Journal of Medicinal Plants and By-products (2020) 1: 79-89

Original Article

Effects of Seed Priming on Germination Characteristics of *Achillea millefolium* Seeds under Different Ageing Treatment

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Article History: Received: 24 September 2019/Accepted in revised form: 30 December 2019

Abstract

The genus *Achillea* L. (Compositae) widely use as medicinal plant possessing several pharmacological effects due to presence of active compounds. In order to study of seed priming effects on seedling growth of yarrow, a factorial experiment, based on randomized complete design with three replications was conducted under greenhouse conditions in Research Institute of Forests and Rangeland (RIFR) for one year. Experimental factors were a) three *Achillea millefolium* L. accessions originated from Kordestan (14303), Semnan (21602) and Gilan (27028), provinces, b) five conservation methods including: medium-term storage (Active Collection at 4 °C,10 or 15 years), long-term storage (Base Collection at -18 °C,10 or 15 years), regenerated seeds in open storage 22 °C for2 years (Control) and aged seed under accelerated ageing (40 °C,98% of relative humidity for 48 and 72h) and c) priming treatments including: non- priming (control), osmo-priming (PEG-0.3Mpa), hormonal priming (gibberellic acid at250 and 500ppm).Data collected for seed emergence percent, root and shoot length, seedling length, vigor index, seedling weight and three peroxidase, catalase and superoxide dismutase (SOD) enzymatic activities. Result of analysis of variance showed significant effects of all factors and their interactions for all of seedling traits and enzymatic activities except for SOD ($P<0.01$).Results also suggested significant differentiation among three accessions of *A. millefolium* for all germination traits and enzymes activities. Results showed that responses of accessions to aging and priming treatments were different. The data demonstrated that catalase is a key enzyme for seed repair against ageing ROS-induced damage during priming treatment.

Keywords: *Achillea*, Deterioration, Priming, Germination, Catalase.

Introduction

Iranian Natural Resource Gene Bank (NRGB) of Research Institute Forest and Rangelands conserves 22000 accessions from wild medicinal plant species. Seed banking is widely used for the *ex situ* conservation of wild plant species seeds now. Generally seed banks stores seeds of plants, according to the gene bank standards [1], whatever, there are no specific standards for the conservation of wild plant species seeds. Although some statistical equations developed to predict deterioration periods of crop species [2], there are less reports for determine the best times of regeneration of wild species in seed banks. FAO [1] recommended monitoring the seeds viability every 10 or 15 years for seeds in medium-or long-term storage, respectively. Storage of orthodox seeds for prolonged period induces their deterioration leading ultimately to loss of their viability. The rate of seed deterioration varies among plant species and seed lots, but higher moisture content and temperature accelerate this process [3,4]. The main theory of ageing is the 'free radical theory' proposed by Harman which postulates that the accumulation of

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damage caused by free radicals is the underlying mechanism by which all living organisms age [5, 6]. Numerous studies report the importance of free radicals in ageing in plant and animals and the importance of reactive oxygen species (ROS) in seed ageing has been shown in various species [4,7, 8]. Oxidative stress is defined as an imbalance between ROS production and antioxidant defense against these ROS. The consequence of oxidative stress is an increase in the formation of oxidized cellular macromolecules. To prevent oxidative damage to cellular components, cells are armed with various enzymatic and non-enzymatic mechanisms for detoxification. Among a number of protective enzymes, SOD remove superoxide anions ($O_2^{\bullet-}$) by catalyzing their conversion into hydrogen peroxide (H_2O_2), which in turn can be broken down by catalase to yield oxygen and water. Other antioxidant enzymes that participates in ROS removal is peroxidase. Observations made in different species show that oxidative damage increases with age in seeds [5,8,9] simultaneous with decreasing deficiency of cellular antioxidant defenses [4,7-10] lending strong support to the free radical theory of ageing. In this context, the importance of antioxidant enzymes has been shown in protection from ROS-induced stress of plant and animals [11-15]. In plants, catalase is considered as a primary enzymatic defense against oxidative stress induced by senescence, chilling, dehydration, osmotic stress, wounding, parquet, ozone and heavy metals [16,17].One of the simple techniques which can improve seedling vigor and establishment and consequently field performance of plants is seed priming [18].This method is useful particularly if the seeds have already aged during storage [19]. Interestingly, priming repairs damage of aged seeds [9,19] or seeds exposed to abiotic stresses such as salinity [20], improving germination performance. Priming treatment consists of soaking seeds in an osmotic of low water potential to control the amount of water supply to the seed. The priming-induced increase in the rate of seed germination has been associated with the initiation of germination-related processes [21,22], repair processes [23,24] and increase in various free radical scavenging enzymes, such as SOD, catalase and peroxidase have also been demonstrated [7,25,26].

