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Scientific Article

Muscari seed germination enhancement by using sulfuric acid, and stratification priming

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Abstract

Muscari with absorbing color is a widespread and commercial species. As a pot and bedding bulb flower has excellent adaptability and vigorous growth in late winter and early spring. The quantity and uniformity of *Muscari* propagation by seed and the importance of seed priming will be measured in current experiment. The selected *M. neglectum* populations which already were gathered in our previews research and *M. armeniacum* were evaluated under separated sulfuric acid (5, 15, and 20 min) and hot water (5, 15, and 20 min) scarification plus 15, 30, and 45 days stratification in a completely randomized design, four replications were planted in separate containers. Seeds of *M. armeniacum* only germinate (70%) by 15 min sulfuric acid plus 45-day stratification treatments. Based on the analysis of variance and mean comparison data that only were reported in *M. neglectum,* their seeds optimally germinated by 5 and 15 min sulfuric acid treatment plus 45-day stratification. In conclusion, *M. neglectum* seeds germination is tuned to take place well in mid-winter under natural conditions; on the other hand, 45 days of lengthy constant stratification and 5 min sulfuric acid priming will accelerate *M. neglectum* seeds germination.
Keywords: germination, hot water, stratification, sulfuric acid, uniformity.

Resumo

Melhoria na germinação de sementes de Muscari usando ácido sulfúrico e *priming* de estratificação

Muscari é uma espécie amplamente difundida e comercial. Como planta de vaso e bulbo, a flor tem excelente adaptabilidade e crescimento vigoroso no final do inverno e início da primavera. A quantidade e uniformidade da propagação de *Muscari* por sementes e a importância do condicionamento fisiológico das sementes serão medidas no presente experimento. As populações selecionadas de *M. negligenciaum* que já foram reunidas em nossas pesquisas prévias e *M. armeniacum* foram avaliadas sob ácido sulfúrico (5, 15 e 20 min) e água quente (5, 15 e 20 min) escarificação mais 15, 30 min, e estratificação de 45 dias em delineamento inteiramente casualizado, 4 repetições foram plantadas em recipientes separados. Sementes de *M. armeniacum* só germinam (70%) após imersão por 15 min de ácido sulfúrico mais tratamentos de estratificação de 45 dias. Com base na análise de variância e nos dados de comparação de médias que foram relatados apenas em *M. negligência*, suas sementes germinaram de maneira ideal por 5 e 15 min de tratamento com ácido sulfúrico mais estratificação de 45 dias. Conclusão, a germinação das sementes de *M. negligenciadum* está ajustada para ocorrer bem no meio do inverno em condições naturais; por outro lado, 45 dias de longa estratificação constante e 5 min de condicionamento com ácido sulfúrico irão acelerar a germinação de sementes de *M. negligencialum*.
Palavras-chave: ácido sulfúrico, água quente, estratificação, germinação, uniformidade.

Introduction

The demand and endeavor for breeding *Muscari* species with great bulbous plants are increase because of its excellent horticultural specifics, economic and pharmaceutical importance (Labbaf et al., 2020). Among the noteworthy species in the *Muscari* genus is *M. neglectum* Guss. *ex* Ten. that forcing bulbs as cut flowers or pots is very probably, also it is beneficial for gardens and landscape (Kamenetsky and Okubo, 2012). *Muscari* flower colors are infinitely varied, and it's worth noting that its natural pigment has many particularly advantageous compounds which may support human well-being (Fawzi et al., 2021). *Muscari armeniacum* with absorbing property

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is a popular and commercial species. It is appropriate as pot, bedding, garden, and woodland owing to greater adaptability, vigorous growth, and salient blue colors in spring (Khaleghi and Khadivi, 2022). *M. neglectum* growth generally in the whole Mediterranean but it distributed into Russia and Iran (Davis and Stuart, 1984). *Muscari* as the endemic flower generally grows in the central area of the Zagros mountainous region of Iran (Candido et al., 2017). Labbaf et al. (2020) investigation showed pretty high values of genetic variation and morphological characteristics within the population and low genetic diversity level among nine genotypes of M. neglectum, belonging to Iran's nine distribution regions.

