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Full Length Research Paper

Seed germination as the major conservation issue of endemic Iranian salvia species

Jaafar Abdollahi[1], Mohsen Ebrahimi[1]*, Hossein Ali Ramshini[1], Ali Ashraf Jaafari[2], Mostafa Eftekhari[1], Yaser Siah Mansouri[3] and Mohammad Ali Sheikh Beig Goharrizi[4]

[1]Department of Agronomy and Plant Breeding Sciences, College of Abouraihan, University of Tehran, Iran.
[2]Research Institute of Forest and Rangelands, Gene Bank Research Division, Tehran, Iran.
[3]Department of Agro Technology, College of Abouraihan, University of Tehran, Iran.
[4]Department of Horticulture, College of Abouraihan, University of Tehran, Iran.

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In Iran, there is a high diversity of salvia species and accessions which includes 70 species that 40% of them are endemic. The objective of this investigation was to find a practical treatment for germination of salvia species, particularly, endemics and to find conservation issues and appropriate approaches. We observed that there was a huge diversity in color (RGB channels), seed area and 1000-seed weights among the population in this study, including 60 accessions (23 species) that thirteen of them (five species) are endemic of Iran. These accessions were soaked in four gibberllic acid (GA3) levels (0, 100, 150 and 200 mg/L). The germination rate and percentage of 62% of accessions were, extremely, increased in response to the GA3 treatment; nonetheless, some accessions did not germinate at all which indicates that there are demands for more efforts to conserve these accessions. Germination percentage of endemic species was significantly lower than non-endemic ones, indicating a serious concern for their conservation. A significant correlation between the 1000-seed weights and area under germination percentage curve (AUGPC) was found that indicates seeds were evolved to have more storage to survive for a long time until germination.

Key words: Conservation, endemic, Iran salvia diversity, germination, gibberllic acid (GA3).

INTRODUCTION

Salvia is an important genus of the Lamiaceae family that includes more than 700 species which are spread throughout the world (Ewans, 1996). Salvia species called Maryam-Goli in Persian (Mozaffarian, 1996) has been famous for its medicinal properties since ancient times (Rivera et al., 1994). Most of salvia species are, commonly, utilized for their essential oils in the foods, medicines and perfumery industries (Goren et al., 2006; Ozcan et al., 2003; Ulubelen and Topcu, 1998). With total of 70 species and 40% endemism, salvia has a consequential center of diversity in Iran. It exhibits an interesting range of morphological variation which is as great as, if not more than, anywhere in the whole world (Rechinger, 1982). Despite this huge diversity in Iran and considering very rare species, natural or even anthropogenic processes are not included within the current conservation management. It is striking that biology researchers have paid so little attention to biological conservation of Iranian endemic species of salvia. For instance, there is a major source of essential oils such as α-pinen (13%), β-pinen (10%), Sabinen (12%) and Bicyclogermacrene (31%) in *Salvia lachnocalyx* (Mirza and Baher, 2007), perennial species that grows only in too narrow region near Eghlid in Fars province which is threatened to extinct in near future (Jalili and Jamzad, 1999). There are some other species like *Salvia eremophila, Salvia mirzayanii, Salvia reuterana*

*Corresponding author. E-mail: mebrahimi@ut.ac.ir. Tel: +989125123848. Fax: +982923040811.

Abbreviations: GA3, Gibberllic acid; **AUGPC,** area under germination percentage curve; **AUGC,** area under percentage germination curve.

and *Salvia sharifii* that were investigated in this research. These species are endemic of Iran which grow only in one or more province *(S. lachnocalyx* is restricted to one region) (Rechinger, 1982).

A major priority in efforts to conserve these rare species is to maintain their evolutionary potential by improving their germination. Many countries have books cataloging rare and threatened species (Jalili and Jamzad, 1999; Stein and Flack, 1997), but red data book of Iran lacks information about some species even for some endemic species like *S. mirzayanii* and *S. reoterana*, and for others, the existing information often lack a lot of necessary meticulous autecological and needs a long-term demographic work. Due to some problems such as destroying pastures and their replacement with farm, uncontrolled use, lack of conservation, extension of urban areas and their narrowness of geographical stretch, some exclusive species of Iran are in threat of extinction and their gene pool is experiencing genetic drift. An effective system to conserve endangered species is supporting seed banks which could preserve viable seeds under cautiously controlled situation. Unfortunately, in Iranian natural resource gene bank which is currently known as the most important institute for this work, there are only seeds of five species out of eighteen endemic species of Iran. Though plants that reproduce by seeds possess highest capacity of genetic diversity in local ecotypes, there is, permanently, the risk of extinction of threatened endemic species or populations that grow only in an exceptional situation or locations (Fay and Muir, 1990; Fay, 1992). The primary global problem of salvia species and accessions has been germination and up till now an integral solution has not been reported for it. Considering the expanding usage of these species at multiplicity parts of the world, finding a solution for triggering their germination is so prominent. In addition, the diversity of germination in Iranian salvia species has not been studied yet.

