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In Vitro and *In Vivo* Performance of Plum (*Prunus domestica* L.) Pollen from the Anthers Stored at Distinct Temperatures for Different Periods

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Abstract: A study was conducted to investigate the effect of different storage periods and temperatures on pollen viability *in vitro* and *in vivo* in plum genotypes 'Valerija', 'Čačanska Lepotica' and 'Valjevka'. *In vitro* pollen viability was tested at day 0 (fresh dry pollen) and after 3, 6, 9 and 12 months of storage at four different temperatures (4, −20, −80 and −196 °C), and *in vivo* after 12 months of storage at distinct temperatures. *In vitro* germination and fluorescein diacetate (FDA) staining methods were used to test pollen viability, while aniline blue staining was used for observing *in vivo* pollen tube growth. Fresh pollen germination and viability ranged from 42.35 to 63.79% ('Valjevka' and 'Čačanska Lepotica', respectively) and 54.58 to 62.15%, ('Valjevka' and 'Valerija', respectively). With storage at 4 °C, pollen viability and germination decreased over the period, with the lowest value after 12 months of storage. Pollen germination and viability for the other storage temperatures (−20, −80 and −196 °C) were higher than 30% by the end of the 12 months. Pollination using pollen stored at 4 °C showed that pollen tube growth mostly ended in the lower part of the style. With the other storage temperatures, pollen tube growth was similar, ranging between 50 and 100% of the pistils with pollen tubes penetrated into the nucellus of the ovule in the genotype 'Čačanska Lepotica'. The results of these findings will have implications for plum pollen breeding and conservation.

Keywords: European plum; pollen germination *in vitro*; pollen viability; pollen storage; low and sub-zero temperatures

1. Introduction

Plums include a large group of closely related *Prunus* species of the *Rosaceae* family. Among them, the European plum (*Prunus domestica* L.) and the Japanese plum (*Prunus salicina* Lindl.) are the most extensively cultivated plum species worldwide [1]. According to FAO production data, in 2020, among temperate fruit species, the plum is in fourth place globally, after apple, pear and peach [2].

Today in Europe, most of the modern plum cultivars belong to *P. domestica*. A previous study [3] reported that the European plum, which includes many old English and Eastern European cultivars, showed the highest level of genetic diversity and on the basis of the fruit characteristics can be divided into several groups: plums, prunes, greengages or Reine Claudes and mirabelles [4]. Plums are mostly used for fresh consumption, but also for drying and processing in different forms [5]. Therefore, in Europe, different plum breeding programmes have been developed focused on defined breeding goals based on the market

requirement. One of the leading plum breeding programmes is that of the Fruit Research Institute, Čačak, Serbia, which is principally aimed at solving the most significant problems in production [6]. Thanks to plum production in Serbia, during the XX century, the former Yugoslavia was one of the largest plum producers in the world [7].

In addition to the common objectives aimed for in plum breeding such as excellent internal and external quality, self-fertility, regular productivity and resistance to *Plum pox virus* (PPV) [8], in recent times, there is an increasing need in plum breeding to obtain genotypes well adapted to different climates and biotic stresses [9]. Conventional methods are still largely used in breeding programs and among them the most important method is hybridization [4–6,8]. Although the results of cross-pollination mostly depend on the female parents, great attention should be paid to the pollen quality. Commonly, pollen quality is assessed on the basis of pollen viability and vigour [10].

Therefore, when there is a need for a readily available supply of pollen in experimental studies, e.g., if there is asynchrony in flowering between parental cultivars or in the case of spatial and temporal isolation between parents, a long period of pollen storage may effectively be utilized [11]. Furthermore, pollen storage is also important in the conservation of genetic material [12]. In an extended period of storage, it is important to preserve the pollen without a significant loss of its viability. Pollen viability includes different features of pollen performance such as germinability, stainability and fertilization ability [13]. A considerable number of methods for testing pollen viability have been introduced so far—germination *in vivo*, *in vitro* and the histochemical approach. Even though *in vivo* methods give the best prediction of pollen behaviour, the seed set may not depend on fertilization alone [14]. Germinability tests *in vitro* have the potential to provide the best basis for fast predicting pollen performance [15]. In the histochemical approach, one of the viability testing methods is based on the fluorochromatic reaction, which tests for the presence of enzyme activity and the membrane integrity of the vegetative cell [16].

Factors affecting pollen viability during storage were the physiological stage of the flower, pollen age and moisture content [17]. Results indicated that when dispersing from the anther, developmentally immature pollen—binucleate (in *Rosaceae* and most other angiosperms) had reduced metabolic activity, low moisture content and can better tolerate desiccation compared to trinucleate pollen [18]. On the contrary, trinucleate pollen grains (in papaya, dianthus, beet, spinach, quinoa, cabbage, radish, flax, carrot, celery, wheat, corn, oat, barley, sorghum, range grasses) are very sensitive to dehydration due to less pronounced exine and decreased level of reserves after the second mitotic division [19].

So far, the study of the viability of stored pollen at different temperatures has been discussed by many authors for various fruit species such as almond [20], pear [21], mango [22], kiwifruit [23], sweet cherry [24] and apple [17]. There is no previous report on the effects of storage periods up to one year at low and sub-zero temperatures on plum pollen viability. The objective of this study was to assess the effect of different storage periods and temperatures on pollen viability *in vitro* and *in vivo* in three plum genotypes. Determination of the most suitable storage period and temperature for pollen preservation can contribute not only to improving breeding efficiency but also to biodiversity conservation.

