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Effects of Sucrose Concentration and Days after Anthesis on *in Vitro* Pollen Germination of Various *Allium hirtifolium* Ecotypes

Jaber Panahandeh*, Sahar Khakpour, Alireza Motalebi Azar

Department of Horticultural Science, Faculty of Agriculture, University of Tabriz, Tabriz, Iran

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*Corresponding author's email: panahandeh@tabrizu.ac.ir

ABSTRACT

In vitro pollen germination offers a promising strategy for accelerating genetic improvement in plant breeding programs. However, to date, no studies have specifically investigated the pollen germination of *Allium hirtifolium*. This study aimed to examine the effects of varying sucrose concentrations and different days after anthesis on pollen viability and *in vitro* germination rates across several *A. hirtifolium* ecotypes. Six populations of *A. hirtifolium* (Persian shallot), collected from diverse regions of Iran, were evaluated for *in vitro* pollen germination potential. Germination assays were conducted using a basal medium supplemented with sucrose at concentrations of 1%, 2%, 3%, 4%, and 5%. Pollen germination rates were assessed at 1, 3, 5, and 7 days post-anthesis. In addition, pollen grains were subjected to deep-freezing storage for 30 days to assess viability under prolonged cold conditions. The results demonstrated that both sucrose concentration and ecotype significantly affected pollen germination, with the highest germination rates observed at a sucrose concentration of 2%. The optimal time for germination was 1 day post-anthesis. Notably, no pollen germination occurred following 30 days of deep-freezing. Across the evaluated time points post-anthesis, sucrose concentration and ecotype remained significantly correlated with pollen germination. This study provides empirical evidence for optimizing medium composition, timing, and ecotype selection to enhance pollen germination of *A. hirtifolium* under *in vitro* conditions.

Introduction

Medicinal plants are essential natural resources that have long been utilized in traditional medicine. Among these, various *Allium* species are known for their considerable nutritional, pharmaceutical, and medicinal properties. In Iran, more than 139 *Allium* species have been identified, approximately 30 of which are endemic to the region (Mozaffarian, 1996). One of the most significant edible *Allium* species in Iran is the Persian shallot (*Allium hirtifolium* Boiss.), commonly known as "Mooseer," which belongs to the family Alliaceae.

Mooseer is a nutritious plant with a distinctive flavor, and its dried bulb slices are widely used as a flavoring agent in yogurt and pickling mixtures. As this species primarily grows in the wild in specific mountainous areas of Iran, comprehensive data on various aspects of its biology, particularly its reproductive biology, remain limited. This lack of knowledge has hindered efforts to cultivate the wild type under controlled conditions. The rising global demand for medicinal and aromatic plant species—driven by increasing human needs and preferences—has led to the continued harvesting of these species from natural habitats. Combined with habitat destruction and unsustainable exploitation practices, this demand has placed many wild species at significant risk of extinction (Gupta et al., 1998).

Mooseer is characterized by a storage organ that typically consists of a single bulb, or occasionally two, each weighing approximately 8–15 times more

than a garlic clove. The plant features a naked, erect scape reaching 80–120 cm in height. Its leaves are green, linear, lanceolate, and range from 20 to 30 cm in length (Asili et al., 2010). Traditionally, Mooseer has been widely used in Iranian folk medicine for the treatment of rheumatic and inflammatory disorders (Asili et al., 2010), in addition to its use as a culinary spice. The reproductive biology of plants plays a critical role in the conservation and sustainable use of herbal species. It encompasses the study of both sexual and asexual reproductive processes, including pollination mechanisms, gene flow, genetic diversity, and the dispersal of propagules within and between populations. An in-depth understanding of these reproductive processes is essential for interpreting the adaptive significance and homology of diagnostic traits used in plant systematics. Furthermore, such knowledge informs the delimitation and classification of species and intraspecific taxa.