Muscari produces many seeds which has a short juvenile period, so their propagation can occur from seeds. Other advantage of seed propagation is that, unlike vegetatively propagated resources, the plants grown from seed are virus-free (Kamenetsky and Okubo, 2012). Therefore, despite their seed propagation significance, there are still problems with their germination. *Muscari* seeds usually germinate better within their native environments. They often take a long time to germinate which is not favorable for propagation. *Muscari* seeds have a hard coating with low water permeability which indicate morphophysiological dormancy with a dormancy period of about 7-8 months (Baskin and Baskin, 2014). In natural environments, seed dormancy induces inactivation and prevents seed germination on a maternal level, allowing for greater dispersal and passage of harmful environmental conditions (Sohindji et al., 2020). Final seed germination in *M. neglectum* was found to be remarkably suppressed by white light (photoinhibition). Therefore, seed dormancy, long germination period, and low germination rate are major problems for *Muscari* species (Doussi and Thanos, 2002; Baskin and Baskin, 2014, Candido et al., 2017). However, before sowing, dormant seeds should be handled. Pre-sowing treatments like cold stratification and chemical or mechanical scarification could minimize seed hardness and increase germination rates in different seeds plant (Baskin and Baskin, 2014; Martinez-Diaz et al., 2018; Sohindji et al., 2020).

M. comosum seeds germinated slowly and steadily at a cool temperature range (5-15 °C). It was seriously inhibited in the dark, over 15 °C (Doussi and Thanos, 2002). Time, uniformity, and synchronization index are important not only for seed technologists and physiologists, but also for ecologists, since they can be used to measure and forecast the dynamics of germination phase (Ranal and Santana, 2006).

The current research aims to fill this information gap by exploring how to enhance the germination of selected populations of *M. neglectum* and *Muscari armeniacum*. Germination parameters for native species, especially rare and endemic species, are critical for population survival and conserve genetic diversity via the sexual reproduction (Fernandez and et al., 2020). The specific goals had achieved: (1) morphological diversity and germinability investigation; (2) to increase germination rate and uniformity, reduce germination period; by (3) introducing laboratory method for breaking seed dormancy.

Material and methods

Plant Materials

The study was conducted with *M. neglectum* seeds resulting from endemic population samples (Table 1) that showed the most morphological characteristics as well as higher flowering percentage in Labbaf et al. (2020) investigation. furthermore, their Genetic diversity investigation indicates that these populations are in the same cluster. Bulbs were screened based on circumference (6.7 cm), and selected bulbs were cultivated in 20 cm diameter potting and moved to the greenhouse after cold treatment (15 weeks at 9 °C) in September 2017. In April 2018, seeds from *M. neglectum* populations were obtained at the same time. *M. armeniacum* seeds were bought from 'www.plant-world-seeds.com'.

Germination

This study was organized in two separate factorial experiments base on a completely randomized design (CRD) with 4 replicates, and each treatment contained 25 seeds. The first and second experiment treatments were acid plus cold stratification and hot water plus cold stratification, respectively. Before providing any treatment, the seeds were sterilized by soaking for 5 min in a 2% NaOCl solution and then rinsing three times with distilled water. Chemical scarification with sulfuric acid (70 percent v v^{-1}) for 0, 5, 15, and 20 min is used in the first experiment. Seeds were rinsed with distilled water after scarification treatments and put on double filter paper disks (Whatman No.1) in 9-cm glass Petri dishes moistened with 7 mL sterilized distilled water and stratified at 4 ºC for 0, 15, 30, and 45 days. They transferred to the germinator (± 0.5 °C -darkness) at the optimum values of 10 °C (Doussi and Thanos, 2002). In the second experiment, the seeds were scarified with hot water (70 ºC) for 0, 5, 15, and 20 min. The number of germinated seeds was counted every day. A seed was considered as germinated once its radicle had protruded about 2mm from the seed coat ((ISTA, 2017). The seed count was concluded when no additional seeds germinated. The following formula was used to measure germination rate and germination percentage (GR and GP), respectively (Olmez and Gokturk, 2007)

$$GP = n/v \times 100 \quad (1)$$

Whereas *n*: the number of seeds that have germinated and *v*: the number of viable seeds that have been initiated,

$$GR = [(n1 \times t1) + (n2 \times t2) + (ni \times ti)] / T \quad (2)$$

Where *ni* represents the number of days in each counting, *ti* represents the number of germinated seeds in each counting, and T represents the cumulative number of germinated seeds.