The *Achillea* species as medicinal plants are used both for pharmaceutical purposes and in folk medicine. These plants contain a complex of different pharmacological compounds like terpenes, flavonoids, alkaloids, bitters, tannins, lignin, etc. [27]. *A. millefolium* subsp. *millefolium* is one of the species with medicinal and industrial valuable of this plant family is found in pastures [28]. This plant grows in areas of Iran and is one of the important medicinal plants used to in traditional medicine [29]. It has anti-inflammatory properties and is used for treat wounds and burns [30]. In Natural Resource Gene Bank of Iran exists about 150 *A. millefolium* accessions that were collected and saved during 25 years. One of main problem in maintenance of seed in gene bank is regeneration of aged seeds that lose their viability over times. For increasing of seed germination traits, it is necessary to apply some seed dormancy breaking and seed priming treatments. Therefore this research was conducted to study effects of different aging and priming treatments on seeds of three accessions of *A. millefolium*in greenhouse condition.

Material and Methods

Seed of three native accessions of *Achillea millefolium* included accession 14303 (Origin: Kordestan, latitude 35°38', longitude 47°07', Storage duration:15 years), accession 21602(Origin: Semnan, latitude 36°04', longitude 53°35', Storage duration:13 years) andaccession 27028 (Origin: Gilan, latitude 37°45', longitude 48°42',Storage duration: 10 years) were provided from Natural Resource Gene Bank (NRGB), Iran. A factorial experiment based on randomized complete design with three replications was conducted to evaluate the effects of naturally and accelerated aging on seed germination traits and enzyme activities for three *A. millefolium* accessions. Naturally aged seeds were provided from Base Collection (stored 15 years at -18 °C) and Active Collection (stored 15 years at 4 °C) of NRGB. A two years harvested seeds of those accessions were aged under moisture of 100% and temperature of 40 °C for 48 and 72 hours. The seeds were primed by incubation for 24 hours at 15 °C in solution of −0.3 MPa Polyethylene Glycol 6000 (PEG6000), gibberellic acid (250 and 500 ppm) for osmo- and hormone-priming respectively. Non-primed and hydro primed (imbibition with distilled water) seeds were used as control. The treated seeds were sown in 15 cm diameter plastic pots filled with sandy soil, and irrigated with tap water in greenhouse at 22 ± 3 °C. The pots were

maintained at field capacity. Data collected for traits including germination percentage, seed vigor index, shoot length (mm), root length (mm), seedling length (mm) and seedling fresh weight (mg) were evaluated at 50th days of experiment [31]. The seed emergence percentage was calculated according to total number of emerged seedlings in numbering final day [32].

Length of rootlet and shootlet: After 50th days of start of the experiment, length of rootlet (mm) and shootlet (mm) (that including five seedlings per pot on random) was measured according to [33].Vigor index was calculated according to Abdulbaki and Anderson [34] that their values obtained from follow equations:

$$Vi = \frac{\%Gr \times MSH}{100}$$

Where, %Gr = Final germination percentage, MSH = Mean seedling height, VI = Vigor Index

Enzyme Activities

Peroxidase

Peroxidase activity was assayed essentially according to the modified method of Shannon *et al*. [35]. The reaction mixture contained 0.1mL of enzyme extract, 2mL of 0.1mol/L Sodium-Acetate buffer (pH 4.5) and 0.5 mL of 20mMGuaicol solution (0.2% in Methanol, freshly prepared). The reaction was initiated with the addition of 0.1mL of0.2 mol/L H2O2. The change in absorbance was recorded at 470nm at an interval of 15 sec for 2 min. The enzyme activity was expressed as units (mg protein^{-1}).

Catalase

Catalase activity was estimated by the modified method of Aebi [36]. The reaction mixture contained 0.6 mL of enzyme extract, 0.1 mL of10 mmol/L H2O2 and 2mL of 30 mmol/L Potassium Phosphate buffer (pH 7.0). The absorbance was read at 240nm immediately after additionof the enzyme extract at an interval of 15 sec for 2 min. The blank was without enzyme extract.

Superoxide Dismutase (SOD)

The SOD enzyme activity was assayed by the modified method of Giannopolitis and Ries [37]. The reaction mixture contained 3mL of 0.1mol/L phosphate buffer (pH 7.8) containing 1.3 µmol/L Riboflavin, 13 mmol/L Methionine, 63 µmol/L Nitroblue Tetrazolium and 0.1mL of enzyme extract. SOD activity was assayed by measuring the ability of the enzyme extract to inhibit the photochemical reduction of NBT. Glass tubes containing the mixture were exposed to light (50 µEm^{-2} s^{-1}).Identical tubes, which were not illuminated, served as blanks. After illumination for 10 min, the tubes were covered with black cloth and absorbance was measured at 560 nm. Log A560 was plotted as a function of the volume of enzyme extract used in the reaction mixture. The volume of enzyme extract, corresponding to 50% inhibition of the photochemical reaction was obtained from the resultant graph. One unit of SOD was defined as the level of enzyme activity that inhibited the photo reduction of NBT to blue Formosan by 50% (expressed as units SOD mg protein^{-1}).

Data analyzed using SAS software and the differences between treatment means were tested using Duncan's Multiple Range Test [38].

