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Imbibition-induced changes in cell membrane on germination and some physiological parameters in aged cress (Lepidium sativum L.) seeds

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Abstract: The deterioration of cellular membranes is the natural phenomenon in seeds exposed to aging. Physiological changes, such as accumulation of reactive oxygen species, mitochondrial dysfunction, and loss of essential metabolites, suppressed antioxidant defense system, and lipid peroxidation occur during the process of seed aging. In the present study, the physiological changes in aged cress seeds and seeds imbibed under different temperatures after aging were examined. In this regard, an array of analysis concerned with the cell membrane leakages and seed viability was performed. For comparison, accelerated aged seed lots with high, intermediate and low viability were used, in relation to the control (not subjected to short-term imbibitions). Accordingly, while the viability in control varied between 43% and 86%, the viability rates of the aged seeds ranged from 41% to 90%. Furthermore, imbibition increased the antioxidant capacity in aged seeds and reduced the lipid peroxidation level, and consequently, reduced the amount of K\(^{+}\), P\(^{3+}\), Ca\(^{2+}\), Na\(^{+}\) ions, soluble sugar, total protein and amino acids in the solute leakage. Hence, this method might be considered as simple, fast, cost-efficient, nondestructive and applicable in many seed lots at the same time.

Key words: Cellular membrane leakage, inorganic efflux, lipid peroxidation, membrane repair mechanism, reactive oxygen species, seed aging

1. Introduction
Seed aging, a natural phenomenon, is a continuous process which results in loss of seed viability and ultimately crop yield is comprised. The decreased seed viability was likely to be consequences of physiological and biochemical changes, which were then translated into or manifested as abnormal growth, delayed germination and seedling emergence. In particular, aging is defined as the disruption of macromolecule and membrane structure, and finally by late programmed cell death (El-Maarouf-Bouteau et al., 2011). In this regard, any attempt to reveal the physiological and biochemical responses against aging process has a great concern in order to reduce or minimize the possible damages. Furthermore, understanding the responses to the aging might contribute to our current practices in increasing the long-term availability of quality seed in the supply chain.

As specific responses to the aging, overaccumulation of reactive oxygen species (ROS), mitochondrial damage, lipid peroxidation, and changes in the antioxidant system are well-reported (Bailly, 2004; Lee et al., 2010; Li et al., 2017). As reported in a quite number of studies, ROS are produced by mitochondria, peroxisomes and chloroplasts during aerobic cell metabolic processes and participate in cell death (Penfield and King, 2009; Waypa et al., 2016). Especially, damage resulting from excessive production of ROS is likely to be the main cause of aging (Harman, 2006). It is important to note that ROS attacks to nucleic acids, lipids, and proteins when the ROS levels are not stabilized by the relevant defense system of the plant (Mittler, 2017) and the membrane integrity loss by the emerged physiological lesions through lipid peroxidation, enzyme inactivation and breakdown, genetic deterioration, and reduced respiration (Kibinza et al., 2006; Mira et al., 2011). The determination of cell membrane leakage is one of the methods that can be used effectively to monitor the aging and death process in seed lots (Kumar and Mishra, 2014; Demir et al., 2019). The leakage rate of intracellular substances depends on several factors such as specific compounds, membrane condition, and the presence or absence of morphological barriers (Taylor et al., 1993). In particular, a correlation between seed viability and seed leakage quantity was revealed, as reported in previous studies (Min and Hong, 2014; Demir et al., 2019). Although cell membrane leakage is of great physiological importance and is directly related to the aging mechanism,

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the mechanisms of cell membrane leakage are not well-understood in detail. Generally, the inorganic ions efflux by the seed into the solute leakage condition indicate a lower density of viable seed and high cellular component losses. Imbibition can act as a seed protective barrier to regulate or prevent leakage (Yu et al., 2015).

Seed imbibition is a critical step related to germination processes. The imbibition process is accompanied by various events such as activation of enzyme systems, initiation of active metabolism and solute leakage, and helps to repair and restore damaged membrane structural integrity (Simon and Mills, 1983). Membrane damage, especially in low-vigour seeds, is higher during maturation and drying and subsequently cell death may occur due to rapid water uptake. The increase in electrical conductivity of seed leachates is likely to be linked to the increased leakage of these electrolytes (Simon, 1984; Mir et al., 2021).