The mean germination time (MGT) and mean germination rate (MDR) were determined in this study according to the methods defined by Dorneles et al. (2005). In usual, during germination activity, more or less deviation from normality can be fined in the context of time. Other drawback of this assessment is that for comparisons, the mean germination period of samples or treatments should be the same, also variances and standard deviations depending on the mean's magnitude (Ranal and Santana, 2006). The variation coefficient of germination time (CVt) was recommended to calculate the uniformity or instability of germination compared to the mean germination time (Dorneles et al., 2005). GV "germination value" was mentioned as GV = PV MDG, where PV: peak value of germination, and MDG: mean daily germination which means the number of seeds that germinate per day (Czabator, 1962). PV stands for the highest cumulative germination percentage divided by the number of days it took to get there. This investigation was approved out by adopting a set of instructions including uncertainty (U) and synchronization index (Z). Seed germination as a synchronized characteristic can be quantified by synchronization index (Z) (Ranal and Santana, 2006). The value of U depends on one seed germination and means that U determines the distribution degree of germination via time, and the synchronization index determines germination overlapping degree (Ranal and Santana, 2006; Dorneles et al., 2005). When at least two seeds can germinate, one behind the other means Z=0 and, Z=1 indicates all seeds germination simultaneously happen. Treatment effects were determined by analysis of variance according to the general linear model procedure in Statistical Analysis System (SAS Institute, Cary, NC, USA). Mean comparisons were performed with Duncan's multiple range test at the 1% level of significance. Because of *M. armeniacum* seed germination results, analysis of variance and mean comparison data only reported in *M. neglectum*.

Results

Stratification, chemical, and hot water scarification were performed on a selected population of *M. neglectum* and *M. armeniacum* (Table 1) to increase germination rate, reduce germination period, and breaking seed dormancy.

The variance analysis table showed that sulfuric acid, hot water, and stratification treatments significantly modified the germination characteristic of *Muscari neglectum* seeds (Table 2). Moreover, sulfuric acid and stratification interaction, as well as hot water and stratification interaction, had a significant effect on germination characteristics.

The germination time courses at stratification and the effect of sulfuric acid and hot water scarification on germination aspects for *M. neglectum* (Table 1) are illustrated in Figure (1a, b).

Table1. In various provinces of Iran, accessions of *M. neglectum* were collected.

***Muscari* Species**	**Country/Province/CS**	**Latitude**	**Longitude**	**Altitude (m)**
M. neglectum	IRAN/Isfahan/ Golpaygan A	50° 19› 25"	33° 25› 55"	1851
M. neglectum	IRAN/Isfahan/ Golpaygan B	50° 17› 71"	33° 28› 23"	1823
M. neglectum	IRAN/Isfahan/ Golpaygan C	50° 20› 31"	33° 28› 42"	1811
Muscari Species	Country/Province	Website		
M. armeniacum	United Kingdom's	www.plant-world-seeds.com		

CS: Collecting Site

Table 2. Variance analysis of Sulfuric acid and hot treatments on germination characteristic of *Muscari neglectum* seeds.