In this experiment the germination and response of Iranian accession to GA3 was studied. Gibberellins treatments were chosen because previous works have revealed that this factor improves seed germination in salvia and other plant species (Chan and Lambers, 1970; Finch-Savage et al., 1991; Kosikova, 1960; Thompson, 1969). The present work focused on *ex situ* need for seed germination of salvia accessions growing in Iran, comparing the germination of endemic and non-endemic species and studying their response to GA3 as an initial step to their conservation. Additionally, our other aims were to (1) find a practical protocols for germination of salvia species and accessions, specially, for *S. lachnocalyx*; (2) the investigation of diversity for seed germination and GA3 response among growing species in Iran and finding differences between endemic and non-endemic species; (3) determination of species and accessions that their germination and, consequently, conservation is not acceptable, especially, for endemic species and (4) the study of genetic diversity in salvia species and accessions growing in Iran.

MATERIALS AND METHODS

Seeds of 60 salvia accessions (23 species) comprises of 13 endemic (5 species) were used in this experiment during the spring to autumn of 2010. There was a limited amount of seeds available for accessions, especially, in five endemic species of Iran (*S. reuterana, S. lachnocalyx, S. sharifii, S. mirzayanii* and *S. eremophila*) due to the small number of seeds in natural resource gene bank in Iran. These seeds have been collected from different regions of Iran for several years at this institute. In general, fresh seeds are naturally dormant, requiring cold treatment and a period for ripening and maturity to germinate (Budvytyte, 2001; Hashemi and Estilai, 1994). Seeds used in this study were matured and dried, underwent a period of storage in darkness at -4°C. Two accessions were collected by researchers from Beyram region (in south of Iran) and stored for a year at a situation similar to others. *S. lachnocalyx* has been classified as nationally endangered because of its endemism, extremely localized allocation and exposure to disastrous events that could commence swift extinction (Jalili and Jamzad, 1999).

Also, there is no information about endangeredness of *S. mirzayanii* and *S. reoterana*. Seeds were surface sterilized in 4% sodium hypochlorite solution containing a few drops of the surfactant Tween for 12 min and then rinsed three times with sterile distilled water; then placed on wetted Whatman No. 3 filter paper discs (Cerabolini et al., 2004) in Petri dishes containing 0 (control), 100, 150 and 200 mg/Lof GA3 (filter-sterilized and added to the growth media after autoclaving). For each accession, seeds were allocated to four replicate Petri dishes, each containing 10 seeds in a completely randomized design. Seeds were incubated for 12 days in a germination chamber in the following environmental regime: 14/10 h light/dark cycle at 22±1°C. All Petri dishes were sealed to prevent from desiccating with parafilm and to ensure no systematic influences due to position within the chamber re-randomizing of Petri dishes was done every other day (Yang et al., 1999). Seeds with at least two millimeters radicle emergence were recorded daily as ‘germinated’. To examine the viability of any non germinated seeds, 20- seed samples from all accessions were sown in pots containing Coco peat and Perlite (3:1) and maintained in the suitable condition for 270 days in greenhouse in plastic trays with daily irrigation. For calculating the 1000-seed weight, for each accession four replications of 100 seeds were used. The weight was measured by a scale with the accuracy of 0.0001. The correlation between 1000-seed weight and the area under germination percentage curve was computed. A total of sixty samples of salvia seed were separated randomly from each accession and were situated in the image. The images of seeds were taken by a camera (SONY SyberShot DSC-H9 color digital camera with 8.1 Mega Pixels of resolution) installed on binoculars.

The magnification coefficient was 1.5 and 15 for binoculars and camera, respectively. Two kinds of information were used to classify accessions, their seed area and RGB channels. The data for this part are from another research about the identification of salvia species and accessions using RGB analysis, seed area and forms. Here, the means of data for RGB channels and seed area are presented showing the amount of diversity among accessions. Germination percentages of accessions in which it was not normal were arcsine transformed before statistical analyses. Differences in germination means among GA3 treatments were analyzed by one-way ANOVA, separately, in each accession. Orthogonal comparisons to show germination differences of endemic and non-endemic in all treatments (α=%1) were performed. Germination rates in different clusters and treatments were analyzed. These