2. Materials and Methods

2.1. Field Site and Plum Genotypes Used

The study was conducted in the plum orchard of the Fruit Research Institute, Čačak located in Serbia at 43°55′24″ N and 20°26′51″ E at an elevation of 497 metres. Three plum genotypes, developed at the Fruit Research Institute, used for investigation were chosen on the basis of the flowering time—'Valerija' (early), 'Čačanska Lepotica' (mid-early) and 'Valjevka' (mid-late) [25]. Branches of the chosen genotypes with flowers in the late balloon stage were taken on 28 and 29 May 2017 and collected pollen was used for storage experiments as well as for pollination of the selected female parent. The average air temperature and humidity on May 28 were 9.26 °C and 47.02%, while on the other day, they were 12. 38 °C and 46.99%, respectively (data were provided by the

local Experimental Meteorological Station located near the experimental orchard). Trees of the selected genotypes were healthy and free from diseases and pests. A study on pollen behaviour *in vivo* (in the pistils) was performed after one year of storage at different temperatures. The genotype 'Čačanska Lepotica' was used as the female parent.

2.2. Anthers' Collection and Storage

In the laboratory, anthers from the flowers in the late balloon phase (BBCH61) of the chosen genotypes were collected into paper dishes [26]. Anthers were left to desiccate at room temperature until their dehiscence and release of pollen grains (up to 48 h). In order to determine moisture content (MC), anthers with pollen grains were oven-dried to a constant weight (Memmert GmbH + Co.KG, Büchenbach, Germany) for 48 h at 65 °C and 1 h at 105 °C. Moisture content was determined from the constant dry weight of anthers and weight measured immediately after dehiscence. Data presented in Table 1 are the mean values of three independent moisture measurements in each genotype. After desiccation, the anthers were placed in marked vials and stored in darkness under the following storage conditions: at 4 °C in a refrigerator; at −20 °C (Artiko PR700, Esbjerg, Denmark); at −80 °C (Artiko ULUF GG, Esbjerg, Denmark) in a freezer; and at −196 °C (Cryo Diffusion B2020, Lery, France) by the direct plunging of closed cryotubes with desiccated pollen in liquid nitrogen (LN). The viability of the pollen was estimated every 3 months during the storage period, namely at day 0 (fresh dry pollen) and after 3, 6, 9 and 12 months (until the full blooming time of the female parent). Each of the storages included four tubes with pollen per each genotype (one tube for each storage period). After defrosting, pollen stored at −20 °C was incubated for a short period at room temperature, while after storage at −80 °C and in LN, fast thawing was done in a water bath at 38 °C for 2 min and 5 min, respectively.

2.3. In Vitro Pollen Germination

A nutrition medium for pollen grains containing 12% sucrose and 1% agar was poured into Petri dishes (10 mL per dish). Fresh pollen taken immediately after desiccation as well as pollen stored at different temperatures were dusted onto the nutrition medium with a fine brush. The incubation period was 24 h at 23 °C. *In vitro* germination was observed under a light microscope (Olympus BX61, Tokyo, Japan) with a 20× ocular by counting germinated pollen grains in three different ocular fields, with an average of about 80 pollen grains in each. A Pollen grain was considered as germinated if the length of the pollen tubes exceeded their diameter [27]. Germinability of pollen was tested for each genotype, storage period and temperature.

2.4. Pollen Viability Test

To determine pollen viability the FDA test was used [15]. Fresh staining solution was poured into vials, after which, pollen was stirred into the solution USING a stainless steel needle. Vials were shaken and left in the dark for 20 min. Then, 1–2 drops of the solution were put into microscope slides, covered with a cover slip and immediately observed under UV light with an Olympus BX61 fluorescence microscope. Pollen grains which showed bright green fluorescence were considered viable, while those with light exine fluorescence and slight adsorption were considered non-viable grains. For each genotype, storage period and temperature, a similar number of pollen grains were observed (on average 50 in three different ocular fields).

2.5. Stigmatic Germinability and Fertilization Ability of Stored Pollen

For testing pollen germinability and fertilization ability after one year of storage at different temperatures, the genotype 'Čačanska Lepotica' was used as the mother parent. At the late balloon phase, flowers of this genotype were emasculated and isolated with paper bags. At the anthesis (BBCH65), pollination was carried out using pollen defrosted as previously described (Section 2.2) and performed by finger (two touches of stigma). Branches were then re-bagged and marked. Sampling of 30 pistils in FPA solution was carried out on

the 3rd, 6th and 10th day after anthesis (DAA) for each tested storagetemperature. FPA solution was prepared using 70% ethyl alcohol, propionic acid and formaldehyde, 90:5:5 by volume. Samples were kept at 4 °C until further observation.

