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# Effect of Pollen Genotype, Temperature and Period of Storage on In Vitro Germinability and In Vivo Seed Set in Chrysanthemum—Preliminary Study

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**Abstract:** Among many challenges in chrysanthemum cross-breeding, the access to viable pollen for hybridization of cultivars distant in location and different in flowering time is required. Low pollen viability along with incompatibility are mainly responsible for low seed set in modern chrysanthemum cultivars. The aim of the study was to test various temperatures and periods of pollen storage of *Chrysanthemum* × *morifolium* in order to elaborate the method of chrysanthemum pollen preservation for cross-breeding purposes. In the first experiment, in vitro pollen germination of four cultivars was investigated following storage at 20 °C, 4 °C, −20 °C, and −80 °C, for one, four, and eight weeks. The second experiment focused on in vivo seed set after one week pollen treatment with 20 °C, 4 °C, −20 °C, and −80 °C (three pollen donor cultivars tested). Pollen in vitro germinability, as well as seed set efficiency, was generally low and cultivar dependent. Independent of the period of storage, stored pollen germinability was lower (5.30–6.63%) than fresh pollen (8.15%). Incubation of pollen in −80 °C significantly increased pollen germinability (9.80%), as well as seed set efficiency in comparison to control (19.28% and 10.21%, respectively) provided the cultivars are compatible. Among cultivars, the highest germinability of pollen was found in 'Brda' and 'Donna' (8.2% and 8.23%, respectively), while 'Bydgoszczanka' showed the lowest germinability (2.97%). There were also pollen genotype dependent effects in in vivo seed set efficiency, which was highest in 'Brda' (17.57%) and much lower in 'Jutrzenka' and 'Polka' (1.34% and 0.39%, respectively), which contributed to the incompatibility of crossed cultivars rather than pollen viability.

**Keywords:** *Chrysanthemum* × *morifolium* (Ramat.); pollen viability; hybridization; breeding; seeds; storage



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## 1. Introduction

Numerous reasons hamper seed setting following hybridization of modern chrysanthemum *Chrysanthemum* × *morifolium* (Ramat.) cultivars and make the classical breeding of this species somewhat inefficient [1]. Sporophytic self-incompatibility (SI), ubiquitous in chrysanthemums, prevents inbreeding, but S alleles are widely distributed in the genomes of greenhouse cultivars, which lead to the decrease in successful fertilization and poor seed production [2–4]. Moreover, centuries of breeding, including mutation breeding most recently, resulted in the inbreeding depression and the negative genetic load, which reduces the overall fertility of the species [1,5]. Most studies focus on the SI phenomenon to explain, as well as to overcome, fertility bottlenecks in chrysanthemum [4]. The development of female and male reproductive organs is well studied, as is the role of their mutual interaction in successful fertilization [6]. Lack of viable pollen or inability of pollen to germinate in the style were also identified among the reasons of seed set failure [3]. Nonetheless, little research has been conducted to develop methods of enhancing pollen viability and germination to gain better seed production in chrysanthemum.

There are hundreds of chrysanthemum cultivars, differing in ornamental traits, as well as growth habits and flowering earliness. Cross-breeding performed between individuals representing desired traits of distant values can be a source of new variability, being of high demand in the world of ornamentals [7]. However, possessing parental plants at a similar stage of development, suitable for hybridization, may be challenging for breeders, since differences in flowering time among cultivars can reach several weeks [8]. Therefore, elaboration of a safe and convenient approach for chrysanthemum pollen storage to increase pollen availability for crossing activities for an extended period of time would be of great value.

This study aimed at finding sufficient temperature and period of storage of freshly collected chrysanthemum pollen grains for breeding purposes. Following the first in vitro experiment, the in vivo seed set efficiency was examined with pollen treated with different temperatures to test the potency of the application of low-temperature pollen treatment, not only in pollen storage, but also in the increasing of seed set in cross-breeding of chrysanthemum.

