A simple and efficient method for onion pollen preservation: germination, dehydration, storage conditions, and seed production

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ABSTRACT

The preservation of viable pollen is essential to overcome the problems related to the asynchronous flowering of the parental lines in onion hybrid breeding programs. The aim of this study was to establish a simple, inexpensive, and easily reproducible protocol for medium-term onion pollen storage. First, the conditions for assessing the *in vitro* pollen germination were optimized. The liquid medium favored the counting of germination of pollen grains in comparison to the solid medium. The addition of 75 mg/l $Ca(NO_3)_2 . 4 H_2O$ to the medium did not improve pollen germination, while that of 150 mg/l $Ca(NO_3)_2 . 4 H_2O$ inhibited pollen germination. The highest germination percentage was achieved by incubation at 30-35 °C in the dark. Second, fresh or dehydrated pollen (maintained in a desiccator with silica gel at 25 °C for 18 h) was stored at 4 °C, –20 °C, and –80 °C for two

years to study pollen preservation. In addition, the viability and germination capacity of stored pollen were periodically evaluated at 0, 15 and 30 days; 2 and 6 months; and 1 and 2 years. Pollen viability was best retained at low relative humidity and temperatures below zero. Dehydration was essential for pollen preservation at –20 °C and –80 °C. The results showed that dehydrated pollen stored at –20 °C could be used, with guarantees, for pollination throughout the flowering season. However, the highest viability and *in vitro* germination percentages after two years of storage (29 and 32%, respectively) were achieved with dehydrated pollen stored at –80 °C. Finally, the capacity of stored pollen to produce seeds was confirmed in crosses with male sterile lines. In this way, dehydrated pollen stored at –80 °C for two years produced an average of 47.9 seeds/100 flowers, representing 43% of the seed in the control crosses. This is the first report in onion research of seed production after pollination with preserved pollen at –80 °C for two years.

Keywords: *Allium cepa;* pollen;, dehydration; storage at –80 °C; seed production; hybrid.

1. Introduction

Onion (*Allium cepa* L.) is one of the most valuable vegetable crops in the world, ranking second after tomato, with a production of over 100 million tons on 5.5 million ha in 2020. The European Union (EU) countries produce approximately 6.6 million tons on 176,500 ha. Spain is the largest producer of dry onions in the EU, with 1.3 million tons on 25,300 ha (Faostat, 2020). Both dried and fresh onions are widely consumed in cuisines around the world, either as food, an ingredient, or a spice, and the trend of their consumption has increased over the last fifty years (Faostat, 2020). Currently, it is well known that onions are a source of natural antioxidant and bioactive compounds with a large number of health-related properties (Fayos et al., 2018; Charles, 2013).

The onion is an allogamous species from which both open-pollinated cultivars and hybrids are cultivated. Hybrids provide many advantages for commercial use, such as higher yield, genetic uniformity, and seed production (Campion et al., 1995; Foschi et al., 2009). Onion hybrid seed production requires the flowering synchronization of the parental lines (Peters, 1990), or pollen availability in the absence of synchrony (Padmani et al., 2007). In this sense, the ability to store viable pollen has a great interest to breeders not only to overcome flowering asynchrony, but also for the preservation, distribution, and exchange of high-elite germplasm (Towill and Walters, 2000).

To estimate the quality of stored pollen, the fluorescein diacetate reaction (FCR) (Heslop-Harrison and Heslop-Harrison, 1970), and the quantification of *in vitro* pollen germination

have been routinely used as simple and time-saving tests (Towill and Walter 2000; Shivanna and Johri, 1985). Although the quantification of seeds is the the most accurate test to evaluate pollen preservation, it is also the most laborious and time-consuming (Shivanna and Johri, 1985). However, this test has not been used widely used for this purpose.

In onion species, several factors influence the *in vitro* germination capacity of pollen, such as sucrose, boric acid, calcium concentrations, and incubation temperature (Kwan et al., 1969). In this sense, a higher germination percentage is achieved with 20% sucrose than with 15% sucrose, and 300 mg/l $Ca(NO_3)_2 . 4H_2O$ inhibits germination compared with 0 mg/l (Gomes et al., 2000). However, in previous studies, concentrations of 600 mg/l or 1000 mg/l $Ca(NO_3)_2 . 4H_2O$ were used (Nomura et al., 1994; Ockedon and Gates, 1976). To date, no studies are available regarding the optimal temperature for *in vitro* germination, although 24 °C was used by Gomes et al. (2000).

The main factors affecting the longevity of stored pollen are the water content and the developmental stage at the time of storage, and the relative humidity and temperature during storage (Buitink et al., 2000; Gomes et al., 2003, Ganeshan et al., 2008). In onion species, pollen germination takes place at the binucleate stage (Ockedon and Gates, 1976). Generally, the longevity of bicellular pollen can be greatly extended by dehydration and by lowering the temperature of storage, with cryopreservation being the best system (Towill and Walters, 2000). Thus, cryopreserved onion pollen had a higher germination percentage than pollen storage at –18 °C after two years (Gomes et al., 2003). Likewise, similar germination percentages of fresh and cryopreserved pollen for one year have been reported in *Allium* species (Kanazawa et al., 1992). Additionally, high fertility profiles have been

reported with pollen cryopreserved for 11 years in onion (Ganeshan and Rajasekharan, 2005). However, cryopreservation in liquid N_2 requires specific and expensive equipment, which is not available in all onion breeding laboratories.