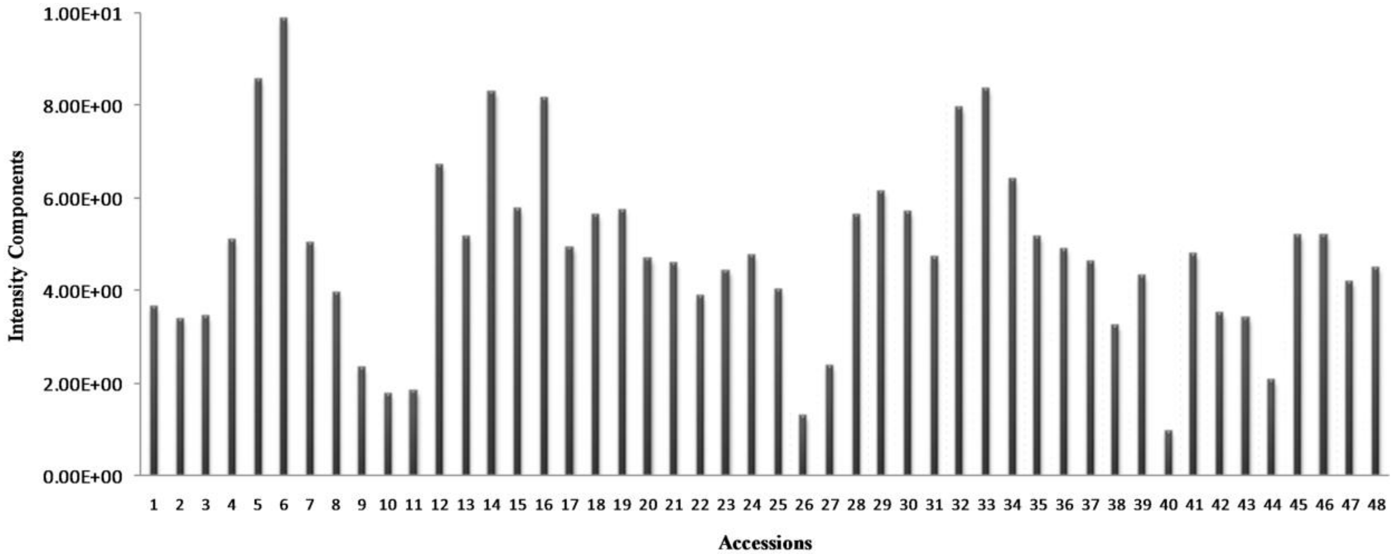

Figure 1. Diversity with the intensity component obtained by average of R, G and B channels mean of seeds for 48 accessions of salvia used in this study, calculated by MATLAB.

statistical analyses were performed with statistical analysis systems (SAS, 1998) and SYSTAT 12 (SSPS, 2007). A dendrogram on germination percentages in different treatments was constructed based on WARD linkage method and Euclidean distance analysis and the Pearson correlation between 1000-seed weight and area under percentage germination curve (AUGC) were calculated by Minitab (2003). The RGB channels and seed area values were calculated by digital images of seeds using MATLAB 7.1.4. This study reported diversity with the intensity component and is given by this equation (Gonzalez et al., 2004):

$$I = (R + G + B)/3 \quad (1)$$

Where I = intensity component, R = the average of red channel, G = the average of green channel and B = the average of blue channel obtained by image processing.

RESULTS

Image processing technique demonstrated that diversity in intensity component (Figure 1) and seed areas (Figure 2) of salvia accessions were very high, likewise there was a huge diversity in 1000-seed weights among species and accessions (varying from 0.625 to 12.725 g with difference magnitude of more than 20 times). Exogenous application of GA3 resulted in a wide spectrum of changes in the percent and rate of germination. A wonderful variation of germination responses to GA3 was found. Different accessions were grouped in four clusters based on seed germination response to four levels of GA3 (Figure 3). Clusters, obviously, separated accessions by their similarities so that it is easier to discuss about the differences of models and diversity in germination and response to GA3 based on these clusters instead of individual accessions (Figure 4). The majority of accessions required a stimulus to trigger germination and most of them failed to germinate in the absence of GA3. Without GA3 treatment, the seed either failed to germinate (34 accessions) or germinated at a very low percentage (Figure 5) and rate (Figure 6). The Germination percentage of accessions which were treated by GA3 was significantly increased particularly for those in Clusters 2 and 3 (Figure 4) and smoothly increased for some accessions of Cluster 4 (Figure 4). However, in 40 accessions both germination rate and percentage increased considerably during 12 days. Sixteen GA3 treated accessions failed to germinate (Cluster 4) because seed dormancy was not broken or twelve days were not long enough for their germination.

All these accessions germinated and grew in pots in 270 days with different rates and percentages that confirmed the seeds had no problem of viability (data not shown). Germination percentages of more than 60% of the accessions (Clusters 1 to 3) reached above 60% in 200 mg/LGA3 medium (Clusters 1, 2 and 3). GA3 had a highly significant effect on germination percentage of many accessions (Figure 5). Germination of 44 accessions was significantly stimulated by GA3 but the effect was very diverse in different species and accessions. Accessions in Cluster 2 responded very well and for 100, 150 and 200 mg/Lof GA3, their responses were nearly the same (Figure 4), but in Cluster 1, the effect of GA3 treatment was intangible. Germination percentage of 23 accessions in Cluster 3 positively responded to the increase of GA3 (100 to 200 mg/l), but comparing with other clusters, there were more differences among individuals of this cluster (Figure 4). In all germinated accessions, the rate of germination,