Source of variation	Df	Mean Sq						
		Ge	MGT	MGR	CV	U	Z	GV
Sulfuric-Acid	3	2431.58**	316.27**	0.01**	677.92**	5.04**	0.004**	3.539**
Stratification	3	15254.9**	1101.17**	0.05**	9867.82**	26.22**	0.016**	17.980**
Sulfuric-Acid × Stratification	9	2955.8**	314.55**	0.007**	908.74**	3.90**	0.002**	3.513**
Error	48	80.08	2.70	0.00009	43.71	0.10	0.0003	0.07
CV %	-	16.9	13.9	14	19.5	13.5	3.2	11.7
Hot Water	3	3.90**	1.62**	0.04**	47.62**	0.29**	0.03**	0.014**
Stratification	3	3.70**	2.24**	0.12**	49.36**	0.25**	0.02**	0.050**
Hot Water × Stratification	9	0.47**	0.26**	0.02**	7.73**	0.04**	0.05**	0.006**
Error	48	0.02	0.01	0.0001	0.60	0.003	0.002	0.00007
CV %	-	16.8	15.8	2.3	20.8	12.2	8.1	19.8

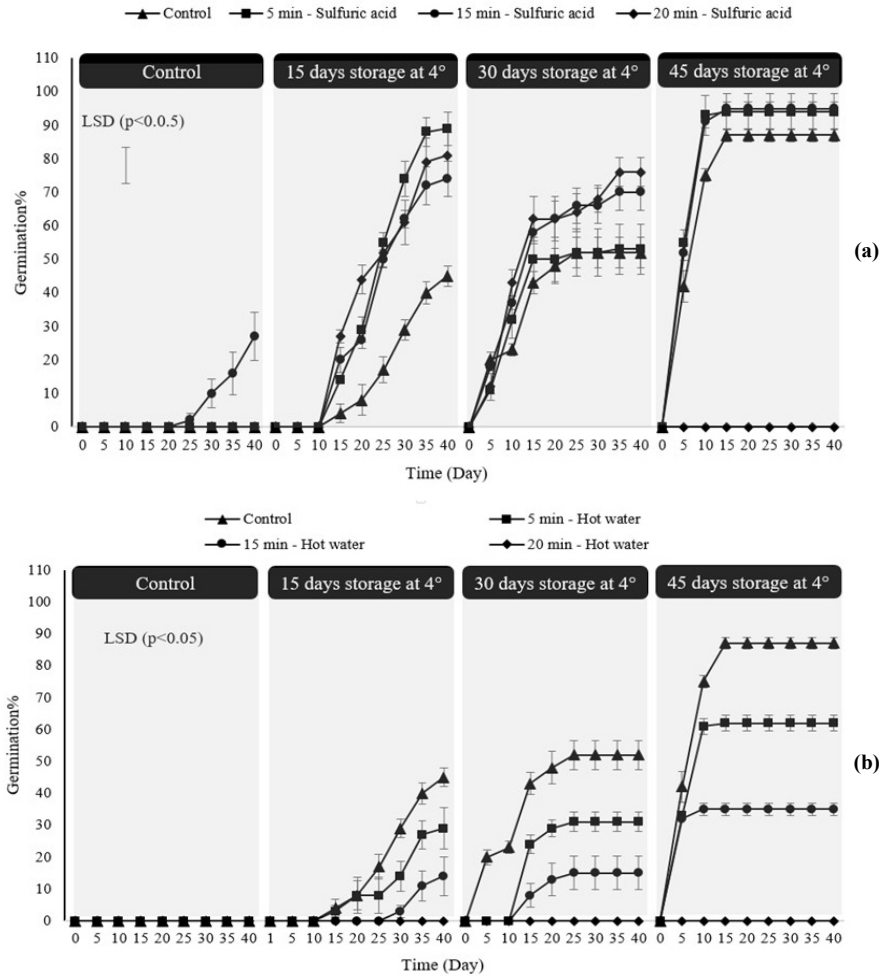

Figure 1. Time courses of seed germination and final seed germination in chemical scarification plus cold stratification treatment (a) Scarification with hot water and cold stratification, for *Muscari neglectum* (b).