Pistils were washed in water for a short time and softened in 8N NaOH for 24 h. Test tubes were occasionally shaken to gently mix the pistils. After softening, pistils were stained with 0.1% aniline blue to view the pollen tubes according to the protocol described by Preil [28] and Kho and Baër [29]. On the microscope slides, the ovaries were separated from the pistils and opened along the suture. The pistils were divided into two parts with needles, covered with cover slips and gently squashed. One or two drops of glycerol were put on two sides of the cover slips to avoid the drying of the sample. The samples were observed under UV light with an Olympus BX61 fluorescence microscope.

For each genotype, the dynamics of pollen tube growth through the different parts of the style and ovary were presented as a percentage of the longest pollen tube penetrating a certain part of the pistils.

2.6. Statistical Analysis

Arcsine root square transformation was used for data in percentage and ANOVA analysis was performed within Statistica 8.0 (StatSoft, Inc., Tulsa, OK, USA). Results were analysed by two-way analysis of variance and the means were separated using the LSD test at $p \leq 0.05$. Data were shown as means ± SD.

3. Results

3.1. Anthers Moisture Content

The results indicate that in all tested genotypes, the percentage of moisture in anthers after drying was above 6%, with the lowest value in 'Valerija' and the highest in 'Čačanska Lepotica' (Table 1).

Table 1. Anthers moisture content measured immediately after dehiscence.

Genotype	Moisture Content (%)
'Čačanska Lepotica'	6.78 ± 0.31
'Valerija'	6.15 ± 0.18
'Valjevka'	6.72 ± 0.65

Values in the table show mean ± SD.

3.2. Pollen Germinability and Viability

Germination and viability of fresh pollen for all tested plum genotypes was over 40% (Tables 2 and 3). Pollen germination was rated from 42.35% in 'Valjevka' to 63.79% in 'Čačanska Lepotica'. The viability percentage ranged from 54.58% to 62.15% ('Valjevka' and 'Valerija', respectively). For fresh pollen, the difference in genotype germinability and viability was found to be significant. Only with pollen of 'Čačanska Lepotica', was slightly lower viability observed compared to germinability, while in the other two tested genotypes, the opposite situation was found.

Table 2. *In vitro* pollen germination stored at different temperatures for different periods.

Genotype	Temperature	Fresh Pollen/Day 0 of the Storage	3 Months of Storage	6 Months of Storage	9 Months of Storage	12 Months of Storage	
'Čačanska Lepotica'	4 °C −20 °C −80 °C −196 °C	63.79 ± 0.87 a/A	57.73 ± 1.06 a/BCD 59.60 ± 0.98 a/ABCD 59.83 ± 0.71 a/ABC 62.04 ± 0.93 a/AB	55.38 ± 0.70 a/DEF 57.61 ± 1.19 a/CD 56.61 ± 0.98 a/CDE 58.94 ± 0.57 a/BCD	48.56 ± 0.64 b/F 56.81 ± 0.45 a/CDE 55.73 ± 0.68 a/CDEF 57.68 ± 1.38 a/CD	3.17 ± 1.97 d/G 52.41 ± 0.91 a/EFG 55.27 ± 0.54 a/DEF 51.77 ± 0.89 a/FG	genotype *, temperature *, genotype × temperature *
'Valerija'	4 °C −20 °C −80 °C −196 °C	44.73 ± 0.94 b/A	33.93 ± 0.38 b/E 41.32 ± 1.08 b/BCD 40.30 ± 1.08 b/BCD 43.87 ± 1.14 b/AB	25.05 ± 0.78 d/F 38.33 ± 0.19 bc/CD 38.24 ± 0.68 bc/CD 41.03 ± 2.57 b/ABC	18.40 ± 0.19 f/G 37.71 ± 0.03 cd/CDE 37.74 ± 0.07 cd/CDE 40.87 ± 0.56 c/ABCD	0.00 ± 0.00 f/H 37.41 ± 1.43 bc/DE 36.77 ± 0.27 bc/DE 40.77 ± 0.36 b/ABCD	genotype *, temperature *, genotype × temperature *
'Valjevka'	4 °C −20 °C −80 °C −196 °C	42.35 ± 0.92 c/A	30.65 ± 1.18 c/C 40.30 ± 1.03 c/BC 37.80 ± 0.76 c/AB 41.92 ± 0.94 c/A	21.43 ± 1.08 e/D 34.63 ± 1.55 c/BC 36.80 ± 1.57 bc/B 41.75 ± 0.76 b/A	13.66 ± 0.36 g/D 33.49 ± 0.54 e/BC 34.57 ± 1.37 de/BC 36.45 ± 1.02 cd/AB	1.68 ± 1.28 e/E 35.27 ± 0.83 c/BC 35.58 ± 0.52 c/B 33.81 ± 1.21 c/BC	genotype *, temperature *, genotype × temperature *
		genotype *, temperature ^{ns}, genotype × temperature ^{ns}	genotype *, temperature *, genotype × temperature ^{ns}	genotype *, temperature *, genotype × temperature *	genotype *, temperature *, genotype × temperature *	genotype *, temperature *, genotype × temperature *	ANOVA

Values in the table show mean ± SD, ns—non-significant; * significant at $p \leq 0.05$. Means followed the same lowercase in columns (comparison between all genotypes and all temperatures within one storage period) and capital in the rows (comparison between all temperatures and all storage periods within one genotype) are not significantly different.

Table 3. Viability (FDA staining) of pollen grain stored at different temperatures for different periods.