Few studies have compared the preservation of dehydrated pollen at temperatures above –196 °C in *Allium* species. In one study, storage of onion pollen above 0 °C showed merely 10% of the initial germination after 10 to 55 days, whereas pollen stored at –18 °C maintained 40% after six months (Kwan et al., 1969). Other studies in diverse *Allium* species have showed that pollen preserved at –30 °C had higher germination than that stored at 5 °C (Nomura et al., 1994). In addition, pollen of *A. chinense* x *A. thunbergii* hybrids stored at –20 °C and –40 °C for one year showed similar germination percentages (Dubouzet et al., 1993). To our knowledge, no studies have been performed on the preservation of onion pollen at –80 °C.

The objective of this study was to identify a simple, inexpensive, and easily reproducible protocol for onion pollen storage that allows the production of hybrid seeds after 1-2 years of pollen preservation. For this purpose, we first optimized the temperature and the germination medium composition to assess pollen quality after storage. Second, we evaluated the effect of pollen dehydration and storage temperature on the quality of pollen, by studying pollen viability, *in vitro* germination, and seed production over two years.

2. Materials and Methods

2.1 Plant material and growth conditions

For pollen preservation studies, bulbs of *A. cepa* L. 'Fuentes de Ebro' (average weight 345 g and diameter 102 mm) were transplanted directly to the soil in a field under natural conditions for inflorescence development in November 2017 and 2018. 'Fuentes de Ebro' is a landrace grown in the northeast of Spain, known for its mild and sweet flavor (Mallor et al., 2011; Mallor and Sales, 2012); it has a high commercial value due to its differentiated quality, provided by the Protected Designation of Origin (PDO) label, according to the European Union Regulation (EEC) 1146/2013.

For cross-pollination, bulbs from two *A. cepa* accessions from the Vegetable Germplasm Bank of Zaragoza (BGHZ, CITA, Zaragoza, Spain) were transplanted into the greenhouse in November 2018 and 2019. Specifically, the male sterile accession 'BGHZ4552' (average weight 199 g and diameter 79 mm) and its maintainer 'BGHZ4553' (average weight 132 g and diameter 71 mm) were used.

2.2. Pollen collection

Umbels with more than half of the flowers opened were harvested between 11:00 am and 1:00 pm. Mature anthers from several umbels collected on the same day were pooled and placed in the lid of a Petri dish.

2.3 Optimization of *in vitro* pollen germination conditions

To study the influence of medium composition on pollen germination, freshly collected pollen was used. First, a modified medium described by Gomes et al. (2000) containing 50 mg H_3BO_3, 200 g/l sucrose, and 10 g/l Sea Plaque agarose (Lonza) (M1A), as well as the same medium without agarose (M1) were tested for pollen germination. Pollen grains were

placed on the surface or inside the solid medium (M1A-S, and M1A-I, respectively), or in the liquid medium (M1). Pollen was embedded in solid medium and gently mixed with the agarose medium at 35 °C. Second, the influence of calcium on pollen germination was studied by adding 0 (M1) mg/l, 75 (M2) mg/l, and 150 (M3) mg/l $Ca(NO_3)_2$. $4H_2O$ to the liquid medium described above. These two experiments were carried out at 25 °C in the dark.

Furthermore, the influence of temperature on the percentage of pollen germination was also studied. Pollen grains were germinated in M1 medium and incubated at 25 °C, 30 °C, and 35 °C for 4-5 hours in the dark.

To determine the *in vitro* pollen germination ability, six to ten anthers were deposited on 1 ml of germination medium as mentioned above. Pollen grains were gently released into the medium; otherwise, a scalpel was used to facilitate the release of pollen from the anther wall. In most cases, three replicates were used. Germination was evaluated after 5 hours according to preliminary experiments, which showed no increase in germination percentage after that time. A pollen grain was considered germinated when the tube length was at least 2.5 times the pollen grain length.

2.4 Pollen storage

Four and six pools of pollen were harvested on different dates in 2018 and 2019, respectively. In 2018, each pollen pool was divided into two portions, namely, a fresh, nondehydrated (non-DH) portion that was stored directly after collection and a dehydrated (DH) portion in a desiccator containing silica gel at 25 °C for 18 h before storage. Both

types of pollen were distributed in 20-25 individual Eppendorf tubes and stored at 4 °C, –20 °C, and –80 °C until preservation analyses and crosses were performed. Pollen to be stored at –80 °C was frozen in liquid N_2 before being transferred to the freezer, whereas pollen to be stored at 4 °C and –20 °C was directly transferred to a refrigerator or freezer. All pollen pools collected in 2019 were dehydrated and stored at –80 °C. Each pollen pool was aliquoted into five to six individual tubes.

The *in vitro* germination and pollen viability percentages were studied to evaluate pollen preservation. Eppendorf tubes were removed from the freezer or refrigerator, and anthers were plated in small Petri dishes at room temperature for 30 min before testing.

In 2018, four pollen pools were collected from June 18th to July 3rd (A-18, B-18, C-18, and D-18), and in 2019, six pools were collected from June 4th to July 2nd (A-19, B-19, C-19, D-19, E-19, and F-19). The viability and germination of pollen gathered in 2018 were studied at 0, 15, and 30 days; 2 and 6 months; and 1 and 2 years of storage in non-DH and DH pollen, while pollen pools from 2019 were evaluated after dehydration at 0 days and one-year of storage at –80 °C. To gain insight into the longevity of frozen pollen after thawing, the viability and germination of pollen collected in 2019 were studied at 4, 7, and 11 days after removing the pools from the freezers.

The *in vitro* germination ability of all pools was evaluated as described above using M1 medium. Pollen viability was studied by the fluorescein diacetate (FDA) assay (Widholm, 1972). To estimate the viability of each pool, pollen from fout to six anthers was released into 27 µl of 0.3 M mannitol and 3 µl FDA (1 mg/ml dissolved in acetone). Three replicates

per pool were used. Germinated pollen and viability were studied under inverted Epifluorescent Nikon Eclipse-T300. EX 450-490, DM 505, and BA 520 filters were used for FDA analysis. Images were recorded with the Digital sight DS 5MC camera and processed using NIS-Elements D (AR 2.10 Laboratory Imaging System, Ltd.).