Seeds of *M. neglectum* optimally germinated by 5 and 15 min sulfuric acid treatment after 45 days cold stratification, while the germination percentage was sharply suppressed after 20min sulfuric acid treatment (0%) (Figure 1a). The final germination percentage for 5 or 15 min sulfuric and 45-day stratification treatment upon transfer to the optimum condition is about 95% (Figure 1a). Almost none of the seeds of *M. neglectum* germinate under control (without stratification) conditions, except those of seeds within15 min sulfuric acid treatment (30% germination) (Figure 1a). The average number of *M. neglectum* seeds germination is reduced 13%, 38%, and 65% by hot water 0, 5, and 15 min respectively plus 45-day stratification, except those of seeds, within 20 min hot water treatment (Figure 1b) which destroyed the embryo. Therefore 45-day stratification plus 5 or 15 min sulfuric acid can only accelerate the germination percentage, and approximately all seeds were germinated after 15 days (Figure 1a). Seeds of *M. armeniacum* only germinate (70%) by 15 min sulfuric acid treatment, plus 45-day stratification, while germination percentage was wholly suppressed after hot water treatment with and without stratification (Figure 1); therefore, analysis of variance and mean comparison data only reported in *M. neglectum*.

The weighted mean of germination time is used to measure the mean germination time (MGT). MGT significantly decreased under 15, 30, and 45- days stratification respectively (Figures 2 and 3a).

MGT decreased 88% under 45-day stratification plus 5 and 15 min sulphuric acid compared to 15-day stratification indicating that 45-day stratification substantiality improved germination for *M. neglectum* seeds. On the other hand, the MGR increased significantly compared to control under 45-day stratification (Figure 2b). The highest MGR significantly achieved by 5 min sulphuric acid and 45-day stratification (Figure 2b). Furthermore, 15 min hot water plus 45-day stratification shows the highest MGR, which means there is the shortest mean germination time during these treatments (Figure 3a and b).