Genotype	Temperature	Fresh Pollen/Day 0 of the Storage	3 Months of Storage	6 Months of Storage	9 Months of Storage	12 Months of Storage	
'Čačanska Lepotica'	4 °C −20 °C −80 °C −196 °C	59.25 ± 3.98 b/A	56.25 ± 1.51 a/AB 58.02 ± 2.36 a/AB 57.82 ± 0.26 a/AB 57.72 ± 3.82 a/AB	51.09 ± 2.87 c/BC 57.90 ± 0.27 a/BC 56.82 ± 0.93 a/BC 56.24 ± 0.43 ab/BC	48.89 ± 1.03 bc/CD 54.25 ± 2.40 ab/CD 52.24 ± 0.18 ab/CD 55.56 ± 0.71 a/CD	39.31 ± 1.59 c/D 53.36 ± 1.80 a/D 51.97 ± 1.80 a/D 54.30 ± 2.58 a/D	genotype *, temperature *, genotype × temperature ^{ns}
'Valerija'	4 °C −20 °C −80 °C −196 °C	62.15 ± 2.78 a/A	54.56 ± 0.56 a/A 57.19 ± 0.50 a/ABC 59.32 ± 1.25 a/AB 60.01 ± 0.77 a/AB	42.72 ± 0.51 de/E 52.62 ± 0.71 bc/CD 56.28 ± 1.32 ab/ABC 57.20 ± 0.93 a/ABC	33.50 ± 3.70 e/ F 51.67 ± 0.95 ab/ CD 53.76 ± 1.08 ab/ BCD 55.70 ± 2.62 a/ BD	0.00 ± 0.00 e/G 47.67 ± 6.29 ab/DE 51.29 ± 2.62 a/CD 54.59 ± 3.65 a/BC	genotype *, temperature *, genotype × temperature *
'Valjevka'	4 °C −20 °C −80 °C −196 °C	54.58 ± 0.49 b/A	45.74 ± 3.65 b/A 52.52 ± 1.09 b/A 54.17 ± 2.68 b/A 51.59 ± 2.18 b/AB	36.66 ± 0.77 f/E 41.81 ± 2.43 de/CDE 41.33 ± 1.70 e/CDE 45.75 ± 0.47 d/BC	19.20 ± 2.13 f/ F 38.17 ± 1.27 de/ DE 40.54 ± 2.46 d/ CDE 43.39 ± 0.98 cd/CD	17.60 ± 1.09 d/F 36.30 ± 1.92 c/E 38.93 ± 2.00 c/DE 41.43 ± 0.85 bc/CDE	genotype *, temperature *, genotype × temperature *
		genotype *, temperature ^{ns}, genotype × temperature ^{ns}	genotype *, temperature ^{ns}, genotype × temperature ^{ns}	genotype *, temperature *, genotype × temperature *	genotype *, temperature *, genotype × temperature *	genotype *, temperature *, genotype × temperature *	ANOVA

Values in the table show mean ± SD, ns—non-significant; * significant at $p \leq 0.05$. Means followed the same lower case in columns (comparison between all genotypes and all temperatures within one storage period) and capitals in the rows (comparison between all temperatures and all storage periods within one genotype) are not significantly different.

Compared with fresh pollen, after 3 months of storage at 4 °C, declines in pollen germination *in vitro* and viability were observed in all tested genotypes. It was the most evident in 'Valjevka' (30.65% germination *in vitro*, 45.74% viability) and 'Valerija' (33.93% germination *in vitro*, 54.56% viability), while less pronounced in 'Čačanska Lepotica'. As regards the pollen stored at −20, −80 and −196 °C for the same period, the percentage of germination and viability was only 1% lower compared to fresh pollen in all genotypes. For this storage period, the analysis of variance showed that the effect of genotype and storage temperature on pollen germination *in vitro* genotypes was significant.

A decreasing trend in pollen germination *in vitro* and viability after 6, 9 and 12 months of pollen storage at 4 °C was even more evident in all analysed genotypes. Both parameters declined to zero after the longest storage period in 'Valerija'. In 'Valjevka', germination was reduced to 1.68% after 12 months, while in 'Čačanska Lepotica' it was found to be 3.17%. Pollen viability in these genotypes was 39.31% ('Čačanska Lepotica') and 17.60% ('Valjevka') after the longest storage period. Germination and viability of the pollen in all genotypes stored at −20, −80 and −196 °C were almost similar.

Pollen germination and viability after 12 months of storage at sub-zero temperatures were above 51% in 'Čačanska Lepotica', 36% in 'Valerija' and 33% in 'Valjevka'. After 12 months of storage at −196 °C in 'Čačanska Lepotica' and 'Valjevka', only a slightly lower percentage of germination *in vitro* was observed in regard to the values observed after storage at −20 and −80 °C, while in 'Valerija' at this temperature, the highest pollen germination was confirmed (Figure S1). The FDA pollen viability test revealed that, after 12 months of storage, the highest pollen viability was determined in grains preserved at −196 °C in all tested genotypes (Figure 1a–d). For all three storage periods, a highly significant effect of genotype, temperature and interaction between genotype and temperature on pollen germination and viability was observed.