2.5 Crosses with stored pollen

To quantify seed production after pollen storage, male sterile 'BGHZ4552' plants were cross-pollinated with DH pollen from 'Fuentes de Ebro', and stored for one year at –80 °C or two years at –20 °C or –80 °C. Umbels from male sterile plants with unopened flowers were bagged in a paper bag. Eppendorf tubes were removed from the freezers and left at room temperature for 30 min before being used for pollination. Pollen was collected with a brush and deposited on the stigma of mature flowers and the umbel was immediately rebagged. Owing to the asynchrony in flower maturation in an umbel, pollination was performed for 5-10 days as maturation was taking place. Pollen from the same Eppendorf tube was used for pollination for four consecutive days and stored at 4 °C. As control crosses, freshly collected pollen from 'BGHZ-4553', the maintainer line of 'BGHZ-4552', was used for the cross-pollination of 'BGHZ-4552' or self-pollination. The seed production was calculated as the number of seeds per 100 pollinated flowers.

2.6 Statistical analysis

A one-way or a three-way analysis of variance (ANOVA) of the percentage of pollen viability and germination was performed using IBM SPSS statistics version 27.0.1. Significant differences among treatments were determined by the Duncan's method ($p \leq 0.05$).

3. RESULTS

3.1 Optimization of *in vitro* pollen germination conditions

For the optimization of pollen germination assays from onion cv. ‘Fuentes de Ebro’, the main factors considered were: the gelling agent, the germination medium composition, and the incubation temperature. The germination percentages of freshly collected pollen in a medium without a gelling agent (liquid medium, M1) and in a solidified medium with agarose (M1A) were compared (**Figures 1A, 1B**). In addition, the pollen depositions on the surface (M1A-S) and inside the medium (M1A-I) were tested in the solidified medium. Similar germination percentages were observed in the M1 (68%) and the M1A medium, regardless of whether it was layered on the surface or inside the medium (66% and 61%, respectively), (**Figures 1A, 1B**). However, the germination evaluation in the solid medium was difficult, as pollen grains tended to clump together and pollen tubes formed a reticulum (**Figures 1B1, 1B2**). Therefore, the M1 liquid medium was chosen for the following experiments.

The effect of the calcium concentration in the germination medium was also evaluated. Calcium in the form of $Ca(NO_3)_2 \cdot 4H_2O$ was added at 0 mg/l, 75 mg/l and 150 mg/l (medium M1, M2 and M3, respectively) (**Figure 1C**). Similar germination percentages were obtained in M1 and M2 media (46% and 40%, respectively). However, the percentage decreased 3.9 times in M3. Thus, the M1 medium was chosen for further experiments.

Finally, the effect of the incubation temperature on pollen germination was evaluated at 25 °C, 30 °C, and 35 °C in M1 medium (**Figure 1D**). Similar percentages were obtained at 30 and 35 °C (50% and 52%, respectively). However, this percentage decreased to 44% at 25 °C. Thus, germination assays were performed at 32 °C in pollen preservation studies.