## 2. Materials and Methods

### *2.1. Experiment 1: In Vitro Pollen Germinability*

Four cultivars of chrysanthemum *Chrysanthemum* × *morifolium* (Ramat.) were used in the experiment: three cultivars belonging to the greenhouse pot group, 'Brda', 'Bydgoszczanka', and 'Polka'; and one garden chrysanthemum cultivar, 'Donna'; all the cultivars were daisy type; all (in both experiments) except 'Donna' were bred at Bydgoszcz University of Science and Technology. Plants were cultivated in a greenhouse according to the common scheme for greenhouse chrysanthemums and brought to flowering under the natural photoperiod, which was at the end of October and the beginning of November. At the anthesis, pollen was collected from inflorescences of the tested chrysanthemums; the gathering of pollen was performed in the late morning between 10.00–11.00 AM. Pollen grains were carefully brushed from heads directly into 1.5 mL polypropylene Eppendorf test tubes (Figure 1A), which were subsequently tightly closed and placed in four different temperature conditions for given periods. Storage conditions were as follows: room temperature (paper box, circa, 20 °C), 4 °C (common kitchen refrigerator), −20 °C (freezer of the common refrigerator), and −80 °C (laboratory low-temperature freezer, C 101L, New Brunswick Scientific, Edison, NJ, USA). Test tubes with pollen grains were stored in a given temperature for one, four, and eight weeks, in darkness.

Pollen germination liquid medium was prepared according to Yang and Endo [9], modified with 16% PEG 4000, 12% sucrose. Two drops of 50 µL medium were put next to one another on a surface of a standard glass slide, and pollen grains were distributed evenly over them. Per each treatment (cultivar × temperature of storage × time of storage), there were five dedicated slides (ten drops of medium with pollen per treatment). Subsequently, slides were incubated for 24 h at room temperature in tightly closed Petri dishes containing wet blotting paper to provide appropriate humidity. Following incubation, slides were screened under 100× microscope magnification (Nikon SMZ 1500). Two independent photographs of optical fields were taken per slide (single photographs per one medium drop) to prevent double counting of pollen grains. Total pollen grains number, as well as number of germinating grains with pollen tubes twice as long as the pollen diameter, were counted, and the pollen germinability was calculated as the share of germinating pollen grains to total number of grains (%). Average pollen germinability based on calculations taken from two photographs from the same slide was treated as one repetition. As the control served the samples of fresh pollen grains put immediately after gathering onto slides with medium and subjected to germinability tests on the next day after pollen collection.


**Figure 1.** (**A**) Pollen collection from cultivar 'Polka' (Experiment 1 and 2). (**B**) Shoot of female parent cultivar 'Filharmonia' with selected four inflorescences at similar stage of development just before the pollination with 'Polka' pollen stored for one week at 4 °C (Experiment 2).

### *2.2. Experiment 2: In Vivo Seed Set*

For in vivo seed set test, three daisy-type, greenhouse pot chrysanthemums *Chrysanthemum* × *morifolium* (Ramat.) 'Brda', 'Jutrzenka', and 'Polka' served as male parents (pollen donors). Spray cultivar 'Filharmonia' served as the female parent in all the crosses. To ensure the exclusiveness of out-crossing, the self-incompatible cultivar can be used, or emasculation process must be performed. Emasculation in chrysanthemum is commonly carried out by the excision of all hermaphroditic disc florets, leaving behind only ligulate florets (which are gynoecious) within inflorescence. Nonetheless, ligulate florets of the outer whorls are of low receptivity [10], so the results of crossing could be unreliable. We decided to use 'Filharmonia' chrysanthemum, which we had previously tested for self-incompatibility (by the means of self-pollination tests) to confirm the exclusiveness of out-crossing. Parental plants were cultivated according to common scheme for greenhouse chrysanthemum production and flowering occurred under natural photoperiod in the beginning of November.