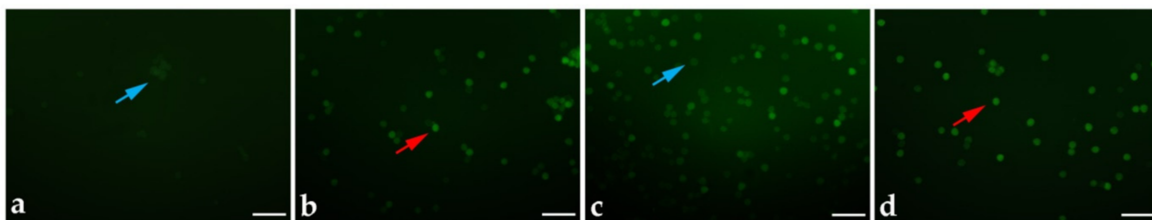

Figure 1. FDA-stained pollen grains after 12 months of storage: (**a**) at 4 °C; (**b**) at −20 °C; (**c**) −80 °C; (**d**) −196 °C. Scale bars = 200 μm. Blue arrows—non-viable pollen grain; red arrows—viable pollen grain.

3.3. Stigmatic Germinability of Pollen and Its Further Growth In Vivo

With pollen of 'Valerija' stored for 12 months at 4 °C, no germination was observed after applying it onto the wet stigma surface, not even on the 3rd or 6th day after pollination. Under the same storage conditions, pollen of 'Čačanska Lepotica' and 'Valjevka' was germinated on the stigma surface, but the growth of pollen tubes on the 10th day after pollination mostly ended in the basal part of the style (in 33.33% and 25% of analysed styles, respectively).

Dynamics of pollen tube growth of pollen stored at −20, −80 and −196 °C for all genotypes were fairly uniform. On the 3rd day after pollination, except for pollen of 'Čačanska Lepotica', the longest pollen tube was evident in the basal part of the style (13.64%). In other genotypes and storage temperatures, pollen tubes penetrated the locule of the ovary in different percentages (Figure 2).

In all tested genotypes and storage temperatures, on the 10th day after pollination, pollen tubes entered the nucellus of the ovule. For 'Čačanska Lepotica' and 'Valerija' the highest percentage of pollen penetration was observed in pollen stored at −80 °C (95% and 66.67%, respectively), while for 'Valjevka' it was noticed with pollen stored at −196 °C (100%) (Figure 3).

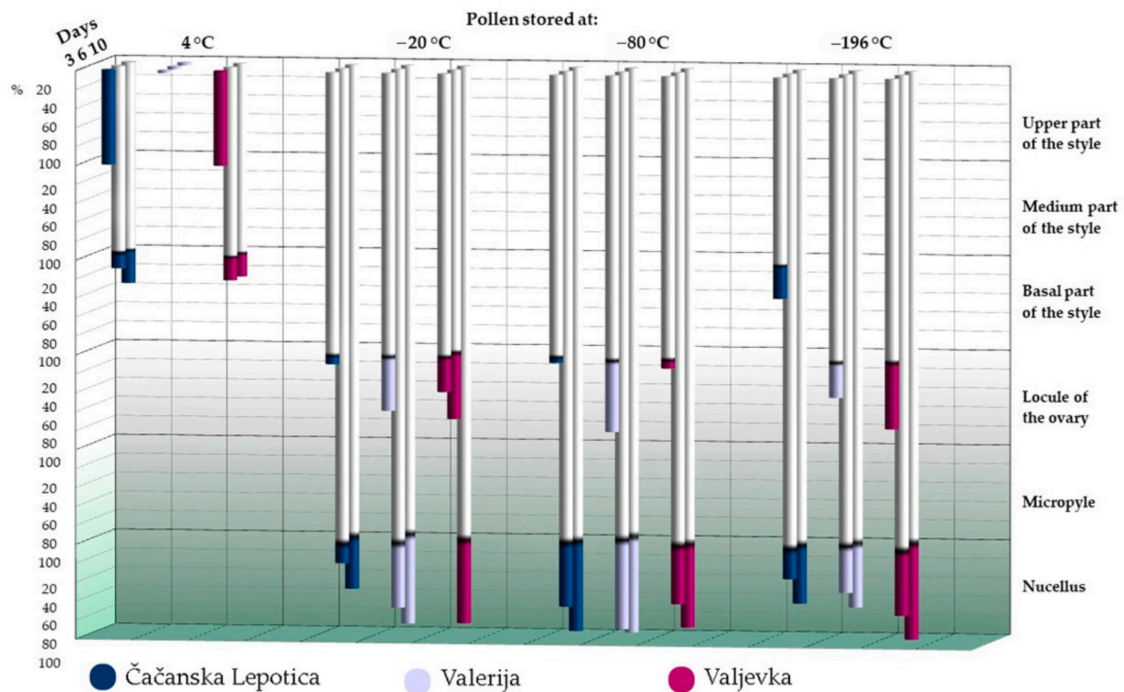

Figure 2. Dynamics of pollen tube growth in the pistils of the 'Čačanska Lepotica' after 12 months of pollen storage at different temperatures.