Temperature plays a role in many vital activities of the plant by sending environmental signals to the stages of the plant's life cycle, regulating seed dormancy and germination rate (Allen et al., 2007; Ucan and Ugur, 2021) and stand establishment (Batlla and Benech-Arnold, 2015; Huo and Bradford, 2015). Although alternating and constant temperatures have been reported to exhibit different impacts on plants (Kumar et al., 2013), alternating temperatures trigger higher germination rate and physiological balance to increase germination than constant temperatures (Liu et al., 2013). There are many studies about imbibition applied in seeds under alternating temperatures on the increase of viability and changes in enzymatic and hormonal balance (Ducic et al., 2003; Goggin et al., 2009; Duclos et al., 2014). In this study, we aimed to underpin how imbibition at alternating temperatures affects the leakage rate in aged seeds as well as its direct effects on seed viability. Furthermore, in this study, it was aimed to examine and interpret the physiological changes both directly and in the cell membrane leakage environment in imbibed and unimbibed seeds after aging. In addition, the main objective is to compare these results with seed viability and to find out the possible solutions. Along with the current study, we hypothesized that if some cellular functions (such as K⁺ concentration and antioxidant capacity) are improved, aging-mediated damage to cell membrane can be partially reduced.

2. Materials and methods

*Lepidium sativum* L. cv. 'Tere' (open-pollinated) fruits were harvested at the mature silica stage in the research area of Department of Horticulture, Agriculture Faculty, Iğdır University, Turkey. The fruits were mechanically sorted and left to dry at room temperature for 1 week after washing, and seed moisture content were determined with dry oven method (ISTA, 2017). Initial seed moisture content balanced 9%, and stored at 5 °C until use. Germination tests were performed on 3 replicates of 50 seeds using the ‘between-paper’ method. Seeds were incubated in the dark for 10 days at 20 °C (ISTA, 2017). Radicle emergence (radicle length > 2 mm) was evaluated for 10 days. We used cress seeds in our study, as they can be separated into different viability groups in a short time by aging tests and allow the ion efflux to the leakage media to be easily monitored compared to other species.

2.1 Accelerated aging (AA) test

The relevant test was carried out by adding 40 mL of distilled water to each plastic aging box (11 × 11 × 4 cm). Three-gram seeds were placed on a monolayer cheesecloth placed on a wire mesh tray (10 × 10 × 3 cm) inside the box (Hampton and TeKrony, 1995). Then, seeds were initially aged at 100% relative humidity at 41 °C. Following 24 h after aging period, the seeds were taken with 8 h intervals from 24 to 144 h in the dark in order to determine the low, intermediate and high seed viability. The relevant seeds were left for drying at ambient temperature for three hours.

2.2 Imbibition

For the imbibition treatments, 2.5 g seeds per group were soaked in 5 mL double distilled water at soaked 9 × 9 cm petri dishes at 5/15 °C – 1/1h and 5/25 °C – 1/1h and 5 °C constant at three different temperatures for 4 h (Table 1). The seeds were dispersed in the petri dish so that they were completely submerged from the surface level. After imbibition, the seeds were surface dried and seed moisture content balanced 9% (ISTA, 2017). Then the seeds were taken to suitable media to determine the viability test and the amount of ion efflux.

2.3 Physiological analysis of aged seeds

In addition to the quantifying the content of malondialdehyde (MDA) and hydrogen peroxide (H₂O₂), the activities of superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX) activities were determined in control and treated seeds.

2.3.1 Determination of lipid peroxidation

For quantification of MDA content in seeds, the thiobarbituric acid (TBA) test as an end product of lipid peroxidation was used (Heath and Packer, 1968). Seeds (0.5 g) were homogenized in 5 mL of 0.1% (w:v) TCA solution. The homogenate was centrifuged at 10,000 g for 20 min and 0.5 mL supernatant in 20% TCA was added to 1 ml 0.5% (w:v) TBA. The mixture was incubated for 30 min in boiling water and the reaction was stopped by placing in an ice bath. The samples were then centrifuged for 5 min at 10,000 g, and the supernatant absorbance was read at 532 nm. Value was subtracted for nonspecific absorption at 600 nm. The amount of MDA-TBA complex was calculated from the extinction coefficient of 155 mM⁻¹ cm⁻¹.
2.3.2 Determination of $H_2O_2$ content
For quantification of $H_2O_2$ content, 500 mg seeds were firstly homogenized in an ice bath with 5 mL of 0.1% (w:v) TCA. Then, the homogenate was centrifuged for 15 min at 10,000 g. After centrifuge, 0.5 mL supernatant was mixed with the 0.5 mL 10 mM potassium phosphate buffer (pH 7.0) including 1 mL 1M KI. After recording the absorbances at 390 nm, the relevant $H_2O_2$ content was quantified using standard curve (Sergiev et al., 1997).