Pollen was collected from inflorescences of male parents (Figure 1A), as described for in vitro germinability tests (Section 2.1), and placed for one week in four temperature conditions: 20 °C, 4 °C, −20 °C, and −80 °C—the same as for Experiment 1.

Four inflorescences of female parent 'Filharmonia' at the same stages of development were carefully selected for pollination in each treatment (male parent cultivar × temperature); the stage of development of inflorescences of the female parents was as follows: three outer whorls of disc florets were fully mature (producing abundance of intense yellow pollen), while the inner whorls of disc florets were not opened yet. The four selected inflorescences were growing from the same shoot; the remaining heads, being of different stages of development, were removed (Figure 1B).

Following storage, pollen grains representing each treatment (cultivar × temperature) were taken out from the Eppendorf tubes, divided into four portions, and loaded with soft brush onto previously selected four inflorescences of the female parent. Control plants were pollinated directly with fresh pollen. Inflorescences were labeled, stems were cut

off 40 cm below the lowest head, and stems were put separately into vases with fresh water and placed in a dry, bright room at ambient temperature (18–20 °C) to develop seeds for one month. Afterwards, dry flower heads were gently cut off, put into separate paper envelopes, and thoroughly examined under stereoscopic microscope, magnification 0.75× (Nikon SMZ 1500). Number of seeds, as well as total number of disc and ray florets, were calculated in each inflorescence. Since florets within heads of *Asteraceae* mature gradually (in chrysanthemum, it takes about two weeks from outer to inner whorls to fully develop) [10] and a single (not recurrent) pollination was performed, it was assumed that only about 15% of florets were receptive on the day of pollination. On that base, the seed set efficiencies were calculated as the number of seeds produced per 15% of the total number of florets. Moreover, the mean number of seeds per head was estimated.

### *2.3. Statistical Analyses*

Experiments were designed as completely randomized with five (Exp.1) and four (Exp.2) replications. Three-way (Exp.1) and two-way (Exp.2) ANOVA were performed, and means were verified with HSD Tukey's test at $p \leq 0.05$. Analyses were performed using STATISTICA 13.3 software package (Tibco, Palo Alto, CA, USA).

## 3. Results

### *3.1. Experiment 1: In Vitro Pollen Germinability*

The total mean number of examined pollen grains was 7639 for cultivar and 477 per each treatment (cultivar × temperature × time of storage). Overall mean germination ability of pollen grains in vitro was low, ranging from 2.97% to 9.97% (Figure 2).

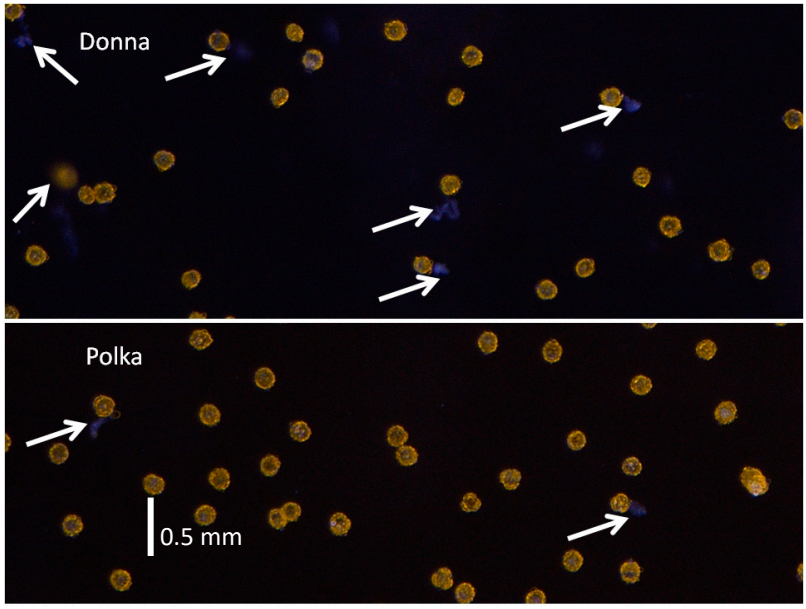


**Figure 2.** Fresh (control) pollen in vitro germination of 'Donna' and 'Polka'. Germinating pollen grains with pollen tubes are indicated with arrows.