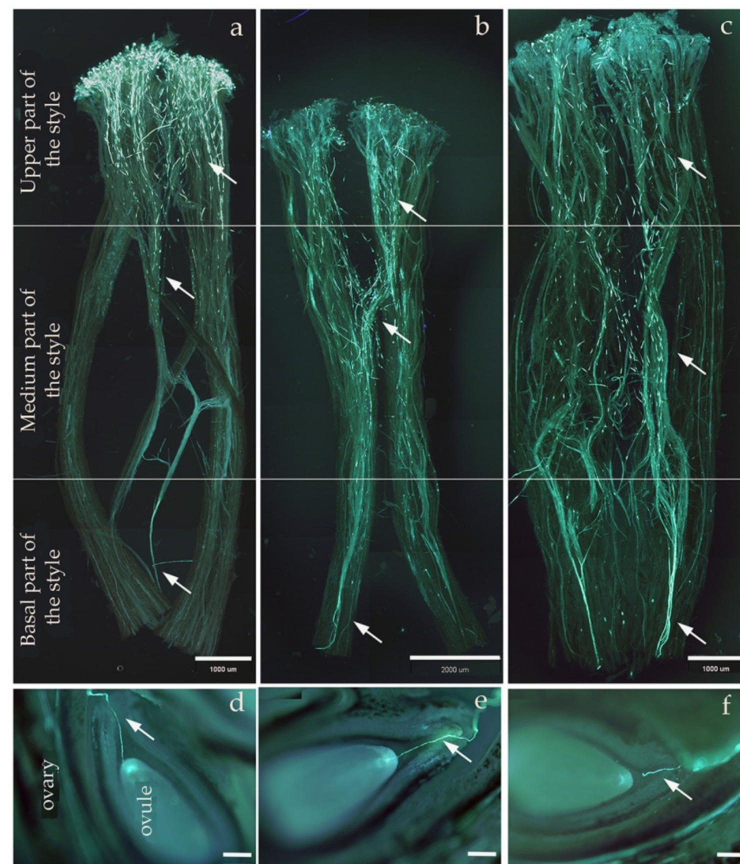

Figure 3. Growth of pollen tubes into certain parts of the pistils of 'Čačanska Lepotica' from the pollen of: (**a**,**d**) 'Čačanska Lepotica' stored at −20 °C; (**b**,**e**) 'Valerija' stored at −80 °C; (**c**,**f**) 'Valjevka' stored at −196 °C. Scale bars: (**a**,**c**)—1000 µm; (**b**)—2000 µm; (**d**,**e**,**f**)—200 µm. Arrows—indicate pollen tubes.

The highest average number of pollen tubes in the upper part of the style was observed in all tested genotypes for pollen stored at −20 °C ('Čačanska Lepotica', 51.53; 'Valjevka', 47.66; 'Valerija', 41.87) (Figure 4). The analysis of variance for the average number of pollen tubes in the upper part of the style indicates that storage temperature and interaction between genotype and temperature had a significant effect. In the ovary, quite surprising results were obtained for the average number of pollen tubes. Namely, the highest average number of pollen tubes was observed with the pollen of 'Valerija' stored at −20 °C (5.73) and 'Valjevka' stored at −196 °C (5.16). As compared to these genotypes, a markedly lower average number of pollen tubes in the locule of the ovary was determined using the pollen of 'Čačanska Lepotica' stored at different temperatures. The analysis of variance showed that the effect of genotype, storage temperature and their interaction on the average number of pollen tubes in the locule of the ovary was significant.