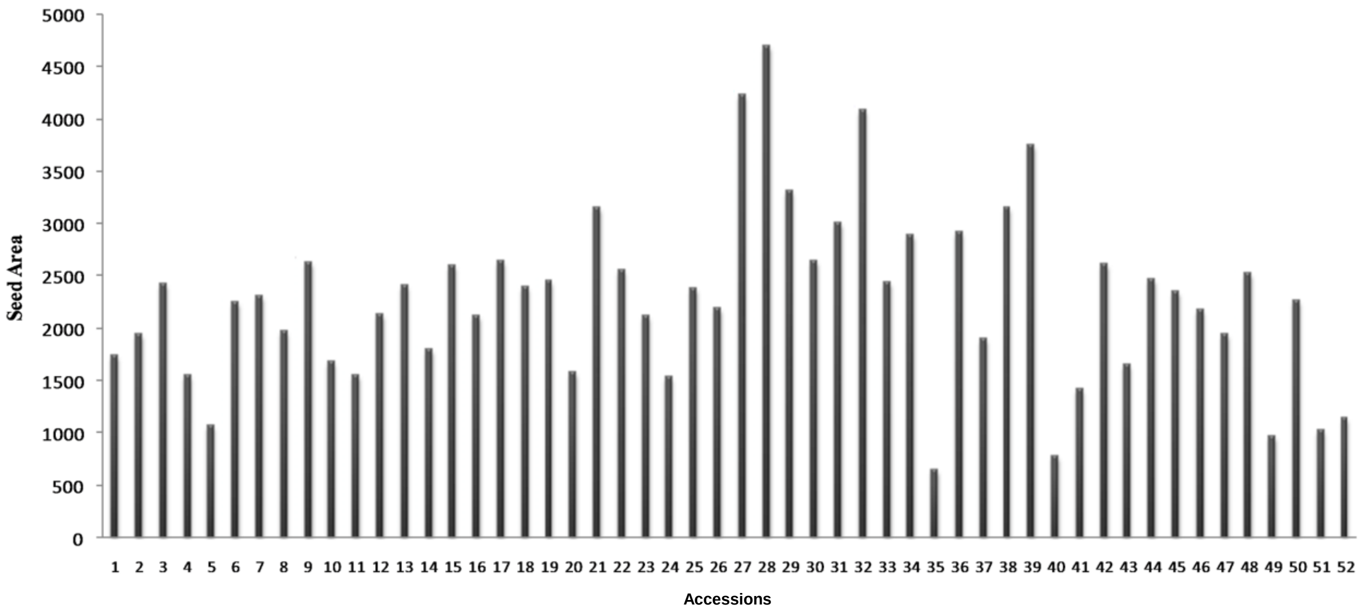

Figure 2. Diversity in seed area values of seed for 52 accessions calculated by MATLAB.

significantly, enhanced with GA3 treatment. The increasing rate of germination by different levels of GA3 was dissimilar in four clusters (Figure 6). The orthogonal comparison of endemic and non-endemic accessions showed that germination percentage of endemic accessions was, significantly, lower than non-endemic ones in all four treatments (α = 0.01) (Figure 7). All *S. mirzayanii* and *S. sharifii* accessions did not germinate at all while most accessions of other endemic species in response to GA3 treatment, relatively, reached acceptable germination percentages. Accessions were categorized into 12 groups based on their 1000-seed weights (including 0 to 1, 1 to 2, 2 to 3, 3 to 4, 4 to 5, 5 to 6, 6 to 7, 7 to 8, 9 to 10, 10 to 11, 11 to 12 and 12 to 13 gr). There was no sample in 8 to 9 group. A negative correlation was observed between 1000-seed weight and area under percentage germination curve ($r = -0.765^{**}$, n = 12). In general, accessions with lighter seeds showed, considerably, lower dormancy than heavier ones.

DISCUSSION

Diversity observed in RGB channels, seed areas and 1000-seed weights proves accessions studied had enough diversity to investigate approximately all kinds of germination and responses to GA3 models and demonstrated that there is a high variation among salvia species and accessions growing in Iran. Accordingly, it seems that their physiological demands are completely different. Therefore, each accession should be investigated, separately, for conservation purposes. In other words, it is not recommended to prescribe any extended result of statistical analysis to all species. For example, the germination percentage of seeds in control had huge differences so that the germination mean for some accessions (like *S. sclarea* Kordestan) was 90% while most of them were zero. Also, the response of accessions to GA3 was, highly, different; there was no response in some species (like *S. sharifi*), but in some others, germination percentage was over 90% (like *S. aethiopis* Ardabil). It corroborates that genetic diversity among accessions is high and consequently their germplasm is valuable for conservation aims.

Study on reproductive strategies is vital for artificial propagation as a result of conservation of diverse accessions. Propagation from seed is, efficiently, economical and common; but germination demands for native species are, in many cases, mysterious, particularly, for thinly distributed or endemic species of which material is additionally difficult to acquire. For these cases, we should use *ex situ* experiments for conservation purposes. For salvia accessions, propagation by seed is a preferred method for preserving their genetic diversity. In addition, in the majority of accessions, *in vitro* methods are not indispensable. For some accessions, these methods are not essential for ideal seed germination and would waste resources if used. Therefore proper methods must be chosen based on the demands of each accession (Benson et al., 2000). Hormonal treatments can effectively be used to increase the rate and the germination percentages. Many salvia species are similar to seeds of some other plant species, demand a stimulus to trigger germination and fail to germinate in the absence of gibberellins treatment (Hashemi and Estilai, 1994). Four separate clusters