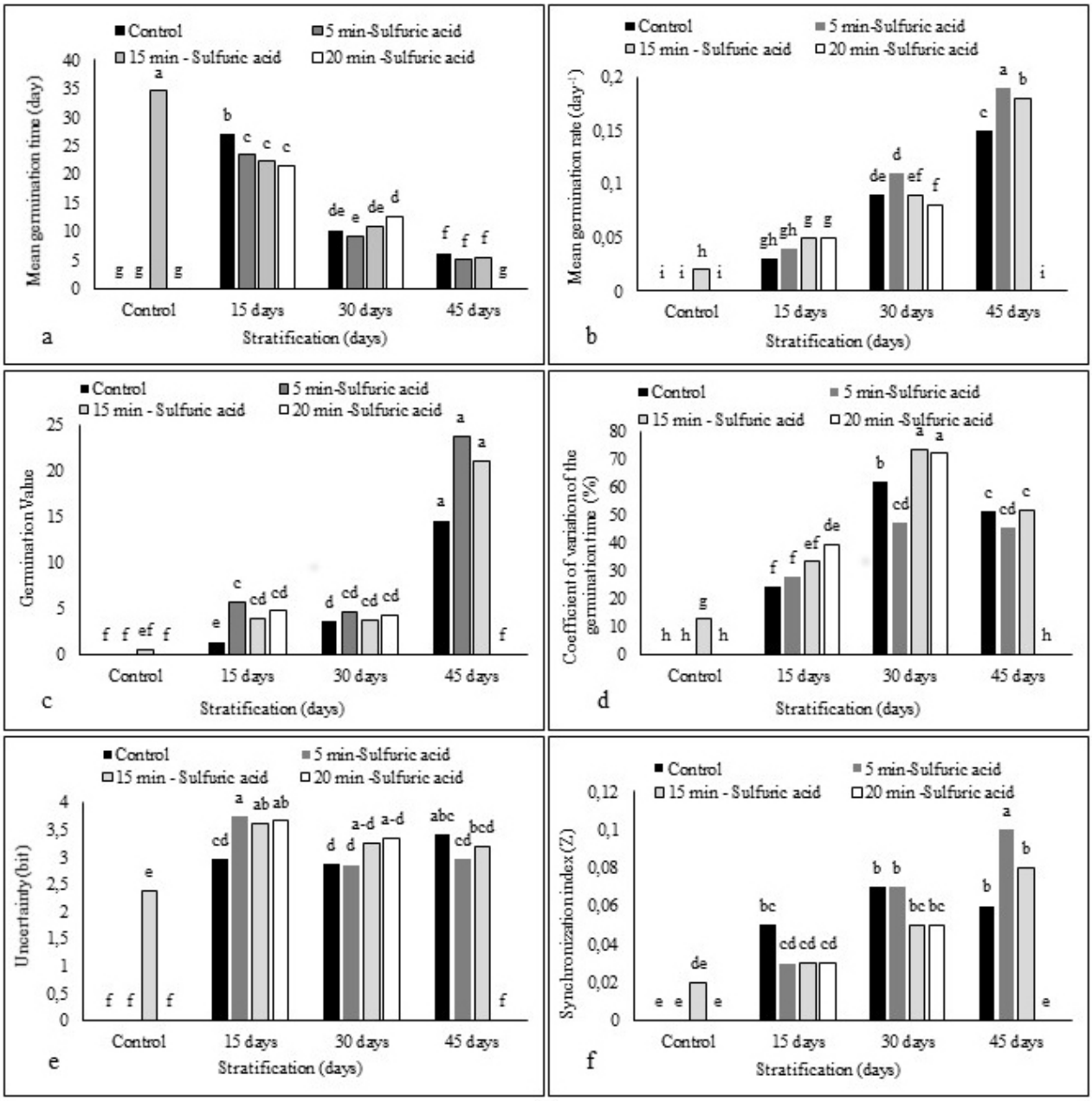

Figure 2. Stratification and sulfuric acid interaction on Mean germination time (MGT) (a) Mean germination rate (MGR) (b) germination value (GV) (c) Coefficient of variation of the germination time (CVt) (d) Uncertainty (U) (e) Synchronization index (Z) (f) in *Muscari neglectum*. Bars with different letters within each preservative and each group are significantly different in the least squares means test