Comprehensive insights into plant reproductive biology are therefore vital for the development of effective conservation strategies, breeding programs, and the sustainable utilization of plant genetic resources. Pollination represents a key step in reproductive success, species diversity, and overall plant fitness. Reproduction is not only a crucial but also a vulnerable phase in a plant's life cycle and is central to its evolutionary trajectory. Consequently, investigating the reproductive biology of a species is essential to understanding the mechanisms by which it may become endangered. One key aspect of reproductive biology is pollen viability, which serves as a valuable tool in artificial hybridization and genetic improvement programs (Soares et al., 2008). Assessing pollen viability, germination capacity, and pollen tube growth is fundamental to both reproductive biology and plant breeding research (Dane et al., 2004; Salles et al., 2016). Given the numerous benefits of pollen storage for germplasm conservation, it is imperative to establish reliable methods for evaluating pollen viability.

Pollen germination potential is defined as the ability of viable pollen grains to germinate under appropriate conditions (Fortescue et al., 2014). It is a critical parameter in the study of various biological processes, including evolutionary (biochemical) and molecular biological phenomena (Dane et al., 2004). Accordingly, the determination of pollen viability is essential for plant breeding and germplasm conservation. This assessment is often conducted through direct methods such as the induction of *in vitro* germination (Sorkheh et al., 2018). *In vitro* pollen germination represents a valuable approach for accelerating genetic improvement in plant breeding. It provides a convenient and effective means to investigate both fundamental and applied aspects of pollen biology (Shekari et al., 2016), particularly in the selection of viable, functional pollen for use in cross-pollination (Sakhanokho et al., 2010). However, the conditions necessary for successful *in vitro* pollen germination vary widely among species and are influenced by a range of internal and external factors (Schueler et al., 2005; Kremer, 2006).

Parameters such as incubation time, temperature, and environmental conditions can significantly impact pollen germination, alongside external factors such as the composition of the germination medium (Lin et al., 2017). A range of organic and inorganic substances—such as sucrose, boric acid, calcium nitrate, potassium nitrate, and magnesium sulfate—have been shown to affect *in vitro* pollen germination (Parton et al., 2002; Kopp et al., 2002; Moutinho et al., 2001). Pollen quality, media composition, and environmental variables all contribute to the success of germination (Sakhanokho et al., 2010). Numerous media formulations have been proposed, incorporating various components to suit the requirements of different species (Shekari et al., 2016; Lin et al., 2017; Wang et al., 2003; Liu et al., 2013). Understanding the specific physiological and environmental requirements for pollen germination is critical for successful cross-pollination and subsequent breeding efforts. These requirements are species-specific, and the preparation of appropriate germination media plays a pivotal role in supporting both pollen germination and tube growth (Dane et al., 2004).

The success of *in vitro* pollen germination is influenced by multiple factors, including humidity, temperature, genotype, the physiological status and vigor of the parent plant, the age of the flower, and the composition of the germination substrate (Shivanna and Johri, 1985). Nutrients such as calcium, magnesium, potassium nitrate, and boric acid have been reported to exert significant effects on pollen germination (Sahar and Spiegel-Roy, 1984). Although prior studies have examined the role of sucrose, boric acid, and calcium ions in pollen germination and preservation under various storage conditions (Fragallah et al., 2019), there remains a lack of comprehensive data regarding the specific effects of sucrose on pollen germination in *Allium hirtifolium*.

This study was designed to investigate the effects of sucrose concentration and days after anthesis on the *in vitro* pollen germination of *Allium hirtifolium*, as well as to assess potential differences in germination response among its ecotypes. The findings aim to improve our understanding of the germination requirements of this under-researched and ecologically important species, thereby supporting its conservation and the development of breeding strategies. Pollen viability can be evaluated through several methods, including staining techniques, *in vitro* and *in vivo* germination tests, and final seed set analysis, with the appropriate method varying

according to the species (Dafni and Firmage, 2000). In many species, successful *in vitro* germination correlates with the inclusion of essential substrates such as calcium nitrate in the germination medium.
In this study, the effects of sucrose concentration, days after anthesis, and ecotype were evaluated in relation to *in vitro* pollen germination in *Allium hirtifolium*. The research further sought to assess pollen fertility across different ecotypes, determine optimal culture medium composition for germination, evaluate pollen viability over time post-anthesis, and identify potential ecotypic differences in pollen viability. This comprehensive approach contributes to advancing artificial pollination techniques and genetic improvement programs for this endangered species.

