. According to species and cultivars, pollen viability rates ranged from 12.83% to 32.04% on day 0 in the study, which assessed pollen viability and germination rates of various chrysanthemum species and cultivar according to varied retention times. It was found that the daily variation ranged from 0.57% to 26.07%. Limited studies on the viability of pollen in chrysanthemums during storage times have been found in the literature searches. Zhao et al. (2008) and Xu et al. (2012), in chrysanthemums, Erbaş et al. (2015) in oil roses, Korkut (2022), Zlesak et al. (2007) and Macovei et al. (2016) in roses, Du et al. (2019), in peony cultivars, Aldahadha et al. (2020) reported that fresh pollen viability rates in pistachio cultivars decreased with the prolongation of the storage period, similar to our study. In the study, stored pollen from natural species of *Chrysanthemum coronarium* and *Chrysanthemum segetum* lost viability at rates of 97.40% and 97.31%,

respectively, while Chic and Haydar variations from commercial cultivars showed viability changes of 90.68% and 95.55%, respectively. In several chrysanthemum species and cultivars, pollen viability rates ranged from 83.5% to 96.3% (Yang and Endo, 2005). Although the results of this investigation are in agreement with those of the aforementioned study, it is believed that the discrepancy between the lower and upper values may be caused by the fact that the aforementioned study determined pollen viability rates using a different method (acetocarmine). Different techniques (TTC, IKI, FDA, acetocarmine, safranin, aniline blue, hanging drop, saturated petri dish, Monnier Culture) have been used for the few studies on chrysanthemum pollen viability determination and various results have been obtained. Pollen viability was reported to be impossible to acquire in some procedures and fairly low in others (Zhao et al., 2005; Li et al., 2020; Dursun et. al., 2023).

The highest pollen germination rates in this study were obtained from fresh (not stored) pollen, just like pollen viability rates, and it was found that pollen germination capacities dropped with longer storage times in all chrysanthemum cultivars and species. In comparison to fresh pollen, the germination rate of pollen stored for 4 days declined by 98.44% and 99.83% in the natural species *Chrysanthemum coronarium* L. and *Chrysanthemum segetum* L., respectively, while pollen stored for 2 days had decreases of 43.24% and 59.86%. In the commercial varieties of Chic and Haydar, the germination rate of pollen stored after 4 days decreased by 99.82% and 100%, respectively, compared to fresh pollen, while the germination rate of pollen stored for 2 days decreased by 78.63% and % 84.34 respectively. According to Yang and Endo (2005), the germination rate of pollen in chrysanthemums was between 69.4% and 76.4% in five different wild chrysanthemum species and three different chrysanthemum cultivars. On the other hand, Zhao et al. (2005) stated that the germination rate of pollen from chrysanthemum species was between 46%-54%, and the rate of chrysanthemums cultivars was between 23%-25%. According to (Zhao et al., 2008), various chrysanthemum species and cultivars have pollen germination rates ranging from 20% to 35%. Twenty-two small-flowered anemone-type chrysanthemums, whose fresh pollen in vitro germination ranged from 0.3% to 25.6%, were also demonstrated to exhibit the genotype impact (Chen et al., 2009). In a different experiment, the 24 cultivars under test revealed significant variations in the pollen germination rate on the pistils, with the highest value of this feature being 36.3% and the lowest being 3.7%, with an average of 14.4% (Wang et al., 2014). In contrast to other plant species, such as watermelon (70%) and olive (18-85%), current greenhouse chrysanthemums have a rather poor overall germination ability (Akutsu et al., 2008; Alba et al., 2011). Yet other plant species, such as Phalaenopsis (4.5%), are also known to have limited germinability compared to chrysanthemums (Yuan et al., 2018). Relatively low pollen germination efficiency and other important factors such as *SI* may contribute to an overall low seed set in chrysanthemums and thus affect the ineffectiveness of cross-breeding (Miler and Wonzy, 2021). However, pollen viability rates and germination rates show significant differences between natural species and commercial varieties (standard, spray, and Santini) (Zhao et al., 2005; Yang and Endo, 2005; Wang et al., 2014).

However, the findings of our study diverge significantly from those of the studies previously stated. This variation can be caused by the species and/or varieties, the climatic conditions of the greenhouse where the plant material is grown (temperature, light, humidity, day length, etc.), the cultivation technique (soil, irrigation, fertilization, pruning, disease and pest control, etc.), the difference in the methods used to determine the germination rates of pollen, the season and time of pollen collection, the storage temperature and time of pollen, etc. Many studies revealed that chrysanthemum species and cultivars have very different pollen germination rates (Yang and Endo, 2005; Chen et al., 2009; Wang et al., 2014; Miler and Wonzy, 2021). The number of studies on the determination of pollen germination rate in chrysanthemum species and/or cultivars is quite limited. Numerous factors, including variety, storage time, and storage temperature, are reported to have an impact on pollen quality in studies on the effects of storage time and temperature on the germination power of pollen in different species and cultivars (Zhao et al., 2008; Sun et al., 2010; Dursun, 2023).

Conclusions

In this study, the highest pollen viability and germination rates were obtained from fresh pollen during storage in all species and cultivars. This situation revealed that pollen should be used immediately after collection in chrysanthemum breeding by hybridization. However, considering that the flowers used as the seed and pollen parent in the breeding greenhouse do not bloom at the same time and the pollen requirement for all hybridizations cannot be supplied on the same day, as a result, it is necessary to store the pollen for a certain period for future use. Considering this situation, the results obtained suggest that *Chrysanthemum coronarium* and *Chrysanthemum segetum* L. species can be used for pollination until the 2nd day, Chic cultivar until the next day, and Haydar cultivar can be used for pollination immediately before storage. These *in vitro* results need to be confirmed *in vivo* as well. The ability to germinate *in vitro* might not always accurately predict the capacity to germinate *in vivo*.

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Author Contribution

EDM: Conceptualization, data curation, formal analysis, investigation, software, original draft preparation, review & editing. **EK:** Conceptualization, data curation. **SK:** Conceptualization, data curation, supervision. **GH:** Formal analysis, investigation.

Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

Data will be available on request.

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