2.3.3 Antioxidant enzyme activities
Enzyme extraction procedures were carried out at 4 ± 1 °C. For each treatment, 0.25 g seeds per sample were crushed in liquid nitrogen in a porcelain bowl. The seeds were homogenized with 5 mL of a cold solution of 0.1 M Na$_3$PO$_4$ (pH 7.5), 0.5 mM Na-EDTA and 1 mM ascorbic acid. The homogenized samples were centrifuged at 4 °C for 30 min at 18,000 g and then incubated at room temperature for 1 h. CAT activity was immediately determined from a portion of the homogenate, and the remaining extract was stored at −20 °C until APX and SOD analysis was conducted. SOD, CAT, and APX activity were determined according to Rahnama and Ebrahimzadeh (2005), Jebara et al. (2005) and Sairam and Saxena (2000), respectively.

2.4 Physiological analysis of solute leakages
2.4.1 Electrical conductivity (EC) test
Corresponding to EC measurements, 100 seeds for each replicate of experimental group were weighed and then soaked in 50 mL of distilled water for 24 h at 20 °C in the dark. The distilled water was used for soaking was kept at 20 °C overnight to equalize the temperature. The EC was measured using a conductivity meter (Schott-Gerate GmbH, Hofheim) and expressed as μS cm$^{-1}$ g$^{-1}$. After EC measurement, the leakages were used for quantifying ions (K$^+$, P$^{5+}$, Ca$^{2+}$, Na$^+$), total protein, total soluble sugar, aminoacids (proline, phenylalanine, glutamic acid, glycine). The remaining solute leakage from three different repetitions were combined, and filtered through crude filter paper to avoid solid residue and kept at −20 °C until used in analysis.

2.4.2 Inorganic solute leakages
K, P, Ca and Na ions efflux were measured by ICP-OES (Perkin Elmer, Optima 2100 DV) after suitable dilutions with slight modification (Kacar and Inal, 2008). In order to determine the amount of ions, 2 mL of solute leakages were taken from the flow water in 3 replications. After adding some deionized water and 2 mL of HNO$_3$ on the leakages, the final volume was completed with the help of deionized water to 50 mL. During reading, the device was set to 25 ± 0.1 °C and the ambient temperature to 25 ± 2 °C.

2.4.3 Total protein content
For total protein content quantification, solute leakage samples firstly were centrifuged at 10,000 g for 30 min at 4 °C. Following the centrifuge, the supernatant was used for protein content quantification according to the method (Bradford, 1976). This method is based on the protein binding of Coomassie Brilliant Blue G-250. The highest absorbance value of the formed complex was read at 595 nm.

2.4.4 Soluble sugars estimation
The sugar profiles were determined according to the method of Xu et al. (2014) with slight modifications. Briefly, the leakages were firstly centrifuged at 5000 g for
5 min at 4 °C in order to remove the residues. Following the centrifuge, the supernatants were filtrated through a PTFE 0.45-µm syringe filter. Then, they were injected into the HPLC system (Agilent 1260 infinity series) for analysis. The detector refractive index (RID) was used and the column was NH2 (250 × 4.6 mm) 5µm (Inertisil). The column temperature was set to 30 °C, and a 20-µL injection volume was used. The flow was isocratic, the flow rate was 1.0 mL/min, and the elution time was 20 min. Aceto-nitrile and distilled water (80:20; v:v) were used as mobile phases. The results were expressed as mg in g FW.