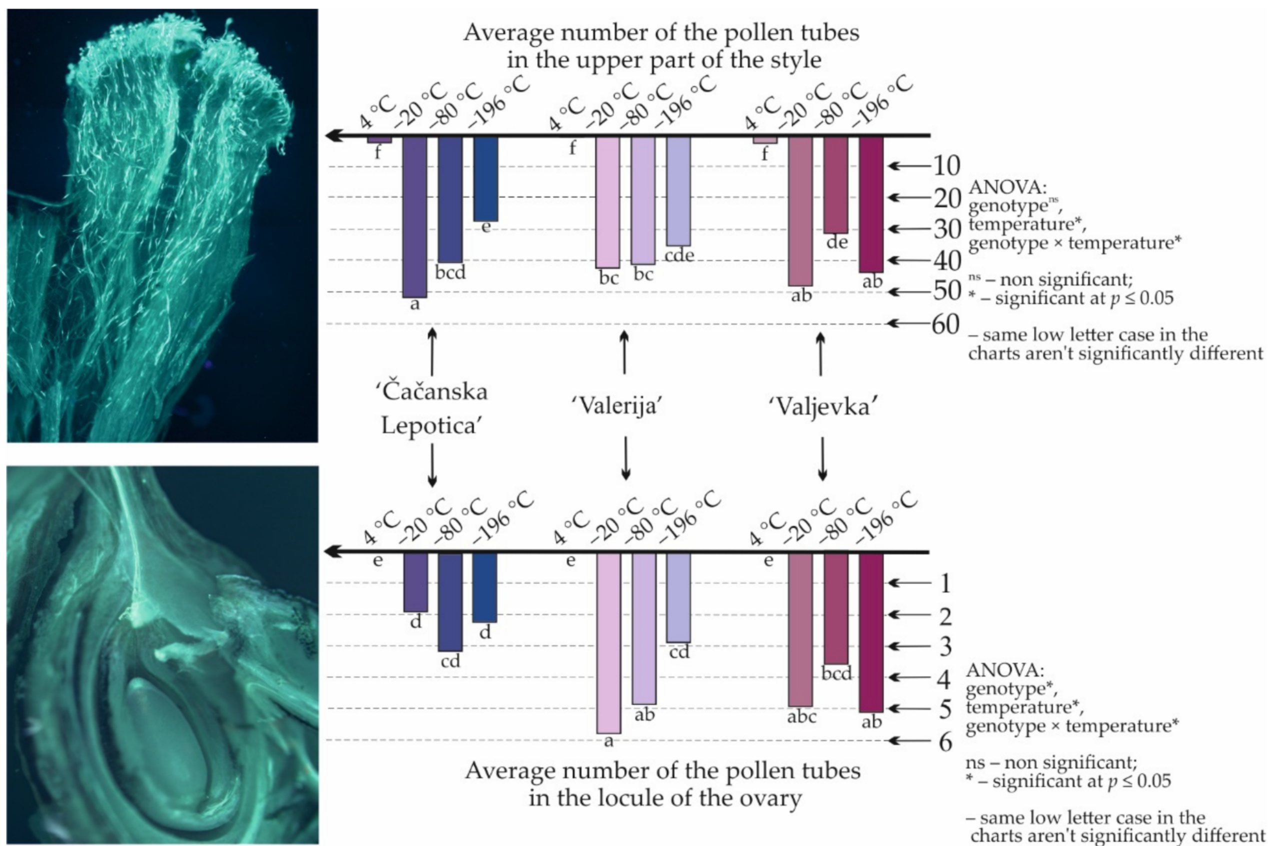

Figure 4. Average number of pollen tubes in certain parts of the pistil of 'Čačanska Lepotica'.

4. Discussion

4.1. Moisture Content of Anthers

Most of the mature pollen grains, when released from the anther, are metabolically inactive and desiccated, with water content ranging from 15 to 35% [30]. Pollen with high initial water content has been observed to be sensitive to stress and is typically short-lived during storage periods [31]. About 70% of plant species, including *Prunus* species, have binucleate pollen classified as tolerant to drying [18]. According to some authors, the longevity of stored pollen may be related to the presence of proteins and starches contained within [32]. Results for successful long-term conservation of desiccation-tolerant pollen indicate that moisture content should be lowered to between 5% and 10% on a fresh weight basis to avoid ice crystals forming during the freezing process [33]. In this current study, the moisture content of anthers after desiccation was less than 7% which can be considered as a low moisture content, which prevents damage during storage at low temperatures and thawing at room temperature.

4.2. Pollen Viability after Different Storage Periods and Temperatures

The obtained results for the germination of freshly collected pollen of genotype 'Čačanska Lepotica' are in agreement with the results from other authors for this genotype [34,35], but this value is higher than reported in the previous study [36], probably due to the fact that in the earlier work the constant temperature during pollen germination was lower. Pollen germination of the other two genotypes 'Valerija' and 'Valjevka' was above 40%, and according to Wertheim [37], these are considered genotypes with good pollen germination rates. Furthermore, other factors, responsible for variation in *in vitro* germination should be taken into account, such as the physiological condition of plants as well as environmental conditions, the time of pollen maturation and shedding that can cause strong variations in pollen germination [38]. Results of some studies [39] indicate that the concentration of some carbohydrates such as sucrose and starch increases in pollen due to the reduction of the metabolism under heat stress. Interaction between exogenous and endogenous factors during pollen grain development may affect its capacity to germinate [35].

With regard to FDA, with fresh pollen of genotype 'Čačanska Lepotica' a slightly lower percentage of pollen was stained in comparison with the percentage of pollen which germinated on the medium, while in the other two genotypes, better value was obtained with FDA.

Generally, the viability of pollen stored for different periods and at different temperatures was lower than for fresh pollen. Similar results were observed with fresh almond [20], mango [22] and apple [17] pollen. After 9 months of storage, pollen viability decreased slightly (but still above 30% in all genotypes), indicating that a temperature of 4 °C is suitable for short-term storage of these plum genotypes. In all genotypes, from 9 months onwards the viability of pollen stored at 4 °C significantly decreased having its lowest value by the end of the storage period. Complete loss of pollen viability, or its gradually decrease, was reported in other *Prunus* species such as almond [20] and sweet cherry [14] at 4 °C after 2 or 12 months of storage, respectively.

The results of this study show that pollen germinability and viability can be preserved for a one-year period by storing at sub-zero temperatures (−20, −80 and −196 °C). Generally, a slight and almost linear decrease in pollen viability was observed in all genotypes at all storage temperatures during the 12-month period. Pollen germination after 12 months of storage at sub-zero temperatures declined by 8.83% to 20.17%, while with the FDA test, this decline was 10% to 33.49%. Pollen germination percentages were similar for pollen stored at −20 and −80 °C, while the greatest differences in pollen viability were observed for those kept at −196 °C. The germination value of pollen stored for 12 months at this temperature was highest in 'Valerija', while in 'Čačanska Lepotica' and 'Valjevka', significantly lower values of this parameter were observed.