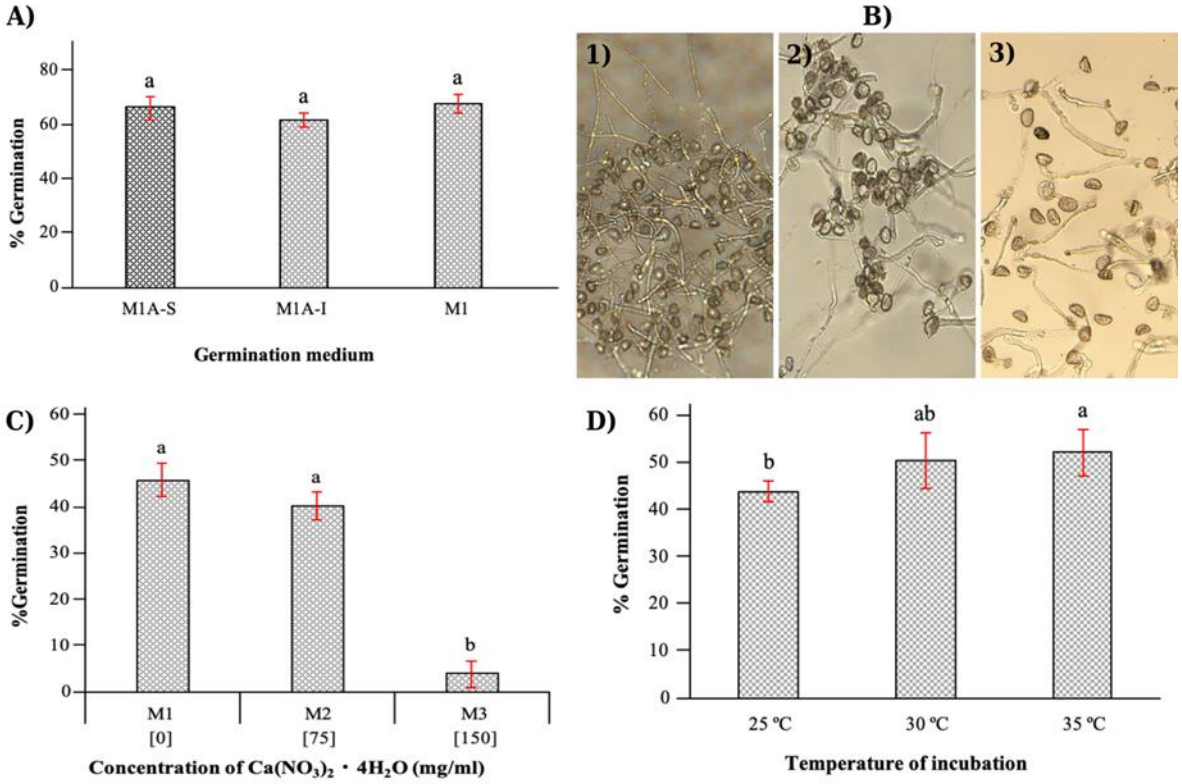


Figure 1. Optimization of pollen germination conditions of onion cv. 'Fuentes de Ebro'. A) Germination precentages from freshly collected pollen in M1 liquid medium (M1), M1 medium solidified with agarose and pollen layered on the surface (M1A-S) and inside the medium (M1A-I). B) Pollen germination in: M1A-S medium (B1), M1A-I medium (B2), and M1 medium (B3). C) Germination percentages in M1 and M1 media supplemented with 75 mg/l and 150 mg/l $Ca(NO_3)_2$. $4H_2O$ (M2 and M3, respectively). D) Germination percentages at 25 °C, 30 °C, and 35 °C in M1 medium.

3.2 Evaluation of pollen storage conditions

Once the conditions for the *in vitro* pollen germination were optimized, assays for short and medium-term pollen preservation were initiated. Four pollen pools from 'Fuentes de Ebro' were collected in 2018, corresponding to different harvest dates (A-18, B-18, C-18, and D-18). Each pool was divided into two portions. one was directly stored after collection (fresh, non-DH), and the other was dehydrated (DH) before storage at 4 ºC, –20 ºC, and –80 ºC. The *in vitro* germination and viability of the two portions of pollen pools were evaluated on the same day of collection (0 days), and after storage at different temperatures for 15 and 30 days; 2 and 6 months; and 1 and 2 years (**Figure 2)**.

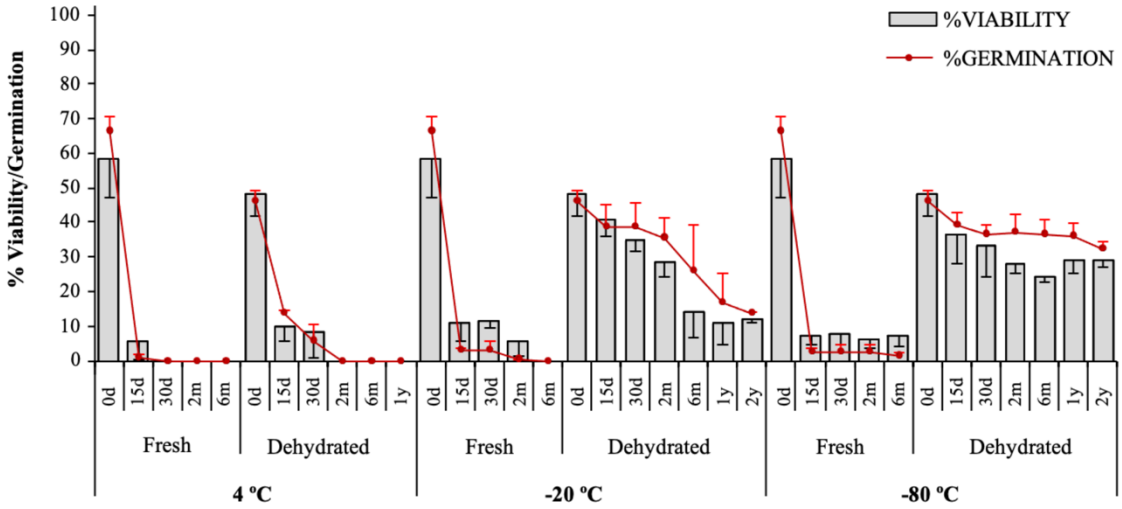


Figure 2. Viability and *in vitro* germination percentages from fresh and dehydrated onion pollen of 'Fuentes de Ebro' on the day of collection (0 days), and after being stored at different temperatures (4 °C, –20 °C, and –80 °C) for 15 and 30 days (d); 2 and 6 months (m); and 1 and 2 years (y). Data are the average of four pollen pools collected in 2018: June 18th (Pool A-18), June 25th (pool B-18), June 27th (pool C-18), and July 3rd (pool D-18).

The average viability and germination percentages of pollen pools at the time of collection (fresh, 0 days) were 58% and 66%, respectively (**Figure 2**). The quality of non-DH pollen decreased drastically after 15 days of storage, independent of the storage temperature, with a reduction of 81-90% in viability and 95-98% in germination (**Supplementary Material 1**). However, a total loss of germination capacity was observed after 30 days at 4 °C, 6 months at –20 °C, and 1 year at –80 °C.

The dehydration procedure itself also slightly decreased the pollen viability to 48% (DH, 0 days), representing 83% of that of non-DH pollen, whereas the *in vitro* germination was reduced to 46% (70% of non-DH). The quality of the DH-preserved pollen depended on the temperature of storage. Hence, the viability and germination of pollen stored at 4 ºC for 15 days were significantly reduced to 10% and 15%, respectively. However, higher viability (approximately 28%) and germination (approximately 36%) were observed in DH pollen stored at –20 ºC and –80 ºC during the first two months (**Figure 2**, **Supplementary Material 1**). Nevertheless, differences in pollen quality between the two freezing temperatures started to become apparent after two months of storage. Viability decreased drastically to values of 14% at –20 °C after six months and decreased slightly to 12% after two years (**Figure 3A**). In contrast, similar viability percentages were observed in pollen preserved at –80 °C after two months (28%) and two years (29%) (**Figure 3C**). Similar results were observed for the germination percentage, showing a 35% in pollen preserved at –20 °C after two months, and decreasing to 14% after two years (**Figure 3B**). However, pollen stored at –80 °C maintained 32% germination after two years (**Figure 3D**), which is considered acceptable enough to be used for onion seed production.