Statistical analysis based on the results of microscopic observation revealed the significant impact of the main factors (cultivar, temperature of storage, and time of storage) on the in vitro germinability of pollen. There were differences between tested cultivars: the highest germinability was recorded for 'Brda' and 'Donna' cultivars, while the lowest was observed for 'Bydgoszczanka' (Figure 3A).

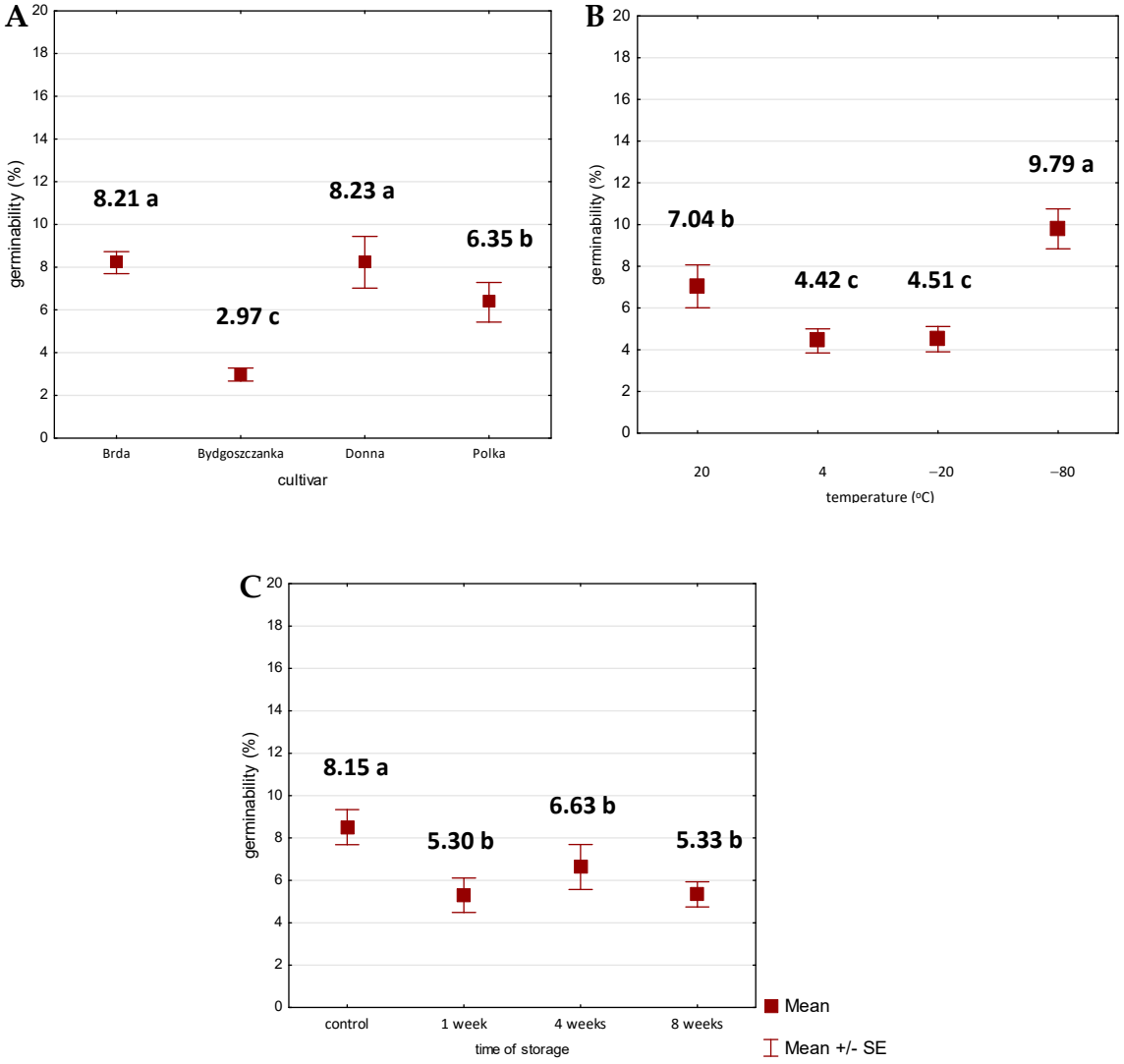


**Figure 3.** Pollen in vitro germinability (%) main factors effects: (**A**) the influence of pollen genotype (cultivar), (**B**) the influence of temperature of storage, (**C**) the influence of time of storage. Means ± SE within each graph followed by the same letter do not differ significantly at $p \leq 0.05$ according to HSD Tukey's test. The graphs show main factors effects; for interactions, see Supplementary File sheet 1.

According to the effect of temperature on pollen in vitro germination it was revealed that the lowest temperature treatment (−80 °C) resulted in the highest germinability (9.79%; Figure 3B). Pollen stored at −80 °C germinated twice as high as pollen stored at 4 °C and −20 °C. Fresh pollen showed higher germinability (8.15%) than stored (5.30–6.63%; Figure 3C).

Detailed analysis of each cultivar's response to time and temperature of pollen storage revealed various germinability effects (Figure 4).

For 'Brda' and 'Donna', storage of pollen grains at 20 °C, 4 °C, and −20 °C considerably decreased the germination ability, while storage at −80 °C enhanced it. Surprisingly high germinability was observed after room temperature storage of pollen in 'Polka'. 'Bydgoszczanka' showed the highest germinability of pollen after four weeks of storage at 4 °C.