In genotypes 'Valerija' and 'Valjevka', higher percentages of pollen grains were stained by FDA in comparison with the percentage of pollen germinated on the medium containing 12% of sucrose. The results obtained in this study are in agreement with previous results for sweet cherry [40] and apple [17], where FDA staining tended to overestimate the ability of pollen to germinate. With the genotype 'Čačanska Lepotica', the opposite situation was observed. A lower value after FDA staining was obtained at all temperatures for all storage periods, which could indicate either a low level of enzyme esterase or its low activity in the pollen grain.

4.3. Pollen Germination In Vivo

Successful pollination and double fertilization are two essential processes in seed production. The progamic phase consists of a number of successive steps started after the pollen lands on the stigma: its adhesion, hydration, germination and production of a pollen tube [41]. This phase is crucial since the process of pollen acceptance/rejection occurs during this period [42,43]. *In vivo* pollen tube growth has been demonstrated as an effective method for assessing pollen behaviour [44]. A strong correlation between the number of pollen tubes in the upper third of the style and pollen germination *in vitro* was

reported in sweet [45] and sour cherry [46], which decreases with the increased distance from the stigma. On the other hand, in plum, [35] no such correlation was found, which was explained by different conditions for pollen tube growth.

Pollen grains of 'Čačanska Lepotica' and 'Valjevka' stored for 12 months at 4 °C germinated on the stigma surface. However, pollen tube growth *in vivo* on the 10th day after pollination mostly ended in the lower parts of the style. Bearing in mind that the average number of pollen tubes in the upper part of the style was lower than 5, we could say that the pollen functionality of these genotypes was lost. Namely, pollen grain can germinate, as the germination test has shown, but could not achieve fertilization. In different plant species, during low-temperature storage, free radicals accumulate due to disorders of the oxidative system which leads to the low energy of pollen and ultimately to the inhibition of pollen germination [47,48]. Furthermore, it is found that during pollen storage, the content of glucose and proline increased when pollen germination decreased. However, storage of pollen at ultra-low temperatures inhibits metabolism, reduces enzyme activity and slows down the respiration rate at which pollen viability declines [49].

The most important findings in this study are that the pollen of all the tested genotypes stored at −20, −80 and −196 °C for 12 months, germinated on the stigma, pollen tubes grew through the style of 'Čačanska Lepotica' and penetrated through the micropyle into the nucellus. Except for the pollen of 'Valjevka' stored at −20 °C, where on the 6th day after pollination the pollen tube entered into the locule of the ovary, tubes of the pollen of the other genotypes stored at different temperatures were observed to enter into the nucellus of the ovary. On the 10th day after pollination, in all tested genotypes, the percentages of pollen tubes entering the nucellus were even higher.

For the genotype 'Čačanska Lepotica', the best dynamic of pollen tube growth was observed with pollen stored at −80 °C. This was followed by the highest average number of pollen tubes entering the locule of the ovary. The dynamics of pollen tube growth for pollen stored at −20 °C and −196 °C are very similar, with nearly the same average number of pollen tubes entering the locule of the ovary. The dynamics of pollen tube growth for the genotype 'Valerija' with pollen stored at −20 °C and −80 °C were similar. The highest average numbers of pollen tubes in the upper part of the style and in the locule of the ovary were observed with pollen stored at these temperatures. As regards 'Valjevka', the best pollen tube dynamics, as well as average pollen tube number in a certain part of the pistils, were observed with pollen stored at −196 °C.

Recent studies investigating the effect of pollinizers on pollen tube growth in the European plum revealed that the best dynamics are achieved in cross-pollination variants [35,36,50]. The results obtained in this work are in accordance with these findings. Good dynamics of pollen tube growth observed in self-pollination of 'Čačanska Lepotica' using pollen stored at different temperatures confirms the self-fertility of this genotype as was previously proved [51,52].

5. Conclusions

To the best of our knowledge, this is the first report on the effect of one-year pollen storage at different temperatures on the behaviour of plum pollen *in vitro* and *in vivo*. This study provides useful information about the possibility of pollen storage for up to one year at different temperatures in plum genotypes 'Čačanska Lepotica', 'Valerija' and 'Valjevka'. These findings are of great importance to plum breeding programmes where there is spatial and temporal isolation between parental genotypes. Furthermore, there is no need for the growth of pollen parent and stored pollen could be a good source of germplasm in different exchange programmes. Based on the observed results, storage of pollen at 4 °C was acceptable for storage up to 3 months in all tested genotypes. Storage of pollen at −20, −80 and −196 °C for a one-year period was possible in all analysed genotypes, although the viability of pollen stored at these temperatures slightly decreased in comparison with fresh pollen. The dynamics of pollen tube growth of pollen from anthers stored at sub-zero

temperatures, which did not differ from the dynamics previously determined using fresh pollen [50,52], is a good indicator that such pollen can be safely used for breeding purposes.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae8070616/s1, Figure S1: Pollen germination *in vitro*: (a) 'Čačanska Lepotica', fresh pollen; (b) 'Valerija', fresh pollen; (c) 'Valjevka', 3 months at 4 °C; (d) 'Valerija', 6 months at 4 °C; (e) 'Čačanska Lepotica', 9 months at −20 °C; (f) 'Čačanska Lepotica', 9 months at −80 °C; (g) 'Valerija', 12 months at 4 °C; (h) 'Čačanska Lepotica', 12 months at −196 °C. Scale bar = 200 µm.

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