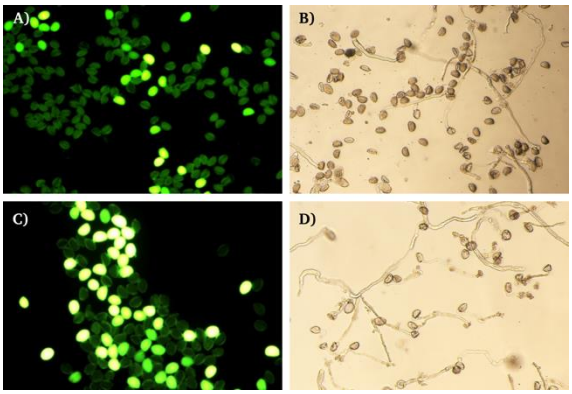


Figure 3. Germination and viability of dehydrated pollen after two years of storage. A) Viability of pollen stored at –20 °C; B) Germination of pollen stored at –20 °C; C) Viability of pollen stored at –80 °C; D) Germination of pollen stored at –80 °C. Viability was evaluated by the fluorescein diacetate (FDA) assay and germination was studied in M1 liquid medium at 32 °C.

Since the highest percentages of viability and germination after one year were obtained with DH pollen preserved at –80 °C, the pools collected in 2019 (A-19, B-19, C-19, D-19, E-19, and F-19) were only stored under these conditions. The quality of the pools was initially studied after dehydration (0 days) and after one year of storage to select the best pools for crosses. A large variation in viability and germination percentages was observed among pools at 0 days (**Figure 4**). The germination percentage ranged from 45% (pool A-19) to 73% (pool E-19), and the viability ranged from 52% (pool A-19) to 86% (Pool D-19). In the pools as a whole, viability and germination decreased by 13% and 14%., repectively, after one year. However, there was no correlation between the initial quality of the pools and their preservation capacity. Thus, pools A and D, which showed low and high germination at 0 days (45% and 70%, respectively), had the lowest reduction after storage (only 5%), whereas pools C and E, which initially showed 60% and 73% germination,

respectively, decreased to 23% and 27%.. Therefore, pools B, D, E, and F with the highest germination percentages after one year of storage at –80 °C were preferentially used for pollination in the 2020 crosses.

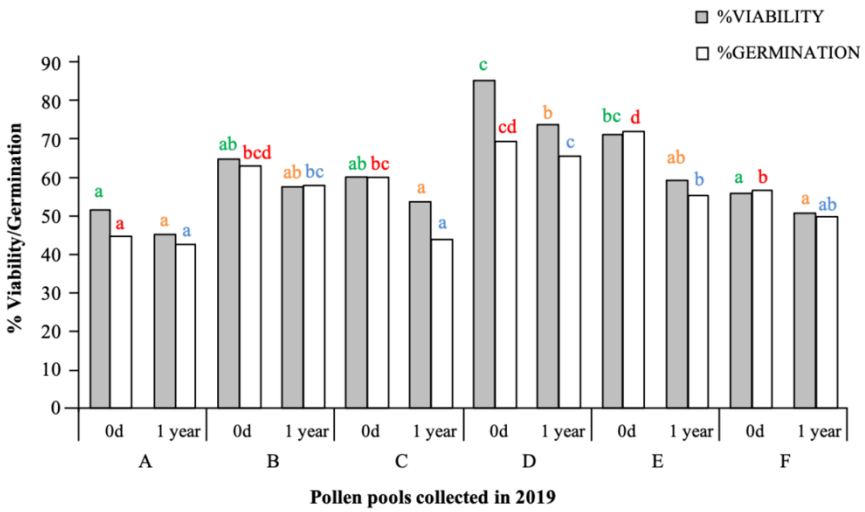
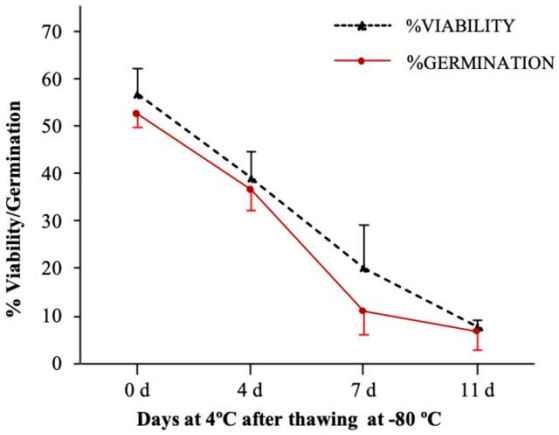


Figure 4. Viability and *in vitro* germination percentages from dehydrated pollen (0 days) collected in 2019 from 'Fuentes de Ebro' and after one year of storage (1 year) at –80 °C. Data were from six pollen pools: June 4th (Pool A-19), June 10th (Pool B-19), June 13th (Pool C-19), June 20th (Pool D-19), June 25th (Pool E-19), July 2nd (Pool F-19). Values followed by the same letter within each condition and variable are not significantly different ($P<0.05$), according to Duncan's test.

The suitability for cross-pollination of pollen thawed and stored at 4 °C was studied with the 2019 pools. The germination percentage decreased from 53% to 36% and 11% after 4 and 7 days, respectively (**Figure 5**). A similar trend was observed for viability. Based on these results, thawed pollen was not used in crosses after four days of storage at 4 °C.



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322 Figure 5. Viability and germination percentages of pollen collected in 2019 and stored at –
323 80 °C for one year, after thawing and storage at 4 °C for 0 to 11 days (average of six pools,
324 A-F).