Result and Discussion

Analysis of variance showed significant differences among accessions, aging and priming treatments for all traits (Table 1). The interaction between accessions, aging and priming methods was also significant (Table 1). In *A. millefolium* seeds, germination decreased significantly with ageing duration at 40 °C (Table 2). Effect of priming treatments (gibberellic acid at 250 and 500 ppm, PEG −0.3 MPa for hormone and osmo-priming) on seed traits and enzyme activities (peroxidase, catalase and SOD) was different in the three accessions (Table 3).

Naturally Aging

Mean comparison at two naturally aging methods (Base and Active Collections) showed that the highest seed germination traits, root length, vigor Index, shoot length, seedling length and seedling fresh weight were obtained in naturally aged seeds at -18 °C, (Base Collection) indicating the effect of low temperature in maintaining seed viability (Table 2). The seeds preserved in Base Collection with low humidity and temperature had low metabolic activity and causes it late deterioration. In contest, in Active Collection there was more traffic of staff and open/close the door and also repeated power fluctuations and humidity cause early seed deterioration. Similar to this research Rincker [39] showed that during the 20 years of storing 37 accessions of alfalfa seeds at (-15 °C)

with a relative humidity of 60%, germination decreased from 91 to 81%, whereas, this value reduced to 50% in open storage conditions during 10 years. Mean comparison of germination characteristics of three accessions showed that stored seeds of accession Kordestan at Base and Active Collections had higher germination percentage, seedling vigor index and shoot length in comparison to control seeds (Table 2). Fig. 1 showed the effects of priming treatments on seeds of different accessions, stored at Base and Active Collections. In general priming did not have a positive effect on seed germination percentage in all three accessions, as well as hormone-priming nor osmo-priming (Fig. 1 a-c). Priming led to enhanced seed aging and reduced seed germination percentage. However the results showed that priming of naturally aged seeds with gibberellic acid (250 ppm) and PEG (-0.3 MPa) had positive effect and efficiency on increasing seed vigor and seedling length (Fig. 1d-i). Undesirable effects of seeds priming with gibberellic acid (500 ppm) on the germination traits were obvious for all three accessions. Farooq *et al*. [40] reported higher effect of priming on root length in rice. Similar to our study, gibberellic acid was effective on recovery of deteriorated seeds of rapeseed [41].