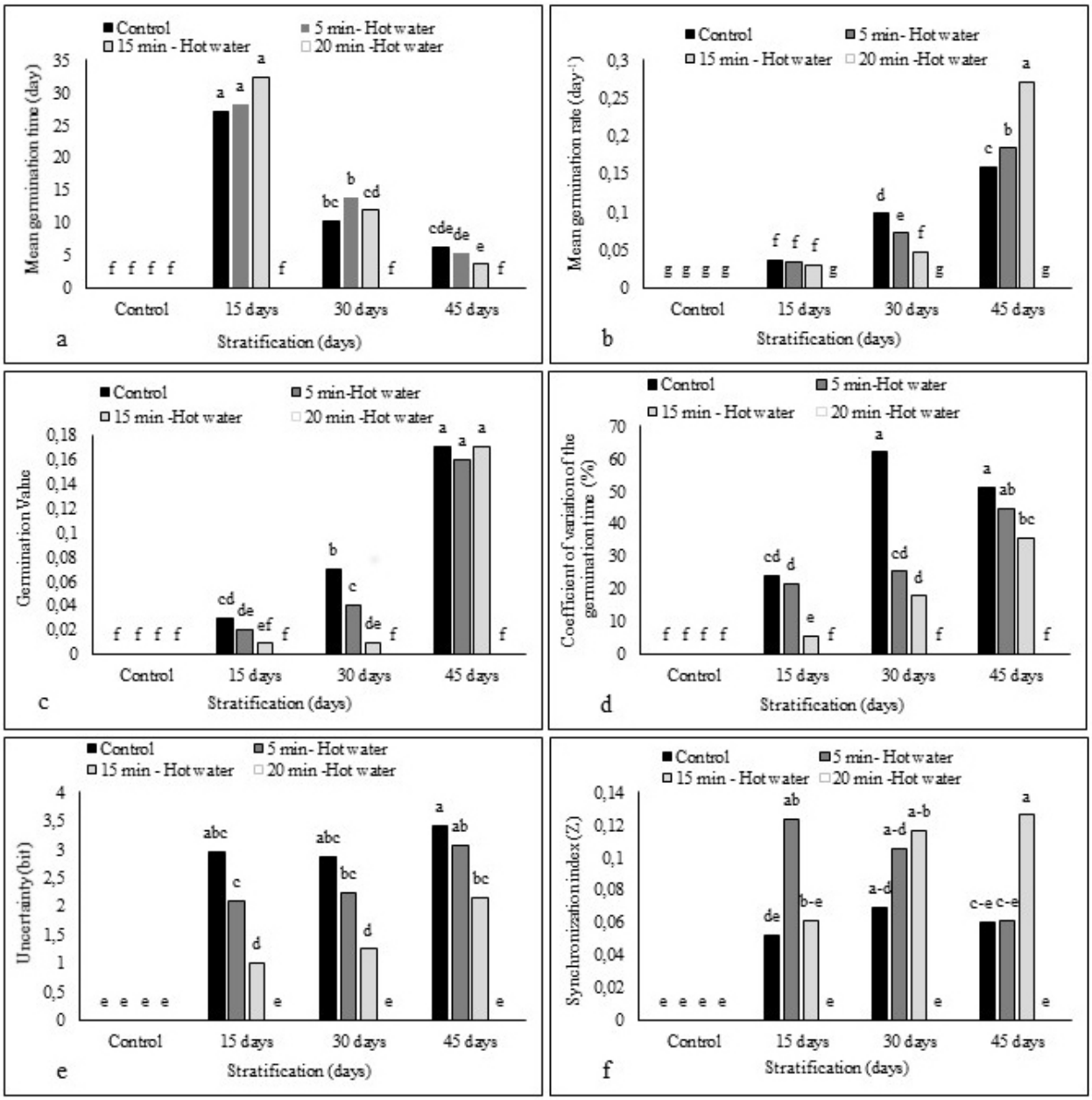

Figure 3. Stratification and hot water interaction on Mean germination time (MGT) (a) Mean germination rate (MGR) (b) germination value (GV) (c) Coefficient of variation of the germination time (CVt) (d) Uncertainty (U) (e) Synchronization index (Z) (f) in *Muscari neglectum*. Bars with different letters within each preservative and each group are significantly different in the least squares means test.

To evaluate the germination value (GV), we report that 5 min sulfuric and 45-day stratification had the highest GV (Figure 2a), which shows no significant differences among control, 5, and 15 min sulfuric acid treatments. Hot water significantly decreases GV, specifically after 15 min hot water treatment (Figure 3a) plus 15, and 30 days' stratification. Moreover, there is no significant difference among control, 5 and 15 min hot water plus 45-day stratification (Figure 3c). GV increases in 45-day stratification groups compared to control (without stratification) (Figures 2 and 3c).

To determine germination variability, we evaluate the variation coefficient of the germination time (CVt). The highest CVt was achieved by the interaction of 15 (73%) and 20 (72%) min sulfuric acid and 30-day stratification; respectively therefore higher germination variability can be interpreted from these treatments (Figure 2d). Because of low germination in hot water treatments (Figure 1b), CVt was significantly suppressed by all hot water treatments (Figure 3d).

The high synchronization index (Z) value shows low variation, and the zero Z index indicates high diversity. Hence, uncertainty (U) correlated by distributing the relative frequency of germination.

Low U values represent frequencies with few peaks indicating more concentrated germination over time. Figure (2e, f) display no difference in z index and U among sulfuric acid treatments in different stratification, except 5 min sulfuric acid plus 45-day stratification (0.1) which significantly reveal the highest Z index. This observation indicates that 5 min sulfuric acid plus 45-day stratification has the highest germination overlapping (Figure 2f). Uncertainty (U) increased in 15-day stratification plus sulfuric acid treatment groups (Figure 2e), which implies a wide range of relative germination frequencies and a low Z index. (Figure 2e, f). Figure (3e) shows U significantly decreased under hot water treatments. Thus, hot water 15 min plus 45-day stratification reveals the highest Z index (0.12), which means more seeds germinated at the same time (Figure 3f).