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326 **3.3 Fertilization and seed production capacity of stored pollen**

327 The fertilization and seed production capacity of stored pollen from 'Fuentes de Ebro' was
328 tested in controlled crosses with male sterile line 'BGHZ-4552'. In 2019, crosses were
329 performed with DH pollen stored for one year at –80 ºC. In 2020, the analysis was extended
330 to DH pollen stored for two years at –20 °C and –80 °C (**Table 1)**. In control crosses, fresh
331 pollen from 'BGHZ-4553' was used for cross-pollination of 'BGHZ-4552' and self-
332 pollination. To estimate the pollination success, the number of seeds obtained per 100
333 flowers was recorded. All crosses performed in 2019 and 2020 with stored pollen produced
334 seeds. In 2019, the number of seeds/100 flowers in crosses with stored pollen at –80 °C
335 varied from 1.7 to 23.6, while those with freshly collected pollen varied from 57.3 to 216.0
336 in 'BGHZ4552' x 'BGHZ4553', and from 0 to 94.6 in 'BGHZ4553' self-pollination. All
337 crosses performed in 2020 with pollen stored at –80 °C produced a higher number of
338 seeds/100 flowers than those obtained in 2019, ranging from 13.9 to 88.3 and 32.9 to 63.0

in crosses with pollen stored for one and two years, respectively (**Figures 6C, 6D**). In addition, pollen stored at –20 ºC for two years also produced 14.8 seeds/100 flowers (**Figure 6B**). On the other hand, the number of seeds/100 flowers in control crosses ranged from 51.4 to 261.6 for 'BGHZ4552' x 'BGHZ4553' (**Figure 6A**) and 19.7 in 'BGHZ4553' self-pollination.

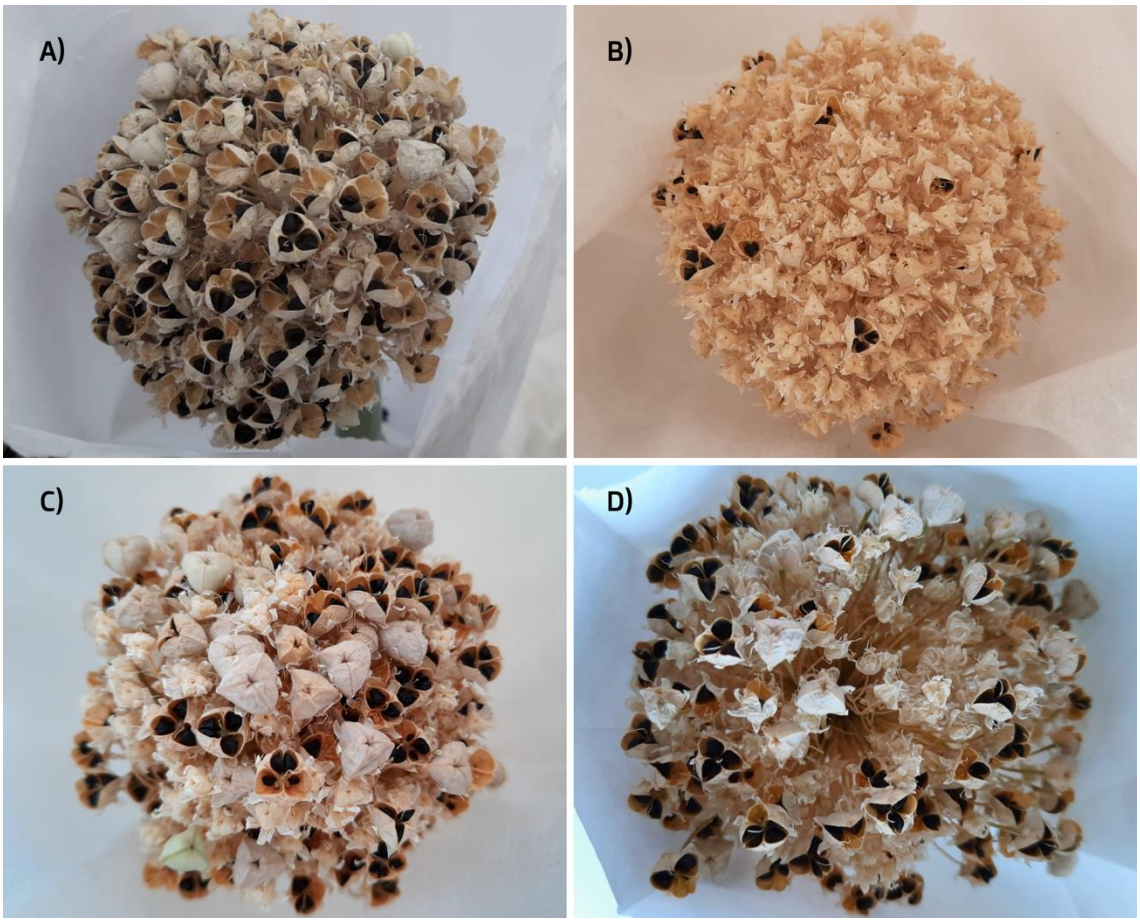

Figure 6. Seed production in crosses performed in 2020. A) Umbel of 'BGHZ4552' pollinated with fresh pollen from 'BGHZ4553' (control); B) Umbel of 'BGHZ4552' pollinated with 'Fuentes de Ebro' pollen stored for 2 years at –20 °C; C) Umbel of 'BGHZ4552' pollinated with 'Fuentes de Ebro' pollen stored for one year at –80 °C; D)

Umbel of ‘BGHZ4552’ pollinated with ‘Fuentes de Ebro’ pollen stored for two years at –80 °C.