2.4.5 Amino acids composition
The free amino acid composition was determined with slight modifications to the method described by Aristoy and Toldra (1991) using HPLC. Single detector (UV) and Zorbax Eclipse-AAA (4.6 × 150mm), 3.5 µm (Agilent PN 963400-902) column HPLC (Agilent 1260 infinity series) were used to determine the free amino acid composition of the samples. OPA (ortho-phthalaldehyde) and FMOC (9-fluorenylmethyl chloroformate) were used as derivatization reagents for amino acids, and 0.4 N Borate (pH 10.2) was used as a buffer solution. As mobile phase in chromatography system; mobile phase A: 40 mM NaH2PO4 (pH 7.8) and mobile phase B: Acetonitrile (ACN): Methanol (MeOH): Water 45:45:10 v/v/v solutions were run. The mobile phase flow rate carried out in the system was set to 2 mL/min and the column temperature to 40 °C. After nitrogen-vacuum cycles, solute leakage samples were hydrolyzed, in glass tubes, in the presence of 300 µL of HCl (6 M) containing 1% (v/v) phenol, for 24 h at 120 °C. The amino acids content was quantified compared to the amino acid standards and the results were expressed in g of amino acid per 100 g amino acids. In the UV detector, the amino acids including glutamic acid, proline, phenylalanine and glycine were quantified.

2.5 Statistical analysis
The experimental groups were compared using one-way variance analysis coupled with Duncan’s multiple range test (p < 0.05) (Minitab 16). Arcsine transformation of germination rates was done prior to analysis. Principal component analysis and heatmap clustering were performed using OriginLab and ClustVis, respectively.

3. Results
The initial viability of the seeds were assayed through different aging periods, i.e. low, intermediate and high viability and control group, ranging between 43.00% and 86.00%. As the aging period prolonged, decreases in initial viability were observed. In relation to the control (C0: Direct), 64h0 (direct), 88h0 (direct) and 104h0 (direct) aging decreased the viability of the seeds by 12.79%, 27.91%, and 50.00%, respectively. Then, the aging process was followed by imbibition for 4 h for all groups. The germination rates of the seeds ranged between 41.00% and 90.00% (Table 2). In comparison to the control group, imbibition after aging treatments, except 104 h treatment, favored for germination rates.

Table 2. Effects of imbibition at different temperatures on seed viability and antioxidant enzyme activity in aged seeds.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Germination (%)</th>
<th>SOD (unite g⁻¹ FW⁻¹)</th>
<th>CAT (mmol g⁻¹ FW min⁻¹)</th>
<th>APX (mmol g⁻¹ FW min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C0</td>
<td>86 ± 1.15 b</td>
<td>8.95 ± 0.16 b</td>
<td>0.356 ± 0.007 ns</td>
<td>2.569 ± 0.05 b</td>
</tr>
<tr>
<td>C1</td>
<td>88 ± 0.58 ab</td>
<td>9.13 ± 0.18 b</td>
<td>0.384 ± 0.024 ns</td>
<td>3.465 ± 0.11 a</td>
</tr>
<tr>
<td>C2</td>
<td>88 ± 1.20 ab</td>
<td>8.81 ± 0.11 b</td>
<td>0.341 ± 0.008 ns</td>
<td>3.612 ± 0.03 a</td>
</tr>
<tr>
<td>C3</td>
<td>90 ± 0.58 a</td>
<td>11.54 ± 0.43 a</td>
<td>0.377 ± 0.005 ns</td>
<td>3.641 ± 0.08 a</td>
</tr>
<tr>
<td>64h0</td>
<td>75 ± 1.15 d</td>
<td>14.15 ± 0.55 c</td>
<td>0.410 ± 0.006 d</td>
<td>2.897 ± 0.09 c</td>
</tr>
<tr>
<td>64h1</td>
<td>78 ± 0.58 c</td>
<td>17.62 ± 0.29 b</td>
<td>0.545 ± 0.010 b</td>
<td>3.685 ± 0.06 ab</td>
</tr>
<tr>
<td>64h2</td>
<td>82 ± 1.53 b</td>
<td>17.99 ± 0.31 b</td>
<td>0.499 ± 0.004 c</td>
<td>3.532 ± 0.02 b</td>
</tr>
<tr>
<td>64h3</td>
<td>85 ± 0.58 a</td>
<td>20.28 ± 0.23 a</td>
<td>0.574 ± 0.008 a</td>
<td>3.788 ± 0.07 a</td>
</tr>
<tr>
<td>88h0</td>
<td>62 ± 1.00 c</td>
<td>13.65 ± 0.39 c</td>
<td>0.525 ± 0.010 c</td>
<td>3.324 ± 0.01 b</td>
</tr>
<tr>
<td>88h1</td>
<td>69 ± 1.53 b</td>
<td>14.89 ± 0.31 b</td>
<td>0.551 ± 0.012 bc</td>
<td>3.697 ± 0.07 a</td>
</tr>
<tr>
<td>88h2</td>
<td>70 ± 0.58 ab</td>
<td>13.77 ± 0.13 c</td>
<td>0.590 ± 0.006 a</td>
<td>3.643 ± 0.03 a</td>
</tr>
<tr>
<td>88h3</td>
<td>73 ± 1.15 a</td>
<td>20.50 ± 0.34 a</td>
<td>0.578 ± 0.005 ab</td>
<td>3.578 ± 0.02 a</td>
</tr>
<tr>
<td>104h0</td>
<td>43 ± 1.15 ns</td>
<td>18.51 ± 0.30 bc</td>
<td>0.712 ± 0.010 b</td>
<td>3.082 ± 0.05 ns</td>
</tr>
<tr>
<td>104h1</td>
<td>42 ± 0.58 ns</td>
<td>19.63 ± 0.61 ab</td>
<td>0.779 ± 0.007 a</td>
<td>3.090 ± 0.05 ns</td>
</tr>
<tr>
<td>104h2</td>
<td>41 ± 1.00 ns</td>
<td>18.17 ± 0.17 c</td>
<td>0.795 ± 0.013 a</td>
<td>3.022 ± 0.01 ns</td>
</tr>
<tr>
<td>104h3</td>
<td>44 ± 1.15 ns</td>
<td>19.81 ± 0.19 a</td>
<td>0.781 ± 0.005 a</td>
<td>3.125 ± 0.03 ns</td>
</tr>
</tbody>
</table>