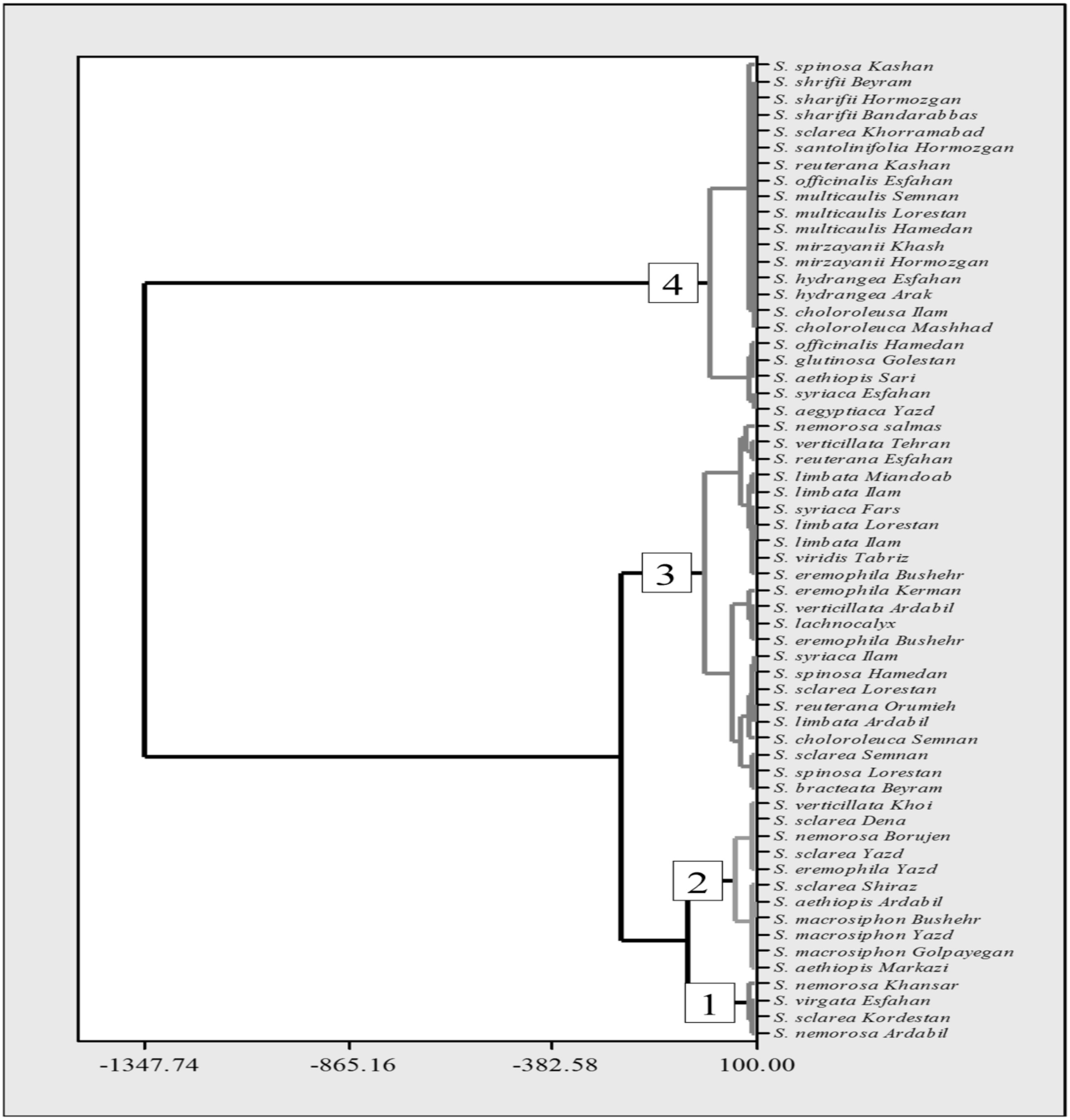

Figure 3. Dendrogram on germination percentages of different treatments of GA3 (0, 100, 150 and 200 mg/L) was constructed based on WARD linkage method and euclidean distance analysis using Minitab statistical package. Sixteen accessions grouped in four distinct clusters. This figure shows the high diversity of accession seeds in germination and response to GA3.

respond differently, to GA3 due to high diversity in germination of accessions.

Probably, in accessions of Cluster 1, there was innate dormancy that with maturing and drying, underwent long time storage in darkness at -4°C has been removed, but accessions in Cluster 2, 3 and 4 contain various strength of induced dormancy. Previous studies have indicated that various responses to germination treatments of accessions in diverse inhabitants are possibly due to environmental differences, in which they have evolved