Material and methods

Study site and plant materials

Six ecotypes of *Allium hirtifolium* (Table 1) were collected from their primary natural habitats across different regions of Iran: (1) Esfahan 1 (Khansar), (2) Esfahan 2 (Fariden), (3) Shahrekord (Sabzeh Kooh), (4) Mashhad (Sabzevar), (5) Kordestan (Dehgolan), and (6) Hamedan. Bulbs were selected based on size and weight, with care taken to standardize experimental conditions by choosing bulbs of similar morphological characteristics (Table 2). All six ecotypes were subsequently cultivated under uniform conditions at the experimental field of the Department of Horticultural Sciences Research Station, University of Tabriz.

Table 1. Geographical distribution of Mooseer populations used in this study.

Number	Province	City/region	Coordinate	Altitude (m)
1	Esfahan 1	Khansar	33,22° N 50,31°E	2215
2	Esfahan 2	Fariden	33° N 51°E	2400
3	Shahrekord	Sabzeh Kooh	31,27° N 50,40°E	1120
4	Mashhad	Sabzevar	36°12′45″N 57°40′55″E	977
5	Kordestan	Dehgolan	35°16′40″N 47°25′03″E	1800
6	Hamedan	Hamedan	34° 47' 46N 48° 30' 57E	1876

Table 2. Characteristics of Mooseer bulbs used in this study.

Number	Province	City/region	Mean Bulb Circumference (cm)	Mean weight (g)
1	Esfahan 1	Khansar	3-3/5	21
2	Esfahan 2	Fariden	3	20
3	Shahrekord	Sabzeh Kooh	3	20
4	Mashhad	Sabzevar	3/5	21
5	Kordestan	Dehgolan	3	20
6	Hamedan	Hamedan	3/5	22

Collection of pollen samples

Flower spikes were collected during the flowering period, which extended from late May to mid-June. Pollen grains were obtained from mature flowers after anthesis. During the experiment, the flower spikes were stored in the laboratory with their stems immersed in distilled water to maintain freshness, and each was labeled separately by ecotype. Samples were collected from all six ecotypes. Fresh pollen grains from each ecotype were transferred into 1.5 mL centrifuge microtubes, which were then placed in silica gel and sealed with Parafilm. The sealed tubes were stored at 4 °C until further use.

In vitro pollen germination

Bulk fresh pollen grains were collected after anthesis, uniformly dispersed into different liquid media, and incubated at 25 °C in darkness for 24 h. The basal culture media consisted of 100 ppm H_3BO_3, 300 ppm Ca $(NO_3)_2$, 200 ppm $MgSO_4$, 100 ppm KNO_3, and varying concentrations of sucrose (Hong et al., 2000). Sucrose was added to the basal media at concentrations of 1, 2, 3, 4, and 5%, following modifications from Tsai et al. (2010). Two drops (2 µL each) of the pollen-media mixture were placed at different points on a glass slide and stored in a Petri dish. To visualize the germinated pollen grains after 24 h, one drop of Acetocarmine stain was

added to the mixture on the glass slides, which were then covered with cover slips. This process was repeated 3, 5, and 7 days after anthesis. Additionally, pollen grains were stored at deep freezing conditions for 30 days.

An *in vitro* pollen germination test was conducted on pollen from six different ecotypes using the basic media. The germination rate was determined by dividing the number of germinated pollen grains per field of view by the total number of pollen grains, expressed as a ratio. The experiment was carried out in a completely randomized design with three replications, each represented by one slide at each 24 h interval. The slides were then examined under a light microscope and photographed using a Nikon camera. A pollen grain was considered germinated when the pollen tube length was equal to or greater than the grain diameter (Kakani et al., 2005). All pollen grains in each photograph were counted to calculate the germination rate. Germinated and non-germinated pollen grains were counted on each slide after 24 h of incubation. The pollen germination rate was determined by dividing the number of germinated pollen grains per field of view by the total number of pollen grains, as described by Kakani et al. (2005). Statistical data analysis was performed using a factorial experiment with a completely randomized design, and differences between treatments were compared using Duncan's test (P<0.05).