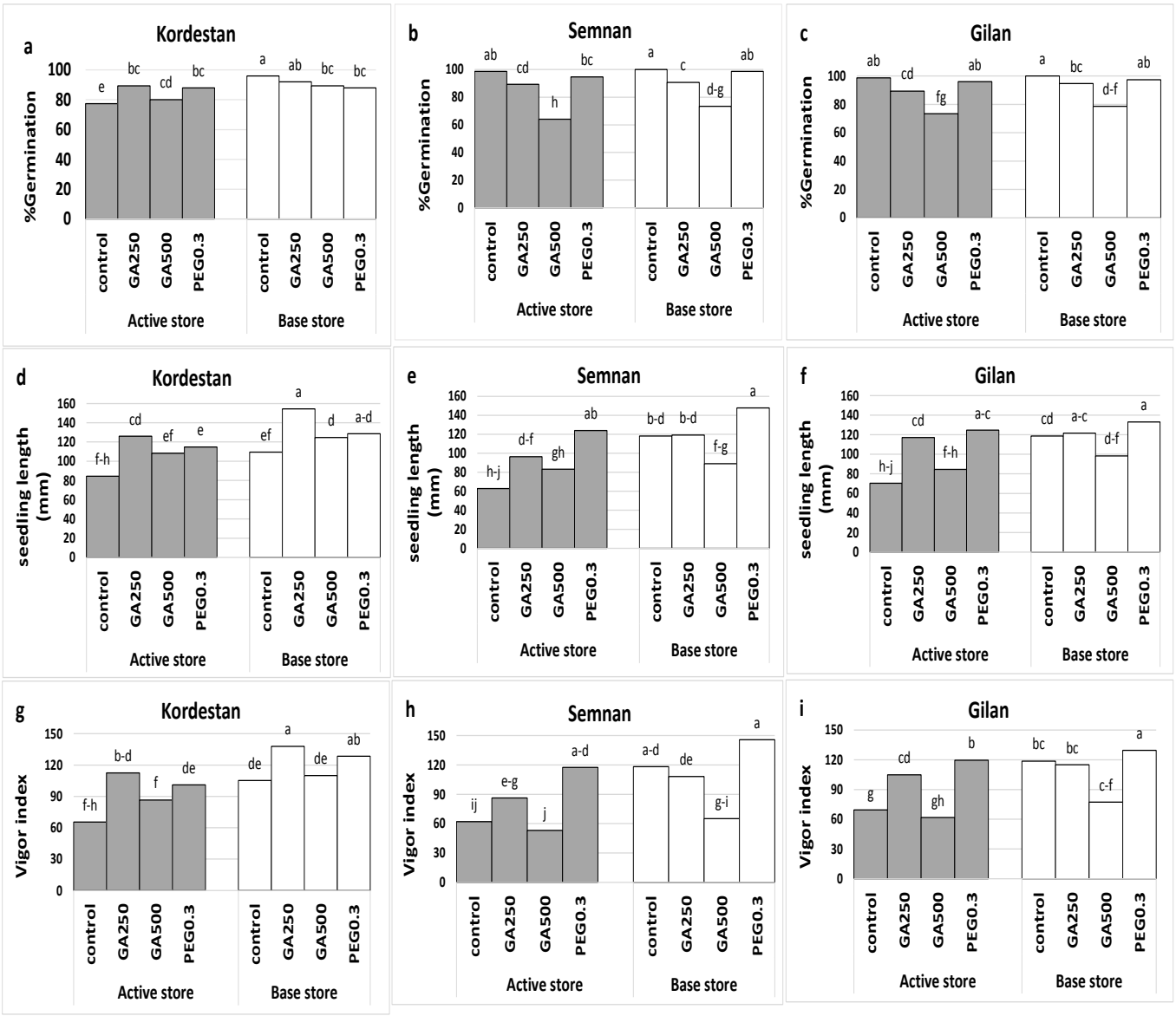

Fig. 1 Effect of priming (gibberellic acid 250 and 500ppm, PEG −0.3 MPa) on naturally aged seeds of different accessions in Active and Base Collections of NRGB.

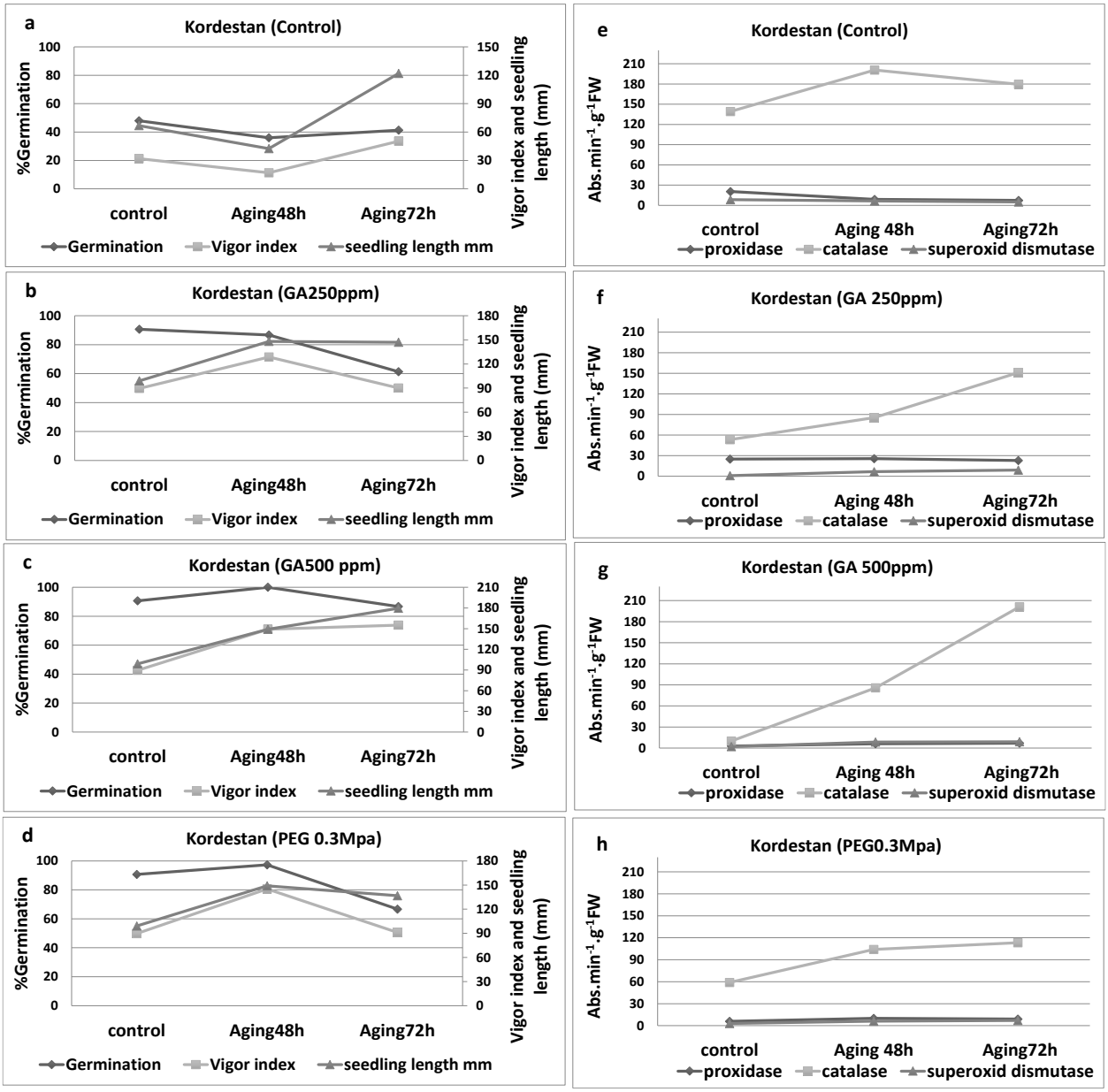

Fig. 2 Effects of accelerated ageing and priming on seeds germination percentage, Vigor Index and seed length (a-d) and enzyme activities (e-h) of accession Kordestan.