Means with different letters in the same column denote significant difference at p < 0.05. The error bars represent ± SEM. ns: nonsignificant.
Following 64h and 88h aging, 5/25 °C – 1/1h imbibition treatments was found to be most effective treatments. The imbibition increased germination rate by 13.33% after 64h, whilst it increased the relevant rate of germination by 17.74% after 88h aging. After a 10-day germination processes, in accordance with the germination rates, enhanced seedling development was observed with the imbibition (Figure 1).

Considering the relevant antioxidant enzymes, the antioxidant enzyme activities increased as the aging period prolonged. The highest SOD activities were observed at 5/25 °C – 1/1h imbibition conditions for all groups. No significant changes in CAT activities were observed in relevant control groups, whilst increased activities of the enzyme were noted for imbibed seeds. APX activities increased in control groups whereas no significant differences in enzyme activities were observed at 104h group. However, as the similar case of the other enzymes, APX activities increased at 64h and 88h groups (Table 2).

It was observed that MDA contents increased proportionally with the aging time in comparison to the control group. When the MDA contents of the groups were examined, it was seen that it started to decrease in the 64h and 88h aging groups, especially in imbibition applications at alternating temperatures. In the 104h aging groups, the highest MDA content was determined in the seeds imbibed at constant temperature (Figure 2A).

With the aging period, H₂O₂ and MDA content increased but the imbibition decreased H₂O₂ content in control group and 88h aging. In particular, in relation to the control (C0) and constant temperature, alternating temperatures after 64h aging significantly affected H₂O₂ content. There was no significant difference between the activities at 104h aging conditions (Figure 2B).

The amount of ion leakage increased as the aging period prolonged. In control, as well 64h and 88h aging, 5/25 °C – 1/1h imbibition significantly decreased the EC level. Nonsignificant differences were observed at 104h aging conditions. Of the quantified ions in solute leakages, K⁺ increased with the aging period, while imbibition decreased the continuous K⁺ efflux. It was determined that 5/25 °C – 1/1h imbibition was the most effective in reducing K⁺ efflux after aging (Table 3).