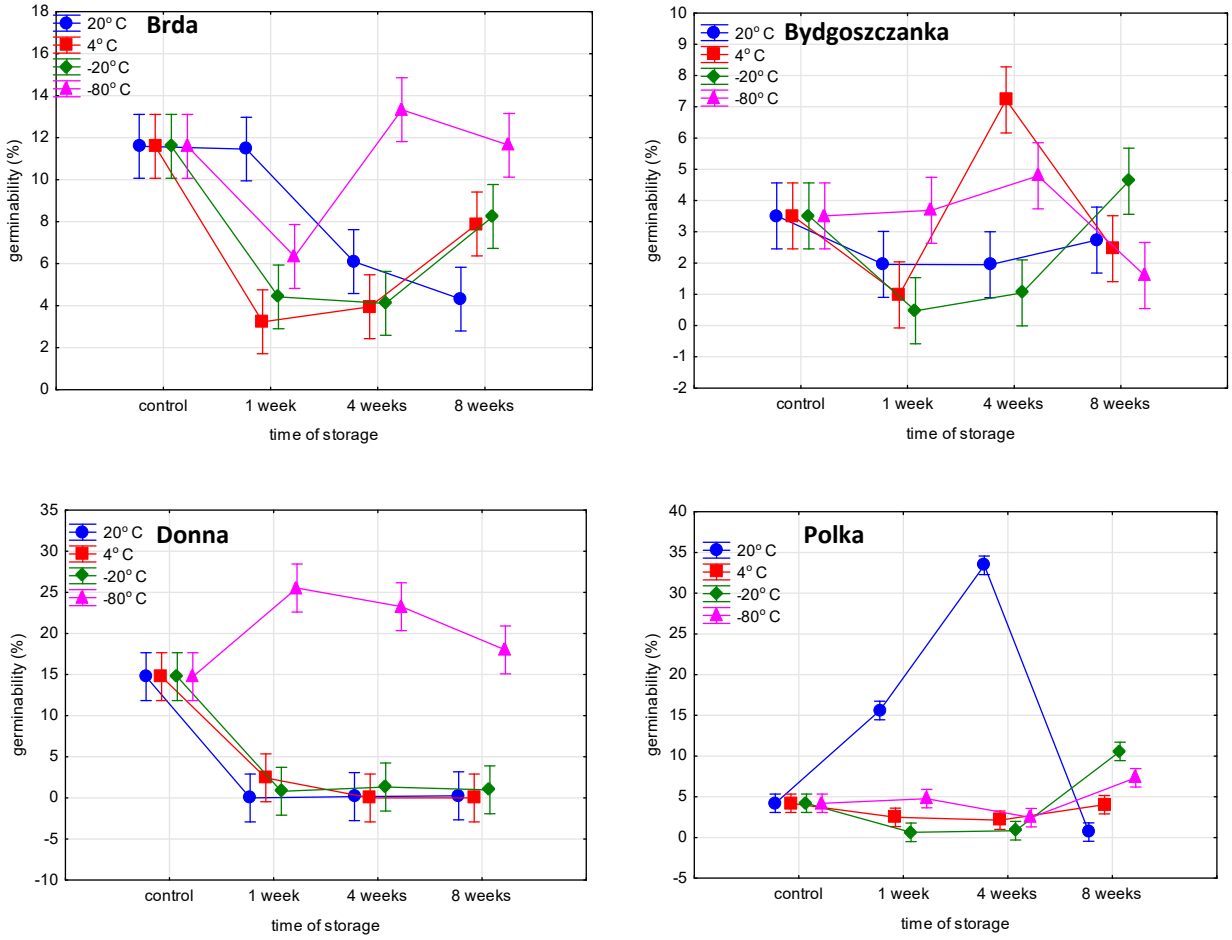


**Figure 4.** Pollen in vitro germinability (%) in four tested chrysanthemum cultivars as affected by the temperature and time of storage, expressed by means ± SE.

### *3.2. Experiment 2: In Vivo Seed Set*

The mean number of florets per inflorescence of the 'Filharmonia' serving as the female parent was 97.8 (72.8 and 24.9 for disc florets and ligulate florets, respectively). The overall seed set efficiency after the pollination with the pollen of different cultivars was low and varied from 0.39% in 'Polka' to 17.57% in 'Brda' (Figure 5; Table 1). The highest seed yield was produced with 'Brda' pollination (45 seeds in total), while seeds number produced with 'Jutrzenka' and 'Polka' pollen were very low (four and one seed, respectively). The seeds obtained from 'Filharmonia' × 'Brda' crosses were germinating in 95.5%, while for the other two crosses, all the seeds germinated.

The mean number of seeds per female parent head was significantly higher after pollination with 'Brda' pollen (2.25) than after pollination with 'Jutrzenka' and 'Polka' pollen. Treatment of pollen with −80 °C significantly increased the number of yielded seeds (2.58) in comparison to fresh pollen application (1.17). Pollen storage prior to pollination at 20 °C, 4 °C, and −20 °C appeared to be ineffective—number of seeds valued from 0.00 to 0.33, irrespective of male parent genotype.


**Figure 5.** Seeds produced by 'Filharmonia' after pollination with 'Brda' pollen. Among many empty, dry florets, few seeds were visible (indicated with arrows).