Results

In the present study, various culture media formulations were evaluated to determine the optimal conditions for *in vitro* pollen germination across six *Allium hirtifolium* ecotypes. Different sucrose concentrations produced varying percentages of pollen germination, and the number of germinated pollen grains differed depending on the media composition. Additionally, the number of days after anthesis was found to significantly influence germination rates. The results indicate that pollen germination is also affected by the ecotype of the tested plants.

Effects of sucrose concentrations on pollen germination

Table 3 presents the mean values of pollen germination percentages after 24 hours of incubation under different sucrose concentrations. The effect of sucrose concentration was statistically significant, with the highest germination observed at 2% sucrose across all ecotypes (Fig. 1). No pollen germination was observed in the control medium (0.0% sucrose), while a sucrose concentration of 5% had a reduced impact on germination rates compared to 2%.

The highest pollen germination rate was recorded in ecotype 4 (94.49%), followed by ecotype 6 (83.50%) when cultured in media containing 2% sucrose. In contrast, under 5% sucrose concentration, the highest germination rate was again observed in ecotype 4 (72.16%), followed by ecotype 6. The lowest germination rate (9.3%) under this concentration was recorded in ecotype 2. Overall, the optimal pollen germination was achieved with 2% sucrose across ecotypes (Fig. 2).

Effects of days after anthesis on pollen germination

During the experiment, the number of days after anthesis significantly affected the pollen germination rate. Viability and germination tests were conducted on the 1st, 3rd, 5th, and 7th d after anthesis. The results showed that the best germination rates were achieved on the first day, with a gradual decline observed by the 7th d. Pollen grains stored under deep freezing conditions for 30 d exhibited no viable pollen upon testing on the 30th d after anthesis (Fig. 3).

Table 3. ANOVA (mean squares) of pollen germination of 6 ecotypes of *Allium hirtifolium* in different sucrose concentrations during various days after anthesis (P < 0.05).

S.O. V	DF	MS	Sig.
Eco	5	32.850**	0.000
Suc	4	10.656**	0.000
day × eco	20	743.827**	0.000
day ×suc	16	230.491**	0.000
eco × suc	20	150.065**	0.000
day × eco × suc	80	39.396	0.085
Error	300	31.195	
Total	449		

**Significance (P < 0.01), *significance (P < 0.05).

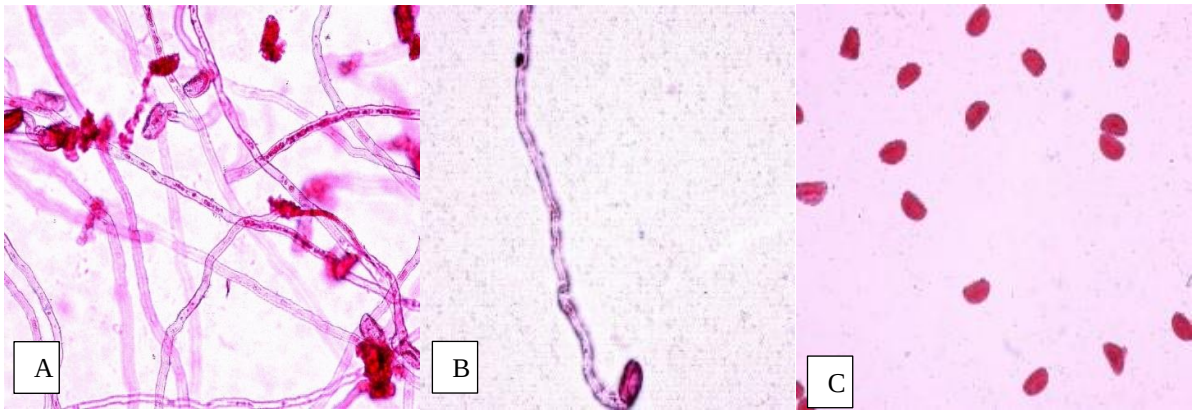

Fig. 1. (**A and B**) Germinated pollen grains and long pollen tubes in the basal media (100 ppm H_3BO_3, 300 ppm Ca $(NO_3)_2$, 200 ppm $MgSO_4$, 100 ppm KNO_3) including 2% sucrose and; (**C**) distilled water in variety number 6 (Hamedan variety) 24 h after culturing, under microscopic view stained by Acetocarmine.