Considering the amount of P³⁺ ion leakage, it was observed that imbibition from control to 88h aging conditions decreases the P³⁺ efflux but as the case of K⁺ efflux, imbibition of 5/25 °C – 1/1h after aging reduced the P³⁺ efflux. There were no statistically significant differences between P³⁺ efflux amounts under 104h aging conditions. While there was no statistically significant difference in the amount of Ca²⁺ efflux under control conditions, it was determined that the 64h and 88h imbibition after aging decreased the Ca²⁺ efflux statistically. Generally, the lowest Ca²⁺ efflux in all aging conditions was found at 5/25 °C – 1/1h imbibition treatment. Also, it was observed that imbibition after aging significantly decreased the amount of Na⁺ efflux (Table 3).

Total protein content in the solute leakage increased as the aging period prolonged. While there was no statistically significant difference in the control group, imbibition

![Figure 1](https://example.com/figure1.png)

Figure 1. Effects of imbibition at different temperatures after aging (64h, 88h and 104h) and Control group on seedling growth
reduced the total amount of protein efflux into the leakage medium under all aging conditions. Especially at 64h, it was seen that 5/25 °C – 1/1h imbibition was effective in decreasing protein efflux (Figure 3A). Fructose, glucose and sucrose contents were determined and the total amount of these three sugars was calculated as the total sugar. As the aging time increased, the amount of fructose, glucose and total sugar in the leakage medium continuously increased (Figures 3B–3D). Of those sugars, in control conditions, only glucose amount was statistically significant (Figure 3D).

It was determined that the amount of fructose efflux into the solute leakage medium decreased with imbibition under 64h and 88h aging conditions, and the highest fructose efflux was observed at 5/15 °C – 1/1h imbibition since the seeds lost their viability in 104h aging conditions (Figure 3C).

Similarly, imbibition reduced the amount of glucose efflux into the solute leakage under 64h and 88h aging conditions, while highest glucose efflux was determined at 5/15 °C – 1/1h imbibition under 104h aging conditions (Figure 3D). In comparison with control, imbibition

![Figure 2](image-url)

**Figure 2.** Effects of imbibition at different temperatures on MDA (A) and H₂O₂ (B) content in aged seeds. Different letters indicate significant differences between treatments in the same aging period according to Duncan's multiple range test at \( p < 0.05 \). ns: non-significant.