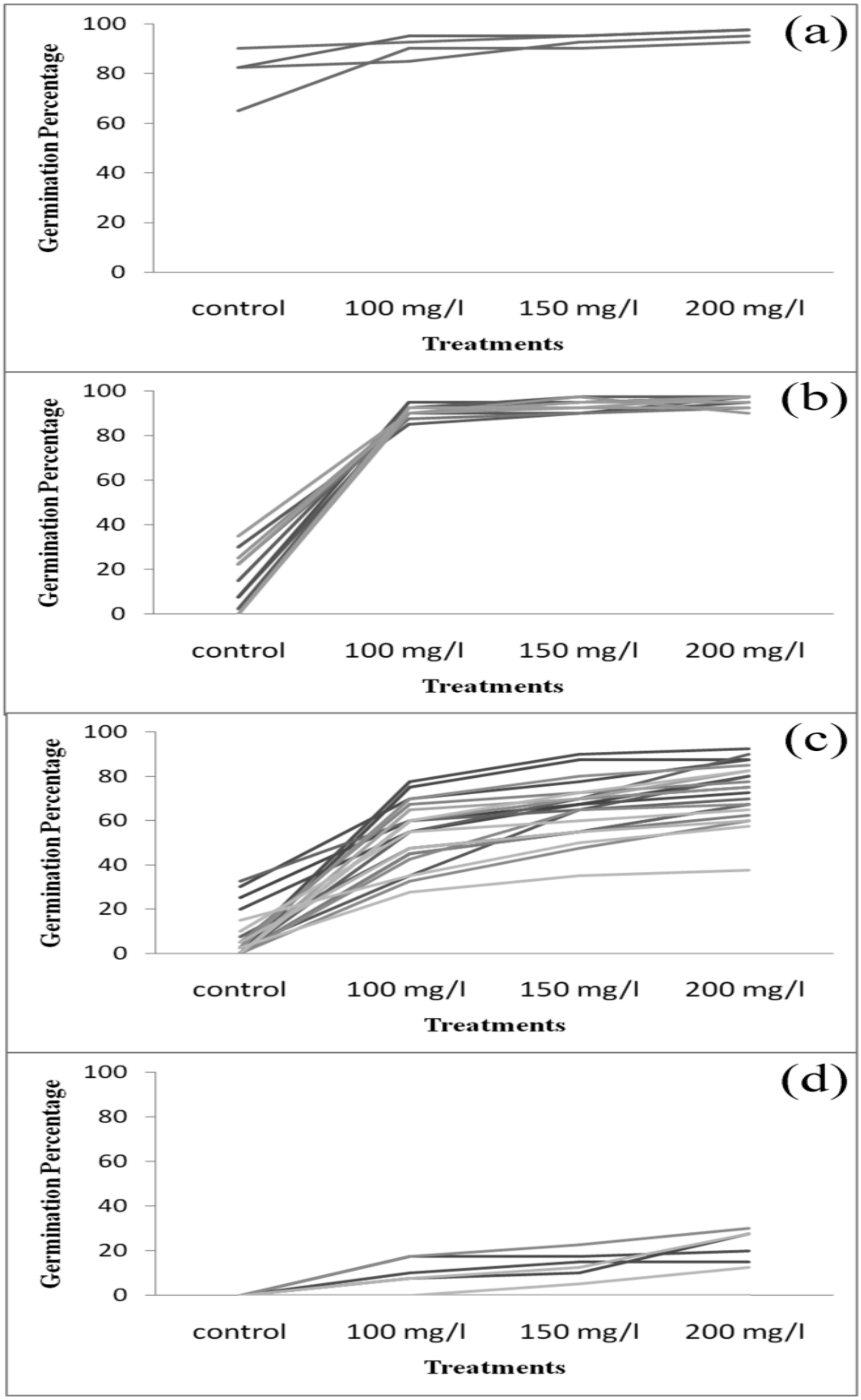

Figure 4. Germination percentage curve of accessions in different treatments of GA3 (0, 100, 150 and 200 mg/L) in Cluster 1a, 2b, 3c and 4d. This figure demonstrated that clusters, obviously, separated, properly, accessions by their similarities and clustering accessions to four groups was appropriate for showing their diverse mode of germination and response to GA3.