**Table 1.** The effect of temperature of pollen storage and genotype of pollen (male parent cultivar) on the efficiency of seed set (%); the mean number of seeds produced per chrysanthemum inflorescence after single in vivo (greenhouse) hand pollination. *Chrysanthemum* × *morifolium* cultivar 'Filharmonia' served as mother cultivar (female parent) in all crosses. Means ± SE in columns followed by the same letter do not differ significantly at $p \leq 0.05$ according to HSD Tukey's test. The table shows main factors effects; for interactions, see Supplementary File sheet 2.

| Temperature of Storage Effect | | | | Genotype of Pollen Effect | | | |
|---|---|---|---|---|---|---|---|
| **Temperature** | **Seed Set Efficiency (%)** | **Mean No. of Seeds per Head** | **Total No. of Seeds** | **Male Parent** | **Seed Set Efficiency (%)** | **Mean No. Of Seeds per Head** | **Total No. of Seeds** |
| control | 10.21 ± 5.55 b | 1.17 ± 0.60 b | 14 | Brda | 17.57 ± 6.09 a | 2.25 ± 0.75 a | 45 |
| 20 °C | 0.00 ± 0.00 c | 0.00 ± 0.00 c | 0 | Jutrzenka | 1.34 ± 0.73 b | 0.20 ± 0.11 b | 4 |
| 4 °C | 2.02 ± 2.02 bc | 0.33 ± 0.33 bc | 4 | Polka | 0.39 ± 0.38 b | 0.05 ± 0.05 b | 1 |
| −20 °C | 0.64 ± 0.65 c | 0.08 ± 0.08 c | 1 | | | | |
| −80 °C | 19.28 ± 8.76 a | 2.58 ± 1.11 a | 31 | | | | |

## 4. Discussion

Stored pollen, provided it recovers with acceptable pollination competence, is of great value in plant breeding and production as an aid in overcoming difficulties arising from differences in flowering time, season, location, and availability of partners [11]. Moreover, the elaboration of methods for pollen storage is important for the conservation genetic resources [12].

In vitro pollen germination testing is a reliable method for indication of pollen viability, since it usually reflects the real fertilization ability [13]. The in vitro germination of chrysanthemum pollen in the present study was cultivar dependent—it was lowest in 'Bydgoszczanka' (2.97%) and highest in 'Brda' and 'Donna' (8.21% and 8.23%, respectively). The genotype effect was also shown for 22 small-flowered anemone-type chrysanthemums,

which fresh pollen in vitro germination ranged from 0.3% to 25.6%, according to the cultivar [14]. In another experiment, big differences were also shown among the 24 cultivars tested in terms of pollen germination on pistils: the highest value of this trait was 36.3%, and the lowest was 3.7%, with an average of 14.4% [3]. In traditional Chinese chrysanthemum cultivar 'Fubaiju', the pollen viability was assessed as high as almost 40%; such high pollen germinability can be attributed to the preserved genotype of this particular cultivar [10]. Considerably higher germination rates for fresh pollen were observed by Yang and Endo [9], namely around 70% in four tested cultivars. The data show, however, that overall germination ability in modern greenhouse chrysanthemums is rather low, as compared with other plant species, e.g., watermelon (70%) and olive (18–85%) [15,16]. Nonetheless, there are also known plant species with similarly low germinability as chrysanthemum, e.g., *Phalaenopsis* (4.5%) [17]. Relatively low pollen germination efficiency, along with other important factors such as SI, may contribute to overall low seed set in chrysanthemum and thus affect the ineffectiveness of cross-breeding.

Conditions of pollen storage play a key role in keeping high pollen fertilization ability [12]. Application of low temperature for pollen storage appears to be the most reliable method of pollen preservation, although in some species, pollen grains pretreatment is required—e.g., dehydration or atmosphere modification [15]. Low temperature of storage prolongs pollen viability. For example, *Phalaenopsis* pollen stored at −20 °C and −80 °C for 40 weeks showed germinability at about 1–2%, while no germination was observed after as much as only four weeks of storage at room temperature [17]. In the present study, the highest germination ability was observed in pollen kept at −80 °C (9.80%), while the lowest value of this trait was recorded in pollen stored at 4 °C and −20 °C (4.43 and 4.50%, respectively). The enhancement of fertilization ability following −80 °C treatment of pollen grains was confirmed with in vivo pollination tests—the seed set and the mean seed number were almost twice as high as recorded after application of fresh pollen (control), provided the crossed cultivars were compatible. Although chrysanthemums possess trinucleate pollen, which is claimed to require dehydration prior to preservation [18], the present study showed that greenhouse conditions of cultivation (stable temperature, lack of precipitation, and low humidity) may contribute in the protection of collected pollen from damage during low temperature treatment; thus, it does not require dehydration procedure. Nonetheless, it should be taken into account that pollen of different cultivars may respond unevenly to temperature treatment.