Sajjadi Jaghoroghi *et al*. [42] studied effect of osmo-priming, hydro-priming and pre-chilling on seed emergence enhancement and seedling vigor of four medicinal species of *Anthemis* under greenhouse conditions.

Their result showed that primed seeds with gibberellic acid and Potassium Nitrate (osmo-priming) improved germination potential and seedlings growth [42].

Accelerated Aging

According to results of variance analysis, effect of accelerated aging treatments on germination traits, were significant among different accessions ($P < 0.01$) (Table 1). In agreement with others results, earlier reports Bailly [43], Goel [44], McDonald [45] and Siad at [46] have shown negative effect of aging on germination characteristics. Our results showed that in all three accessions the highest germination percentage and vigor index were obtained under control condition (Table 3). Increase of aging duration reduced the germination percentage and vigor index, therefore the minimum amount of these traits was attained under 72 hours of aging (Table 3).

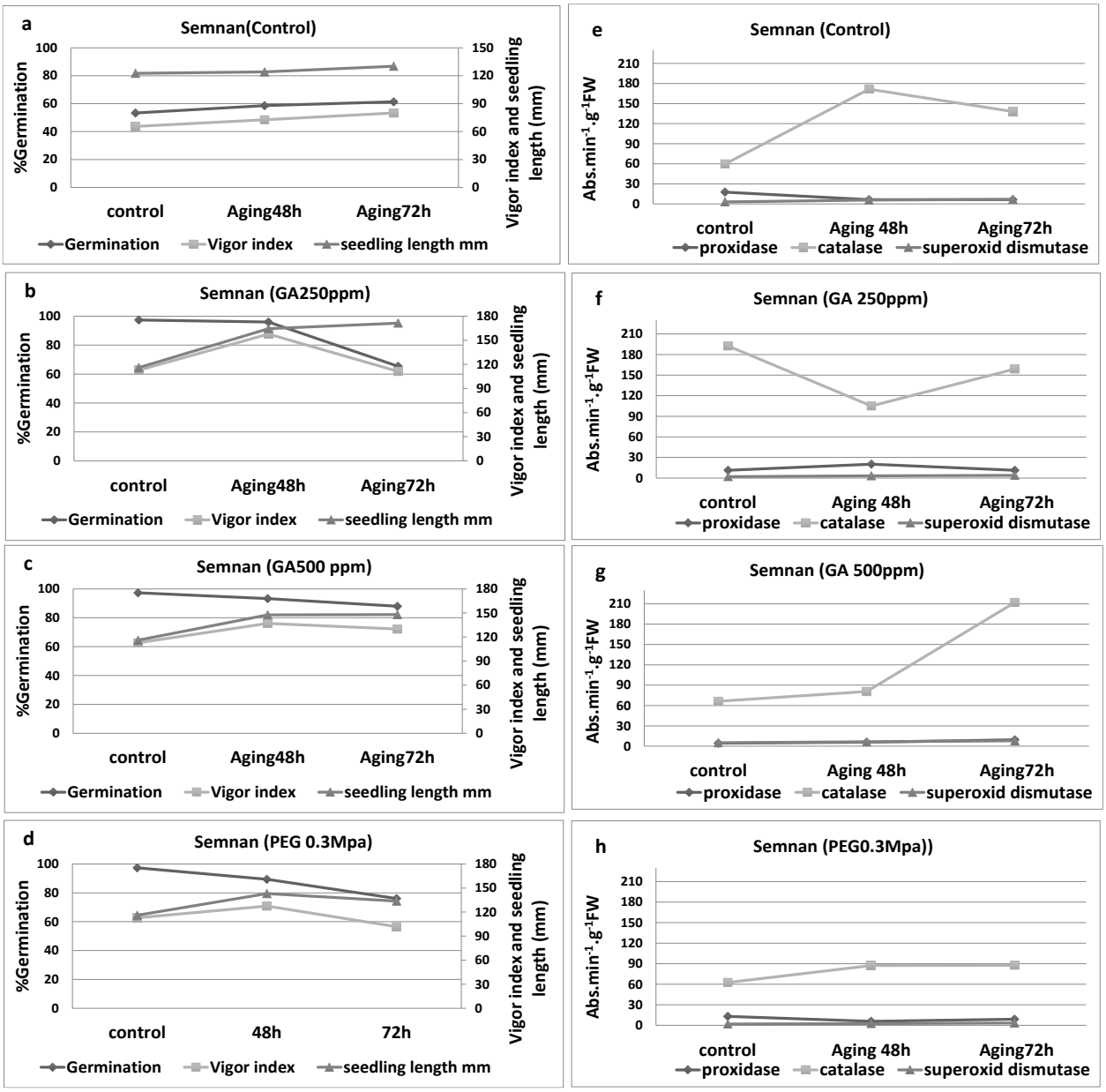

Fig. 3 Effects of accelerated ageing and priming on seeds germination percentage, Vigor Index and seed length (a-d) and enzyme activities (e-h) of accession Semnan.