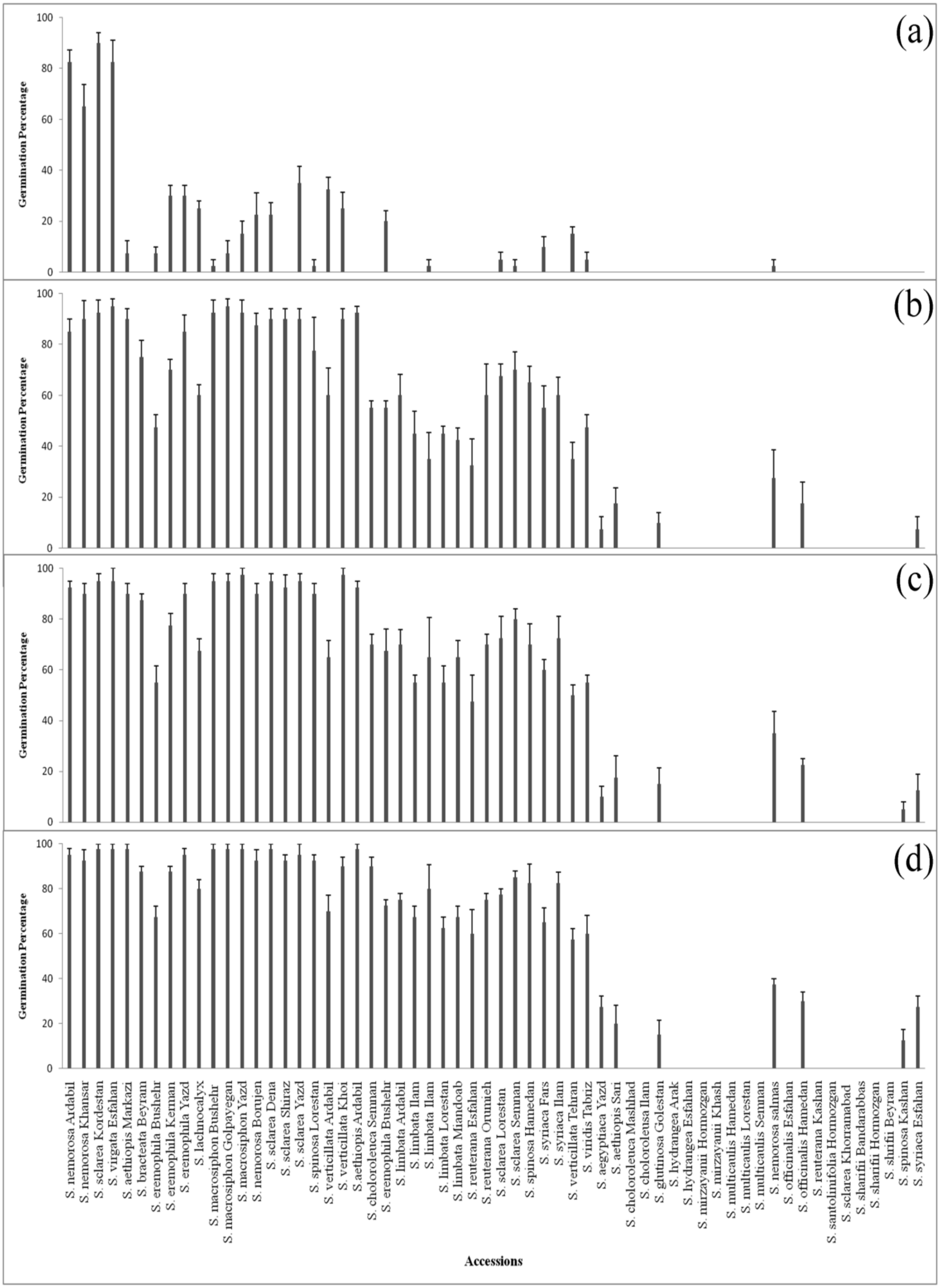

Figure 5. Germination percentage of 60 accessions of Salvia separated in four treatments of GA3; a: control (0 mg/l); b: 100 mg/L; c: 150 mg/L and d: 200 mg/L. The majority of accessions required a stimulus to trigger germination and most of them failed to germinate in the absence of GA3 or germinated at a very low percentage.

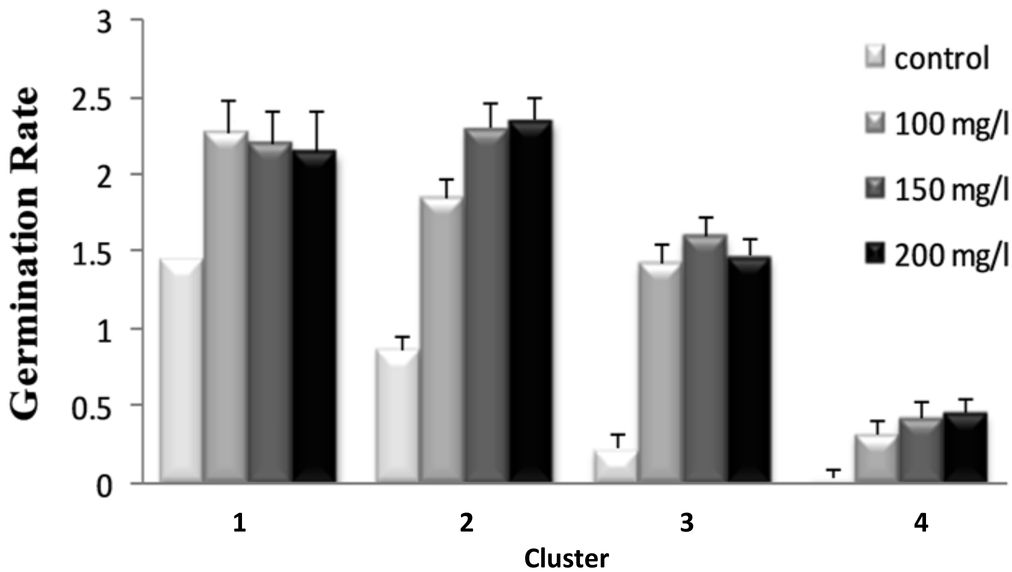

Figure 6. The rate of germination in different levels of GA3 (0, 100, 150 and 200 mg/L), separately, shows for four clusters. In all germinated accessions, the rate of germination, significantly, enhanced with GA3 treatment that the increasing of germination rate by different levels of GA3 was dissimilar in four clusters.

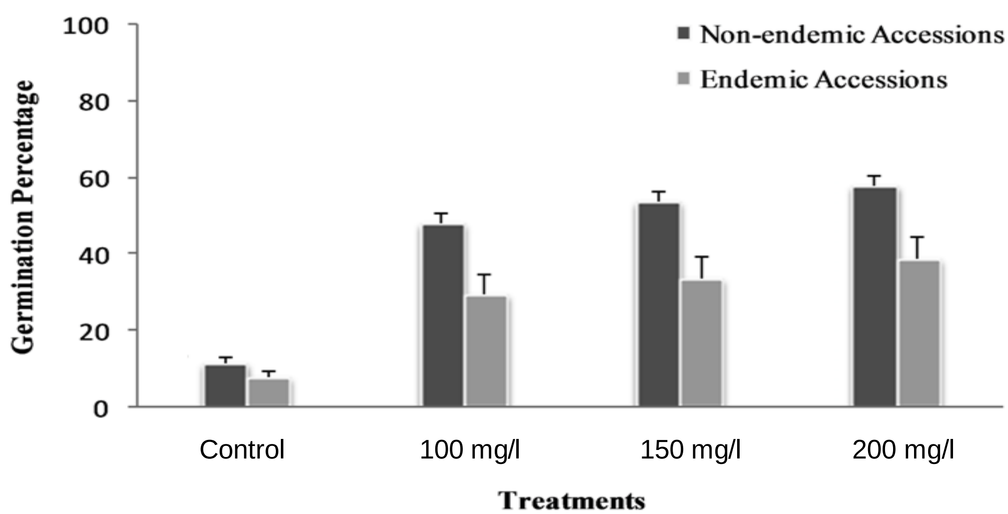

Figure 7. Germination percentages of endemic and non-endemic accessions were, separately, compared for four treatments of GA3 (0, 100, 150 and 200 mg/L). The orthogonal comparison showed that germination percentage of endemic accessions was, significantly, lower than non-endemic ones in all four treatments (α = 0.01).