The pollen genotype effect appeared in the in vivo experiment; there were differences in the seed production after pollination with various male parent pollen. The highest seed yield was produced in 'Filharmonia' × 'Brda' crosses, while seed numbers produced in crosses of 'Filharmonia' with 'Jutrzenka' and 'Polka' were extremely low. This result cannot be directly contributed to pollen viability, since 'Polka' showed moderate pollen viability in in vitro experiments; thus, a similar moderate seed set efficiency would be expected. More likely, genetic factors responsible for incompatibility affected the final outcome and the low seed set in the case of 'Jutrzenka' and 'Polka' male parent application. The calculation of seed set is one of the methods for compatibility assessment in chrysanthemum [19]. Modern greenhouse chrysanthemum cultivars are thought to possess numerous SI alleles distributed widely, even if they do not show any close relationship [1]. Therefore, the presence of incompatibility alleles could hamper the seed setting after 'Jutrzenka' and 'Polka' pollination. On the opposite, 'Brda' seems to be compatible with 'Filharmonia', since the seed yield was higher, and one can draw the conclusion that the seed set efficiency with the application of −80 °C treated pollen is significantly higher than in control.

Predominantly, pollen storage for a certain time results in the decrease in its original germinability. In watermelon, pollen viability dropped from 70% to 50% as a consequence of year-long-lasting pollen storage at −25 °C in liquid $N_2$ or $CO_2$; moreover, incubation at higher temperature (4 °C) for as long as three months completely blocked pollen germination [15]. Similarly, the decrease in pollen viability from 85% to 30% was observed for olive pollen preserved for a year at −196 °C [16]. In chrysanthemum, storage of pollen at

4 °C for a month decreased its in vitro germinability from 69.2% in fresh pollen to 3% after storage [9]. In the present research, the germinability of pollen dropped after storage: from 8.51% for fresh pollen to 5.30–6.63% for stored pollen, independent of the period of storage.

For modern greenhouse chrysanthemums, which possess innate low pollen germinability, the application of −80 °C for pollen storage may contribute not only in prolonging pollen availability for breeding purposes, but also may lead to the increase in seed set, provided the crossed cultivars are compatible. For chrysanthemums, all the methods that help to overcome the after-storage pollen germinability loss are welcome, since they can increase the final hybridization results. Nonetheless, the objective needs more studies with a broader cultivars selection to clarify low-temperature pollen storage effect in different chrysanthemum genotypes.

## 5. Conclusions

In vitro tests of chrysanthemum pollen germinability revealed a generally low share of germinating pollen grains; nevertheless, this trait was genotype- and storage temperature-dependent. Similarly, in vivo tests showed the influence of temperature of pollen treatment onto seed set efficiency, provided crossed cultivars are compatible and able to produce viable seeds. Although different cultivars responded unevenly, the increase in overall germinability in in vitro tests following −80 °C temperature treatment of pollen, as well as the increase in seed set efficiency, encourage us to draw the conclusion that storage of pollen in −80 °C can contribute to better hybridization results. Nonetheless, there is a need to perform more investigations with a vaster selection of tested cultivars.

**Supplementary Materials:** The following are available online at https://www.mdpi.com/article/10.3390/agronomy11122395/s1, File S1: ANOVA details for pollen germinability and seed set.

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