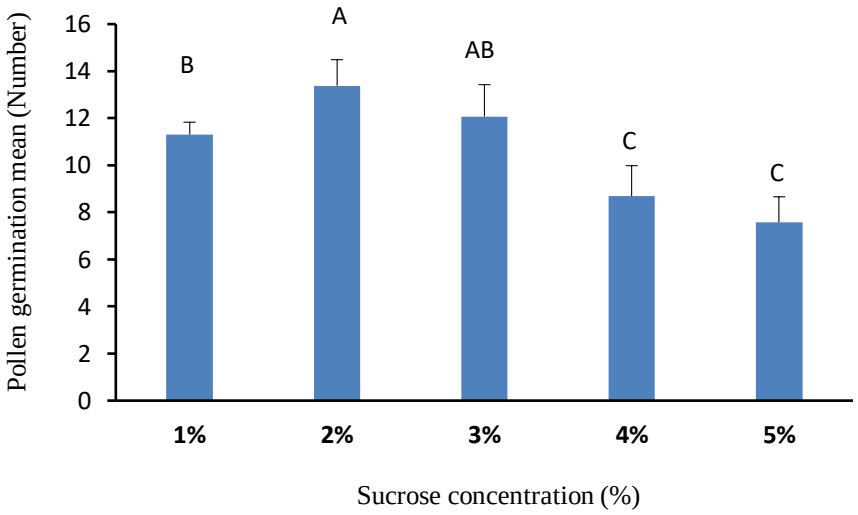

Fig. 2. Effects of sucrose concentration on pollen germination in Mooseer ecotypes in the basal media (100 ppm H_3BO_3, 300 ppm Ca $(NO_3)_2$, 200 ppm $MgSO_4$, 100 ppm KNO_3). Columns with similar letters are not statistically different. Standard Errors used for bar lines.

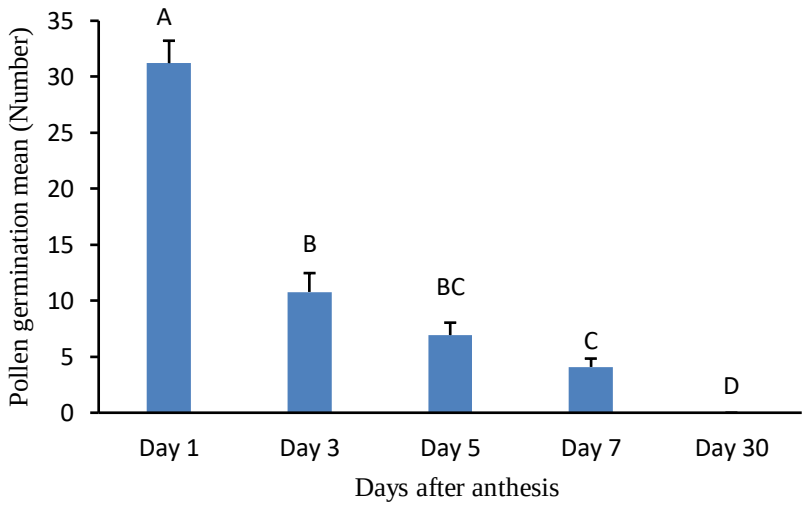

Fig. 3. Negative relationship between days after anthesis and pollen germination in Mooseer ecotypes in the basal media (100 ppm H_3BO_3, 300 ppm Ca $(NO_3)_2$, 200 ppm $MgSO_4$, 100 ppm KNO_3) including 2% of sucrose. Columns with similar letters are not statistically different. Standard Errors used for bar lines.

Effects of ecotypes on pollen germination

A comparison of the six ecotypes of *Allium hirtifolium* was based on pollen germination rate using the basal media including H_3BO_3 100 ppm, Ca $(NO_3)_2$ 300 ppm, $MgSO_4$ 200 ppm, KNO_3 100 ppm, and 2% sucrose (Fig. 4). Notably, ecotype 4 exhibited the highest pollen germination capacity among the examined ecotypes. The number of germinated pollen grains showed that ecotype 4 (Mashhad) and ecotype 6 (Hamedan) had the highest number of germinated pollen grains (Fig. 5). This trend was consistent throughout the experiment on each day.