Discussion

The establishment of a simple and inexpensive protocol for a medium-term pollen storage, that guarantees seed production, is essential in onion hybrid breeding programs that use cultivars with an asynchronous flowering time.

Cryopreservation is the most effective method for long-term pollen storage in *Allium* species (Gomes et al., 2003; Kanazawa et al., 1992; Rajasekharan et al., 2013). However, cryopreservation has some drawbacks, such as the high cost of equipment and maintenance, which are inaccessible to most plant breeders. An alternative and cheaper method for medium-term pollen preservation is storage in freezers (Gomes et al., 2003; Nomura et al., 1994). In this study, the *in vitro* germination and seed production capacity of pollen preserved at 4 °C, –20 °C, and –80 °C was evaluated to establish a simple protocol for onion pollen storage.

Reliable tests are required to evaluate the viability and *in vitro* germination of onion pollen. Germination tests depend on the medium composition and the incubation temperature, and both factors were optimized in this study. The medium described by Gomes et al. (2000), replacing agar with agarose due to its lower level of impurities, was compared with a liquid medium. Similar germination percentages were obtained in both media, but the liquid medium was chosen because it was easier to handle and facilitated counting. Similar results

have been described in pepper (Mercado et al., 1994). However, a higher germination percentage in a solid medium was reported in areca (Liu et al., 2013).

Concentrations of boron, calcium, or sucrose in the germination medium must also be considered (Brewbaker and Beyoung, 1963; Kwan et al., 1969; Kanazawa et al., 1992; Dubouzet et al., 1993). Calcium ions are known to play a crucial role in signaling events that take place during pollen germination, tube growth, and fertilization (for a review, see Zheng et al., 2019). Pollen germination in a medium with 300 mg/l $Ca(NO_3)_2 \cdot 4H_2O$ has been described in 86 species (Brewbaker and Beyoung, 1963). However, 300 mg/l inhibited germination compared with a medium without calcium in onion (Gomes et al., 2000). In our study, 150 mg/l $Ca(NO_3)_2 \cdot 4H_2O$ reduced germination by 90%, whereas 75 mg/l produced similar rates to those of the control. Germination inhibition was also reported with 23.6 mg/l and 236 mg/l $Ca(NO_3)_2 \cdot 4H_2O$ in potato (Říhová et al., 1996). It has been postulated that germination is favoured by the release of Ca^{+2} from the pollen wall in a calcium-free media (Zheng et al., 2019).

Previous studies in *A. victorialis* have shown that the optimum temperature for *in vitro* pollen germination was between 20 °C and 25 °C (Kanazawa et al., 1992). However, in this study, the highest germination was achieved at 30-35 °C. These results indicate that each *Allium* species might have a different optimal temperature for pollen germination, likely adapted to the environmental conditions of its growing area. It should be noted that the flowering of 'Fuentes de Ebro' occurs during June - July, when high temperatures are recorded in the Ebro Valley.

As storage temperature and humidity content are the main factors affecting pollen preservation, the effect of these two factors on the preservation on 'Fuentes de Ebro' pollen over two years was studied. Overall, pollen viability was best preserved at low temperatures and low relative humidity (**Figure 2**). Hence, pollen stored at –20 °C and –80 °C showed higher germination and viability than pollen stored at 4 °C. This is in accordance with previous results in *A. cepa* after six months of preservation (Kwan et al., 1969), and in different *Allium* species and eggplant after one year (Dubouzet et al., 1993; Khan and Perveen, 2006). Furthermore, the reduction of humidity content is crucial when using freezing temperatures, as ice crystals could be formed, thereby damaging structure during storage (de Souza et al., 2014; Sidhu, 2019). Accordingly, non-DH pollen completely lost its germination capacity after six months at –20 °C and 1 year at –80 °C, whereas DH pollen showed 13% and 32 % germination after two years of storage at –20 °C and –80 °C, respectively. Therefore, these results indicate that a dehydration process prior to storage is essential for the preservation of onion pollen at freezing temperatures. However, the dehydration step must be carried out carefully, as pollen may become unviable if the water content is excessively removed (Benson, 2008). In this study, germination capacity was reduced by 31%, placing pollen in Petri dishes inside a desiccator with silica gel at 25 °C for 24 h. resulting in 32-43% relative humidity. Dehydration sensitivity has been attributed to the structural basis (Towill and Walters, 2000), low sugar content (Hoekstra et al., 1989), and carbohydrate type (Paccini, 1996).

Dehydrated pollen preserved at –20 °C showed 35% germination (approximately 75% of the initial percentage) after two months, and 13% after two years. Therefore, DH pollen storage at –20 °C could be used for pollination with guarantees within the same flowering

season. Higher germination (30-37%) has been reported after storage in a dehydrated atmosphere at –20 °C or –30 °C for 2-2.5 years in onion (Nomura et al., 1994; Gomes et al., 2003) and in *A. fistulosum* (Nomura et al., 1994). These differences in germination may be due to initial germination at harvest, the dehydration procedure and/or the sensitivity of the genotype or species to the dehydration or freezing process, as reported in *Allium* and coconut species (Dubouzet et al., 1993; Nomura et al., 1994; de Araujo-Machado et al., 2014). On the other hand, pollen storage at –80 °C could be a good alternative to prolong the viability of preserved pollen. In this study, DH pollen stored at –80 °C had the highest longevity after two years, with 32% germination (70% of the initial). To the best of our knowledge, this is the first report that –80 °C can be used for medium-term pollen preservation in onion. A higher germination percentage after storage at –80 °C than at –20 °C or at 4 °C has been described in litchi, peonies, and pecan (Wang et al., 2015; Du et al., 2019; Wang et al., 2021).