(Maruta, 1994; Martin et al., 1995; Nishitani and Masuzawa, 1996). Results showed that in some species, germination behavior is, intensively, differing between regions (Figure 5). For example, the species of *S. sclarea* from Kordestan have been located in Cluster 1 while the samples of this species gathered from Yazd, Dena and Shiraz grouped in Cluster 2 and the samples of Lorestan and Khoram-Abad have entered into Clusters 3 and 4, respectively. It means that the location is very influential on this species and its accessions have acclimated and adapted to the growth area. On the other hand, the grouping of all accessions of *S. mirzayanii*, *S. hydragea*, *S. sharifii* and *S. multicaulis* in Cluster 4 and those of *S. imbata* and *S. macrosiphon* in Clusters 3 and 2, respectively, suggest that the environment in the evolution of these species had no effect or their environments had similar conditions. Further, researches are, certainly, needed to estimate the heritability of germination percentage and GA3 response. Different levels of GA3 have different effects on different accessions. For instance in Cluster 2, the strength of dormancy is low and the response to GA3 has been very high and there is no significant difference among treatment levels (Figure 4).

So, it seems that the usage of 100 mg/LGA3 for stimulating germination of these accessions is adequate while in accessions of Cluster 3 that possess high dormancy strength, the GA3 effect was less pronounced. Therefore, at least, 200 mg/L GA3 is necessary for their acceptable germination. For accessions with extreme dormancy in Cluster 4, it seems that they need other treatments. Results showed that the amounts of GA3 ranging from 100 to 200 mg/L were optimum and for industrial and scientific purposes, different doses in this range will be necessary for improving germination of majority of accessions. Among endemic species, *S. lachnocalyx* whose territory has been restricted for many years at a small cold region near Eghlid in Fars province and its extinction risk in near future is very high, although its germination percentage and rate were very low at control, but they increased, noticeably, by applying GA3. Despite being, intrinsically, endangered for germination problem, having narrow distribution makes it very susceptible to environment destruction. For *S. reuterana* that completely adopted to cold climates, the maximum germination percentage in one accession was zero and in other two accessions was less than 60% which does not seem acceptable and another treatments should be tested to improve its germination. The *S. sharifii* and *S. mirzayanii* that evolved to grow in arid and semi-arid warm in south of Iran regions with too disperse precipitation, did not germinate at all. Other researchers suggested that extreme environment strength of dormancy increased (Beckstead et al., 1996; Meyer et al., 1997; Meyer and Allen, 1999) and germination with extreme ‘opportunistic’ or ‘cautious’ strategies may be expanded (Gutterman, 2000). Based on the above conclusion it seems there is a sensible evolution in these species to survive against variable environment with delayed germination.

However seeds that remain dormant are subjected to increasing mortality, growth reduction and low or unsuccessful reproduction (Kevin and Andrew, 2001). *S. eremophila* as a desert species has evolved to germinate rapidly during the rainy period. Considering germination percentage in control treatment of all four accessions, it seems that the risk of their extinction due to germination problem is low compare to other endemic species. However, with application of GA3 the rate and germination percentage of its accessions, significantly, increased. Species that subsist in, extremely, specific territory often generate seeds with, exceedingly, specialized adaptations. Iranian endemic accessions had delay in germination. They were grouped in Clusters 2 to 4 whose common characteristics were their lack of ability to germinate in absent of germination stimulators. Most part of Iran has semi-arid climate with highly uneven distribution and dispersed precipitation. Most salvia species existed in natural resource gene bank of Iran has been collected from areas with low precipitation which categorized as arid and semi-arid areas. It can be deduced that endemic accessions have been more, specifically, evolved to these conditions than non-endemic ones, so that they refuse to germinate, easily, with a little or disperse precipitation and delay to germinate because this strategy helps them to keep their soil seed bank.

In wild plants, delays in germination decrease their competitiveness. It also slows the establishment of plants by increasing the probability of site occupation by other plants resulting in reduced seedling growth. This leads to slighter adult mass and the decrease of reproductive productivity (Abul-Fatih and Bazzaz, 1979; Miller, 1987; Ross and Harper, 1972). Consequently a significant decrease in competitive ability and ultimate plant vigor occurs due to germination delay. Thus, for expanding their growth area and their conservation, it is necessary to pay attention to their germination problem exclusively. Investigation on numerous species have proved that seed weight in most cases have significant effects on germination rate and percentage (Navarro and Guitián, 2003). Several studies have revealed that seed weight is a good predictor of different variables, including germination capability (Schaal, 1980; Stanton, 1985). Correlation of seed weight and AUGPC as a suitable criterion for indirect assay of seed dormancy was measured. The AUGPC is preferred to germination percentage at individual levels of GA3 because by using this variable, it is possible to consider the effect of this hormone on germinations, simultaneously, for all levels. In general, accessions with lighter seeds showed considerably superior germination than heavier seeds. It seems that some salvia accessions have evolved in a way that do not easily enter the germination phase due to the semi-arid climate of Iran and disperse precipitations. Since seed is alive, it was evolved to have more material stored to survive for a long time until germination. Likewise, accessions with germination delay evolved to have thicker seed coat. The thicker coat acts as a protection against soil organisms such as decomposers and rodents. The same is true with environmental factors like rain, wind and erosion for several years. Destruction of their natural habitats and industrialization, lack of preservation programs for natural ecosystems and their replacement by agricultural purposes have endangered the existence of these extremely dormant accessions.

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