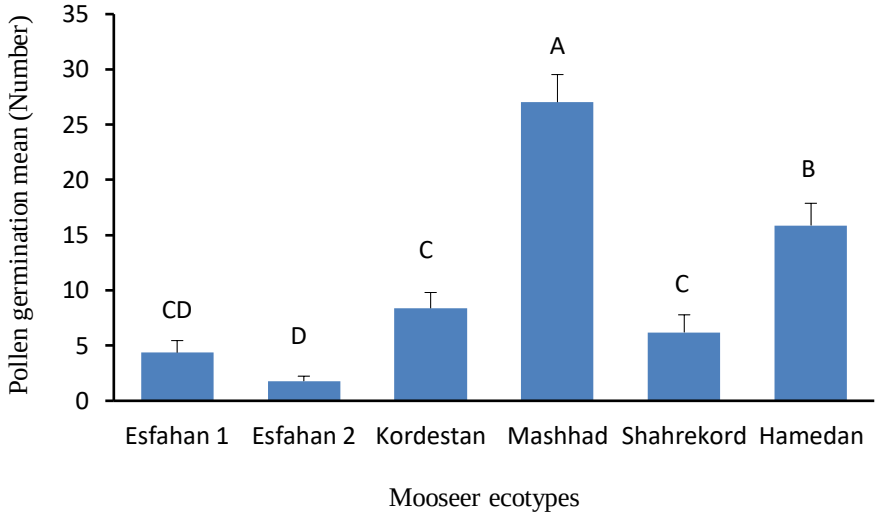

Fig. 4. Different ecotypes showed different results for pollen germination in the basal media (100 ppm H_3BO_3, 300 ppm Ca $(NO_3)_2$, 200 ppm $MgSO_4$, 100 ppm KNO_3) with 2% of sucrose, eco 1 (Esfahan 1 (Khansar)), eco 2 (Esfahan 2 (Faridan)), eco 3 (Shahrekord), eco 4 (Mashhad), eco 5 (Kordestan), and eco 6 (Hamedan). Columns with similar letters are not statistically different. Standard Errors used for bar lines.

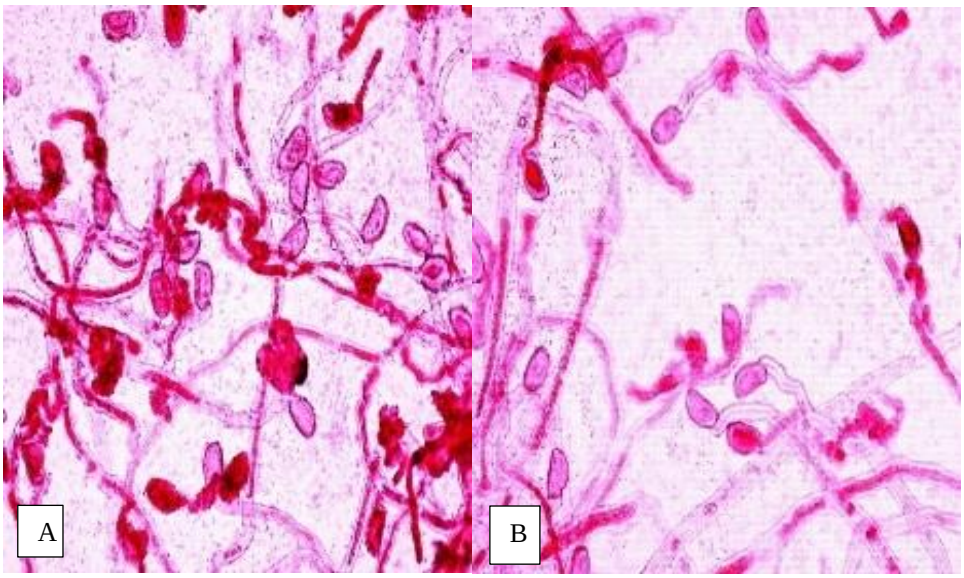

Fig. 5. (**A**) Germinated pollen grains of ecotype number 4 (Mashhad) and; (**B**) ecotype number 6 (Hamedan) in the basal media (100 ppm H_3BO_3, 300 ppm Ca $(NO_3)_2$, 200 ppm $MgSO_4$, 100 ppm KNO_3) with 2% of sucrose.