Pollen longevity after thawing is another important factor for the use of preserved pollen in crosses. A 31% reduction in germination and viability was observed after 4 days of thawing in DH pollen stored at –80 ºC for one year. Greater longevity has been reported in onion pollen cryopreserved for one year, which maintained a similar germination percentage after 10 days of being transferred to 4 °C (Gomes et al., 2003).

Out of the different tests to assay pollen viability, seed quantification is the most time-consuming, but also the most accurate way to determine the fertilization capacity of stored pollen. The results obtained in a number of crosses confirmed previous data of *in vitro* germination and viability tests, ratifying that the highest pollen preservation was achieved

446 at –80 °C. Thus, DH pollen preserved at –80 °C for two years produced an average of 47.9
447 seeds/100 flowers, representing 43% of that in control crosses (**Table 1**). These results are
448 in the range of others previously obtained with cryopreserved onion pollen (25% of that
449 with fresh pollen) (Senula and Keller, 2014). Pollen stored at –20 °C also produced seeds,
450 but at lower rates (14.8 seeds/100 flowers), representing 13% of that in control crosses.
451
452 Interestingly, a significantly higher percentage of seeds was obtained in 2020 than in 2019
453 with pollen stored at –80 °C for one year (41.9 and 8.0 seeds/100 flowers, respectively).
454 This might be due to a higher quality of pollen collected in 2019 than in 2018, as revealed
455 by the germination percentages of DH pollen at 0 days, with 62% and 46%, respectively, or
456 after one year of storage at –80 °C, with 54% and 36%, respectively, (**Figures 2, 4**).
457 Environmental factors, especially temperature before anthesis and during fertilization
458 significantly affect seed production in onion species (Litcher and Mündler, 1961; Chang
459 and Struckmeyer, 1976), and determine pollen germination percentages (Novara et al.,
460 2017).
461
462 **Conclusion**
463 The composition of the germination medium and the incubation temperature were
464 optimized for the *in vitro* germination of 'Fuentes de Ebro' pollen. The highest percentage
465 was obtained in a liquid medium without calcium at 30-35 ºC. A dehydration procedure,
466 prior to storage, was crucial to maintain pollen viability in the medium-term. Pollen was
467 better preserved at –20 °C and –80 °C than at 4 °C. Thus, pollen dehydrated and stored at –
468 20 °C for two months showed germination percentages of 35%, and therefore could be used
469 for pollination within the same flowering season. Additionally, pollen dehydrated and

stored at –80 °C for two years, showed high viability and germination percentages (29% and 32%, respectively), and more importantly seed production (47.9 seeds/100 flowers), thereby allowing its use in breeding programs over two seasons. Therefore, pollen storage at –80 °C could be used as a cheaper and easier alternative approach to cryopreservation in onion breeding.

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Credit Authorship contribution statement

Oreto Fayos: Conceptualization, Methodology, Validation, Formal análisis, Investigation, Visualization, Writing-original draft, Writing-review and editing. **Begoña Echavarri**: Formal Analysis, Investigation, Visualization, Writing-review and editing**. María Pilar Vallés**: Conceptualization, Methodology, Investigation, Resources, Writing-review and editing**. Cristina Mallor**: Conceptualization, Methodology, Investigation, Resources, Writing-review and editing. **Ana Garcés-Claver**: Conceptualization, Methodology, Investigation, Resources, Writing-review and editing, Funding acquisition**.** Project Administration**. Ana María Castillo**: Conceptualization, Methodology, Investigation, Resources, Writing-original draft, Writing-review and editing, Supervision, Project Administration.

Competing interests

The authors declare that they have no competing interests

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674 Table 1. Crosses were performed in 2019 and 2020 with fresh pollen and DH pollen stored
675 at –20 °C and –80 °C for one or two years. Data of seed sets from crosses: number of
676 pollinated flowers, number of total seeds, and the ratio of number of seeds per 100
677 pollinated flowers.

Year	Female genotype	Male (Pool) genotype	Time storage	T[a] storage (°C)	N pollinated flowers	N total seeds	N seeds /100 flowers
2019	BGHZ4552	Pool B-18	1 year	–80	124	6	4.8
2019	BGHZ4552	Pool B-18	1 year	–80	267	12	4.5
2019	BGHZ4552	Pool B-18	1 year	–80	178	42	23.6
2019	BGHZ4552	Pool B-18	1 year	–80	114	6	5.3
2019	BGHZ4552	Pool D-18	1 year	–80	361	6	1.7
2019	BGHZ4552	Pool D-18	1 year	–80	445	36	8.1
2019	BGHZ4552	BGHZ4553	Fresh	-	136	78	57.3
2019	BGHZ4552	BGHZ4553	Fresh	-	25	54	216.0
2019	BGHZ4553	BGHZ4553	Fresh	-	181	0	0.0
2019	BGHZ4553	BGHZ4553	Fresh	-	126	36	28.6
2019	BGHZ4553	BGHZ4553	Fresh	-	241	228	94.6
2019	BGHZ4553	BGHZ4553	Fresh	-	81	24	29.6
2020	BGHZ4552	19-Pools D+E	1 year	–80	406	95	23.4
2020	BGHZ4552	19-Pools D+B	1 year	–80	473	66	13.9
2020	BGHZ4552	19-Pools F+E	1 year	–80	512	452	88.3
2020	BGHZ4552	Pools B+D-18	2 years	–20	445	66	14.8
2020	BGHZ4552	Pool C-18	2 years	–80	527	332	63.0
2020	BGHZ4552	Pool C-18	2 years	–80	410	135	32.9
2020	BGHZ4552	BGHZ4553	Fresh	-	367	960	261.6
2020	BGHZ4552	BGHZ4553	Fresh	-	175	90	51.4
2020	BGHZ4553	BGHZ4553	Fresh	-	137	27	19.7

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