This result is in accordance with reports of Alizadeh [47] in 17 medicinal species, Jan-Mohammadi *et al*. [48] and Ghassemi-Golezani *et al*. [49] in rapeseed, Bhattacharjee *et al*. [50] in common bean and sunflower and Saha and Sultana [51] in soybean. Also, earlier reports Bailly [43], Goel [44], McDonald [45], Siadat [46] Moradi and younesi [52] have shown negative effect of aging in relation to seed performance, germination percentage and seedling indices [53] suggested that decreasing in germination percentage was related to chromosomal aberrations that occur under long storage conditions.

According to Abdalla and Roberts [55] in barley Decreasing of germination percentage in aged seeds can be due to reduction of α-amylase activity and Carbohydrate contents or denaturation of proteins [43,54] and pea seeds treated with different combinations of accelerated aging treatment showed that the amount of genetic damage was solely a function of loss of viability. Effects of priming on aged seeds of studied accessions were significantly different (Fig 2-4 a-d).In accession Kordestan increasing duration of aging to 48 hours is improved germination by priming treatments (gibberellic acid at 250 ppm as hormone-priming, and PEG −0.3 Mpa as osmo-priming) (Fig. 2 a-d).

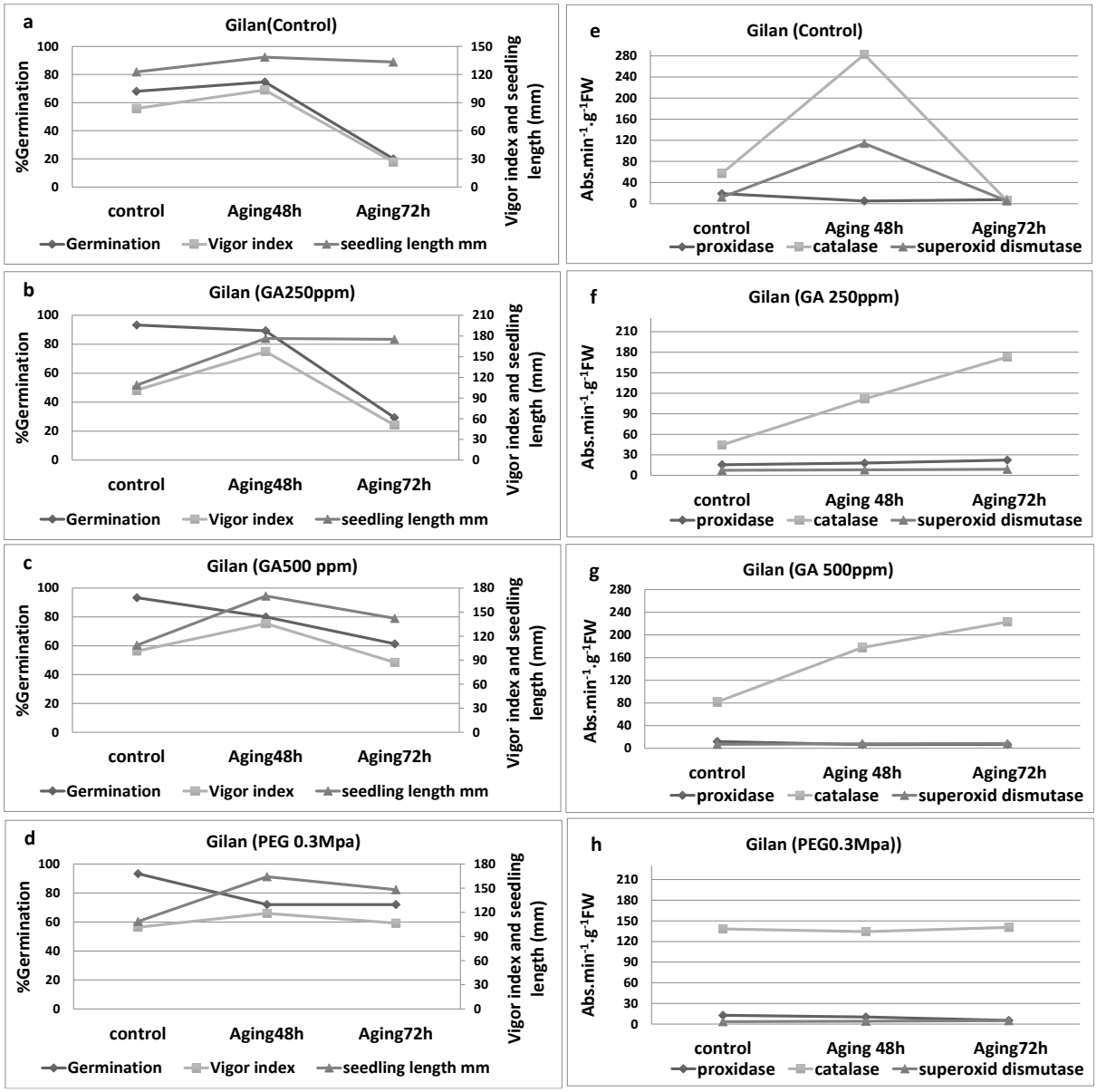

Fig. 4 Effects of accelerated ageing and priming on seeds germination percentage, vigor Index and seed length (a-d) and enzyme activities (e-h) of accession Gilan