Discussion

Pollen germination and tube growth rate are critical characteristics linked to pollen quality, successful fertilization, and seed set. High germination rates and rapid tube elongation are essential for effective fertilization, whereas reduced pollen tube growth may result in ovule degradation before the pollen tube reaches the ovary, thereby reducing seed set (Sharafi, 2011). In this study, significant differences in pollen germination rates were observed among

media containing different sucrose concentrations. In general, pollen germination increased with sucrose concentration up to an optimal point, beyond which further increases led to a decline. This trend is likely due to the role of sucrose in providing metabolic energy and supporting the biosynthesis of organic compounds necessary for cell growth (Boavida and McCormick, 2007; Chagas et al., 2010; Figueiredo et al., 2013).

The requirements for successful pollen germination vary significantly among species, and both the composition of the germination medium and the culture conditions can greatly influence the outcome. As reported by Dane et al. (2004), the preparation of suitable germination media is species-specific and dependent on numerous internal and external factors. These include humidity, temperature, genotypic variation, vegetative vigor, plant physiological stage, flower age, and the specific components of the germination substrate (Shivanna et al., 1985).

However, the findings of this study contrast with those reported by Shen et al. (2010) and Souza-Lang et al. (1997), likely due to differences in sucrose concentrations used in the media as well as genetic variability among the ecotypes examined. The addition of sucrose to the culture medium is crucial, as it serves as a primary nutrient for pollen germination and tube development (Lin, 2017). Nonetheless, excessively high sucrose concentrations can inhibit germination. Optimal sucrose levels vary widely across species (Liu et al., 2013), and inconsistencies in germination within the same medium may result from imbalanced osmotic pressure (Youmbi et al., 2015).

The age of pollen is another critical factor affecting germination percentage and fertilization potential. Maximum germination in many fruit and vegetable species typically occurs on the day of flower anthesis or anther dehiscence (Edwards, 1966). Lorraine and George (1969) reported significant differences in germination among onion pollen samples of three different ages, even at a 1% sucrose concentration. In their study, onion pollen viability declined rapidly after the first day and approached zero by the sixth day post-anthesis, highlighting the importance of pollen age in seed production.

Pollen conservation is a valuable strategy for germplasm maintenance. However, several factors influence the viability of fresh pollen under storage. The current findings indicate that *A. hirtifolium* pollen lacks sufficient viability for conservation at low temperatures. This result contrasts with previous reports (Ganeshan, 1986; Volk, 2011; Doubouzet et al., 1993), which demonstrated pollen longevity exceeding one year in liquid nitrogen or more than 50% germination capacity retained after eight months of storage at 2–3 °C. Such discrepancies may be attributed to differences in plant material. *Allium* is a large genus comprising nearly 800 species distributed widely from the Far East to the Far West, and it exhibits extensive variability in pollen longevity. *A. hirtifolium*, which belongs to the subgenus *Melanochromyum*, differs considerably in its ecological requirements and morphological traits from most commonly cultivated *Allium* species. These differences likely account for its limited pollen storage capacity under conventional low-temperature conditions.

Conclusion

Ecotype, culture media composition, and days after anthesis all had significant effects on the *in vitro* pollen germination potential of *Allium hirtifolium*. Notable differences in germination rates were observed among the ecotypes. Among the evaluated factors, the composition of the culture medium had the most pronounced influence on pollen germination. Specifically, sucrose concentration significantly affected germination, with 2% sucrose identified as the optimal concentration, producing the highest germination rates. The effect of ecotype was also clearly evident, with ecotypes 4 (Mashhad) and 6 (Hamedan) exhibiting the highest pollen germination potential under *in vitro* conditions.

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Author Contributions

Conceptualization, JB; methodology, JB; validation and formal analysis, JB, AM and SK; investigation, resources and writing original draft preparation, SK; review and editing, JB and AM; supervision, JB and AM. All authors have read and agreed to the published version of the manuscript.

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Conflict of Interest

The authors indicate no conflict of interest in this work.

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