Discussion

These results indicate that *M. neglectum* seeds germinate 95 % after 45-day stratification and 5 or 15 min sulfuric acid treatments in optimum temperature (Figure 1a). Seeds of *M. armeniacum* only germinate 69% by 15 min sulfuric acid treatment plus 45-day stratification. Doussi and Thanos (2002) indicated that *Muscari* seed germination took place in a relatively small range of cold temperatures (10 or 15°C) and at an astonishingly slow rate; also, no primary dormancy was reported. Cold stratification and chemical or mechanical scarification seem to have decrease seed hardness and increased low or irregular germination and emergence rates (Baskin and Baskin, 2014; Martinez-Diaz et al., 2018). According to Doussi and Thanos (2002), chipping *M. comosum* seeds resulted in only a small improvement in germination rate without primary dormancy; however, the finding explains the 45-day stratification impact *Muscari* embryos' post-maturing growth requirements. Many Liliaceous species have previously been recorded to have tiny, linear, and underdeveloped embryos (Baskin and Baskin, 2014). We conclude that 5 min sulfuric acid plus 45-day stratification treatment on *M. neglectum* seed decreases germination time to 15 days in optimum temperature. Seeds of different plants require 4-28 weeks of stratification to break their dormancy, depending on the species (Tang et al., 2019). Stratification plays an important role as a stimulator that helps to break dormancy. The oxygen demand of the embryo is best met at lower temperatures because more oxygen is soluble in water (Leo, 2013). Hence, by enzyme activity and generating amino acids needed for embryo use during development, stratification was successful in shortening the seed dormancy time (Saffari et al., 2021). However, there is a report that indicates the chipping without stratification increased the germination rate, and around 100% germination occurred 24 days after plantation of *M. neglectum (*Doussi and Thanos, 2002).

MGT and MGR results indicate 45-day stratification plus 5 min sulphuric acid remarkably improved the germination quality of *M. neglectum* seeds. The number of seeds which germinated during the data collection periods is used as a weight (Ranal and Santana, 2006). Using of the weighted mean is needed in this situation, as it considers that a various number of seeds germinate. GV increases in 45-day stratification plus hot water and sulfuric acid (5 and 15 min) compared to control (without stratification) (Figure 2 and 3c). This result suggests that GV is a measure of germination speed and totality, and their interaction, as defined by Brown and Mayer (1988). On the other hand, 15 and 45 days stratifications significantly decreased CVt, as a consequence, these treatments can be perceived as having a higher germination uniformity.

We found that 5 min sulfuric acid plus 45-day stratification significantly increased synchronization index (Z) (0.1) which has the highest germination overlapping or low uncertainty (U) (Figure 2e, f). This observation, consistent to this treatments' germination course time in Figure 1a. We recommended that 5 min sulfuric acid plus 45-day stratification can be used as the most effective and easily applicable pretreatment for the shortest time to complete germination in *M. neglectum* populations. On the other hand, *M. armeniacum* seeds show 70% germination only by 15 min sulfuric acid plus 45-day stratification treatment.

Conclusions

In summary, the result indicated, *M. armeniacum* seeds only germinate by 15 min sulfuric acid treatment plus 45-day stratification and *M. neglectum* seeds should receive 5 min sulfuric acid plus 45 days' stratification for rapid, uniform, and valuable germination. Based on these experimental results, field germination of top morphological Iranian selected population of *M. neglectum* seed is tuned to take place well in mid-winter and provided a lengthy constant stratification for embryo development and some scarification. We conclude that different seed priming genetic and environmental aspects can influence morphological details and germination characteristics. These observations indicate essential germination features which help discriminate between excellent and poor treatments. Therefore, MGT and MGR introduce germination quality, GV is a measure of germination speed and totality, CVt indicate germination uniformity and synchronization index (Z) which support germination overlapping are the most effective germination features.

Author Contribution

IR and **AMN** designed and directed the project; **NL** performed the experiments; **AMN** analyzed data; **IR** and **NL** developed the theoretical framework and wrote the article.

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