In accessions Semnan and Gilan the osmo- and hormone-priming following the ageing treatment decreased germination of aged seeds whatever the duration of ageing (Fig 3, 4 a-d). However in accessions Semnan and Gilan increasing duration of aging to 48 hours improved the vigor index value (Fig 3, 4 a-d). In all three accessions priming following 48 and 72 hours aging had positive effect on seedling length (Fig. 2-4 a-d). This result was similar with result of [56], they studied effect of seed priming on the enhancement of germination traits of aged seed of *Achillea vermicularis* Trin. Trin Comparison of Peroxidase, Catalase and Superoxide Dismutase activities showed that ageing induced a considerable increase in Catalase activity. (Table 2 and Fig. 2-4 e-h). Hormone priming treatments considerably increase catalase activity of 72 hours aged seeds in accession Kordestan and Gilan (Fig. 2, 4 e-h), indicating that catalase is determinant enzyme in seed recovery and subsequent germination. This result is in accordance with Kibinza *et al*. [57] that reported the Catalase is a key enzyme in seed recovery from aging during priming. This result suggest, in accordance with Butler *et al*. [19] that priming repair is possible if accumulated damage is not irreversible. Irreversible damage at the cellular level was designated as the point of non-return which is the point when the cell becomes irreversibly committed to die [58].

Table 1 Analysis of variance and mean of squares of *Achillea millefolium* L. seeds traitsand enzyme activities (peroxidase, catalase and SOD) under greenhouse conditions.

Source of variation	df	Germination (%)	Vigor Index	Root Length	Shoot Length	Seedling Length	Fresh Weight	Peroxidase activity	Catalase activity	SOD activity
Accession (acc.)	2	829.44 **	3112.97 **	1921.69 **	384.41 **	2171.06 **	0.0039 **	81.41 **	4944.93 **	40.57 **
Aging	4	13871.53 **	10582.86 **	11786.22 **	6139.72 **	4410.80 **	0.0026 **	173.65 **	37867.08 **	57.97 **
Priming	4	6142.47 **	29904.23 **	27133.95 **	3427.60 **	47977.85 **	0.0104 **	689.83 **	10631.07 **	46.85 **
Acc. ×aging	8	549.88 **	3929.31 **	1768.17 **	237 **	3150.29 **	0.0007 **	12.28 **	5923.15 **	0.68 ns
Acc. ×priming	8	554.41 **	1606.08 **	679.39 **	184.08 **	1421.05 **	0.0014 **	85.06 **	8018.33 **	20.41 **
Aging× priming	16	4747.98 **	11230.67 **	8151.58 **	981.51 **	13976.88 **	0.0036 **	135.16 **	16743.94 **	2.12 **
Acc.× aging×priming	32	360.19 **	981.60 **	419.54 **	84.74 **	730.45 **	0.0008 **	51.86 **	3896.55 **	11.16 **
Error	150	24.24	102.54	107.39	15.8	175.64	0.00006	1.01	1.25	0.47
CV%		6.68	11.39	14	10.98	12.02	16.12	8.3	0.87	12.43

ns: no significant; **: significant at 0.01 level, respectively.

Table 2 Mean comparison of three accessions of *Achillea millefolium* L. seeds traits and enzyme activities (peroxidase, catalase and SOD) at naturally and accelerated aging treatments.

Accession	Aging		Germination (%)	Vigor Index	Root Length (mm)	Shoot length (mm)	Seedling length (mm)	Fresh weight (mg)	Peroxidase activity	Catalase activity	SOD activity
Kordestan	Naturally aging	-18 °C	93.07 a	116.04 bc	69.6 ij	55.23 a	124.83 fg	52.93 gh	-	-	-
		4 °C	83.73 cd	87.11 fg	58.67 kl	44.67 cd	103.33 ij	38.6 jk	-	-	-
	Accelerated aging	Control	61.67 gh	59.3 k	61.69 jk	34.6 fg	96.29 jk	34.5 kl	20.4 a	139.2 ef	8.4 a
		48 h	80 cd	118.46 b	109.29 cd	34.13 fg	143.42 d	45.75 hi	8.82 d	200.83 a-c	6.5 b
		72 h	64 e-g	96.67 ef	115.68 bc	30.7 g	146.37 cd	74.25 bc	7.34 de	179.43 de	5.07 bc
Semnan	Naturally aged	-18 °C	92.8 ab	108.69 cd	64.01 jk	52.38 ab	116.39 gh	59.8 g	-	-	-
		4 °C	88.8 b	86.77 fg	54.02 lm	43.4 d	97.42 jk	47.87 hi	-	-	-
	Accelerated aging	Control	85 bc	105.99 d	77.33 hi	47.5 c	124.83 fg	66.25 de	17.6 ab	59.73 hi	3.01 d
		48 h	79 d	126.9 a	122.17 a	40.17 e	162.33 a	64.75 ef	6.42 de	171.63 de	5.88 bc
		72 h	45.67 h-k	67.79 ij	114.53 bc	35.16 f	149.69 bc	86.33 a	6.43 de	138.03 ef	6.84 b
Gilan	Naturally aging	-18 °C	92 ab	108.67 cd	66.75 ij	49.93 bc	116.68 gh	52.2 gh	-	-	-
		4 °C	86.93 bc	79.91 gh	51.19 m	40.47 e	91.65 k	44.27 ij	-	-	-
	Accelerated aging	Control	83.2 cd	107.08 cd	89.7 e-h	38.81 ef	128.51 ef	54.27 gh	19 ab	57.03 hi	0.6 e
		48 h	84.33 c	123.78 ab	116.71 b	28.12 gh	144.83 cd	62.42 ef	4.96 e	282.63 a	5.47 bc
		72 h	72.67 de	105.74 d	118.15 ab	27.67 h	145.82 cd	66.83 de	12.34 c	114.1 fg	5.35 bc

Means with the same letter are not significantly different (P=0.01)

Table 3 Mean comparison of three accessions of *Achillea millefolium* L. seeds traits and enzyme activities (peroxidase, catalase and SOD)under priming treatments (at gibberlic acid 250 and 500ppm as hormone-priming, and PEG −0.3 MPa as osmo-priming).

Accession	Priming	Germination (%)	Vigor Index	Root Length (mm)	Shoot Length (mm)	Seedling Length (mm)	Fresh Weight (mg)	Peroxidase activity	Catalase activity	SOD activity
Kordestan	Control	88.67 b-e	86.76 hi	55.61 j	42 cd	97.61 j	47.33 fg	12.18 e	173.15 a	6.65 b
	GA_{250}	73.87 i	104.44 ef	92.08 bc	47.75 ab	139.82 b	61.13 d	24.55 a	96.62 hi	5.5 bc
	GA_{500}	88 b-e	125.22 a	100.5 a	39.92 de	140.42 ab	71.5 b	6.56 g	143.44 d	8.78 a
	$PEG_{-0.3}$	83.47 f	105.66 e	84.96 cd	39.97 de	124.93 b-e	37.6 i	8.31 fg	92.08 ij	5.42 bc
Semnan	Control	97.33 ab	96.51 fg	55.56 j	43.67 c	99.22 g-j	56.89 e	10.15 ef	123.13 f	5.24 bc
	GA_{250}	78.4 gh	107.92 b-e	95.54 b	47.49 ab	143.03 a	76.87 a	14.16 cd	152.12 cd	3.04 cd
	GA_{500}	73.33 i	88.54 hi	87.68 cd	36.1 ef	123.78 e	61.75 d	6.63 g	119.66 fg	6.5 b
	$PEG_{-0.3}$	85.33 e-f	120.34 b	92.96 bc	49.61 a	142.58 ab	74.07 ab	9.3 f	79.26 jk	2.56 d
Gilan	Control	98.67 a	97.69 fg	59.67 e-j	39.33 de	99 g-j	42.67 gh	12.1 e	151.25 cd	3.8 cd
	GA_{250}	86.93 e	121.25 ab	98.63 ab	42.29 cd	140.93 ab	62.93 cd	18.72 a-c	109.78 gh	8.2 a
	GA_{500}	81.6 fg	93.98 fg	81.2 de	31.18 g	112.38 e-g	51.93 f	8.52 fg	160.97 a-c	7.86 ab
	$PEG_{-0.3}$	88.27 b-e	124.37 ab	99.71 ab	41.37 cd	141.07 ab	68.13 bc	12.47 e	137.68 de	4.17 c

Means with the same letter are not significantly different (P=0.01)

Seeds with irreversibly damaged, whatever the ageing period, were not repaired by priming. The increase of half-cell reduction potential was shown in response to ageing in *Pisum sativum* seeds [8].

This increase was proposed to be related to a programmed cell death (PCD)/DNA fragmentation suggesting an active and genetically regulated cell death in response to ageing in seeds. Interestingly, Catalase was also associated to the reduction of hypersensitive response induction of PCD [59]. If PCD is the common way for seeds to lose their viability during ageing, Catalase involvement seems to be significant. Although the involvement of protective enzymes like SOD or Peroxidase is likely to be probable, our data demonstrated that Catalase is a key enzyme for seed repair against ageing ROS-induced damage during priming treatment.

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