**Table 3.** Changes in EC and inorganic ions efflux measured in the solute leakage medium.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>EC (μS cm⁻¹ g⁻¹)</th>
<th>K⁺ (mg L⁻¹)</th>
<th>P³⁺ (mg L⁻¹)</th>
<th>Ca²⁺ (mg L⁻¹)</th>
<th>Na⁺ (mg L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C0</td>
<td>418.5 ± 4.1 a</td>
<td>131.6 ± 1.9 a</td>
<td>39.6 ± 2.3 a</td>
<td>1.2 ± 0.10 ns</td>
<td>0.9 ± 0.06 a</td>
</tr>
<tr>
<td>C1</td>
<td>365.6 ± 10.3 c</td>
<td>89.7 ± 5.1 c</td>
<td>31.2 ± 2.8 b</td>
<td>1.2 ± 0.06 ns</td>
<td>0.7 ± 0.10 ab</td>
</tr>
<tr>
<td>C2</td>
<td>386.3 ± 2.3 b</td>
<td>103.8 ± 3.2 b</td>
<td>26.7 ± 0.9 b</td>
<td>1.5 ± 0.10 ns</td>
<td>0.7 ± 0.06 ab</td>
</tr>
<tr>
<td>C3</td>
<td>352.4 ± 2.8 c</td>
<td>95.1 ± 1.2 bc</td>
<td>25.3 ± 1.8 b</td>
<td>1.2 ± 0.12 ns</td>
<td>0.5 ± 0.10 b</td>
</tr>
<tr>
<td>64h0</td>
<td>469.4 ± 4.1 ab</td>
<td>227.0 ± 2.9 a</td>
<td>89.5 ± 3.4 a</td>
<td>5.5 ± 0.12 a</td>
<td>2.0 ± 0.06 a</td>
</tr>
<tr>
<td>64h1</td>
<td>474.7 ± 4.1 a</td>
<td>169.5 ± 2.5 b</td>
<td>85.1 ± 2.7 ab</td>
<td>3.9 ± 0.12 b</td>
<td>1.4 ± 0.06 b</td>
</tr>
<tr>
<td>64h2</td>
<td>463.2 ± 10.8 b</td>
<td>163.1 ± 2.7 b</td>
<td>80.1 ± 1.3 bc</td>
<td>2.8 ± 0.10 c</td>
<td>1.6 ± 0.10 b</td>
</tr>
<tr>
<td>64h3</td>
<td>442.5 ± 3.3 c</td>
<td>144.4 ± 5.9 c</td>
<td>75.2 ± 1.2 c</td>
<td>1.9 ± 0.10 d</td>
<td>1.1 ± 0.06 c</td>
</tr>
<tr>
<td>88h0</td>
<td>513.1 ± 7.4 a</td>
<td>319.6 ± 2.1 a</td>
<td>106.6 ± 3.7 a</td>
<td>8.6 ± 0.12 a</td>
<td>3.2 ± 0.15 a</td>
</tr>
<tr>
<td>88h1</td>
<td>492.6 ± 9.0 ab</td>
<td>302.5 ± 4.6 b</td>
<td>95.9 ± 2.5 ab</td>
<td>8.1 ± 0.10 b</td>
<td>2.1 ± 0.12 b</td>
</tr>
<tr>
<td>88h2</td>
<td>496.7 ± 7.5 a</td>
<td>286.2 ± 2.8 c</td>
<td>99.7 ± 3.5 a</td>
<td>6.4 ± 0.06 c</td>
<td>1.7 ± 0.10 c</td>
</tr>
<tr>
<td>88h3</td>
<td>471.8 ± 2.5 b</td>
<td>251.2 ± 4.7 d</td>
<td>86.4 ± 3.0 b</td>
<td>5.3 ± 0.12 d</td>
<td>1.8 ± 0.06 bc</td>
</tr>
<tr>
<td>104h0</td>
<td>587.5 ± 9.1 ns</td>
<td>418.3 ± 2.0 a</td>
<td>121.1 ± 2.8 ns</td>
<td>9.6 ± 0.12 a</td>
<td>4.7 ± 0.06 a</td>
</tr>
<tr>
<td>104h1</td>
<td>580.1 ± 5.7 ns</td>
<td>410.2 ± 4.5 ab</td>
<td>124.3 ± 3.3 ns</td>
<td>9.4 ± 0.06 a</td>
<td>4.4 ± 0.12 b</td>
</tr>
<tr>
<td>104h2</td>
<td>594.0 ± 3.1 ns</td>
<td>399.8 ± 1.6 bc</td>
<td>115.9 ± 1.9 ns</td>
<td>9.4 ± 0.10 a</td>
<td>4.4 ± 0.06 b</td>
</tr>
<tr>
<td>104h3</td>
<td>585.8 ± 4.1 ns</td>
<td>389.4 ± 7.1 c</td>
<td>118.6 ± 1.6 ns</td>
<td>9.1 ± 0.06 b</td>
<td>4.1 ± 0.06 c</td>
</tr>
</tbody>
</table>

Means with different letters in the same column denote significant difference at \( p < 0.05 \). The error bars represent ± SEM. ns: nonsignificant.
treatments after 88h and 104h aging conditions did not affect the amount of sucrose efflux into the medium but imbibition at alternating temperatures after 64h aging conditions significantly decreased the amount of sucrose efflux into the medium (Figure 3E). Especially at 64h and 88h aging conditions where viability rates are high, it was determined that the total amount of sugar efflux into the solute leakage medium decreased significantly after imbibition (Figure 3B).

Regarding the amount of amino acid efflux of imbibition after aging, it was observed that especially the amount of proline efflux increased with aging but imbibition after aging decreased the amount of leakage (Figure 4A). After aging period, phenylalanine amino acid efflux increased in comparison with the control but only imbibition after 64h aging significantly decreased the phenylalanine amino acid efflux (Figure 4B).

Aging did not affect the glutamic acid enough, which was the most abundant amino acid in the leakage. Concerning imbibition treatments, only imbibition after 88h aging significantly reduced the efflux and it was observed that 5/25 °C – 1/1h imbibition decreased the most amount of glutamic acid efflux (Figure 4C).

Similar to glutamic acid, it was observed that similar amounts of glycine amino acid efflux were found in aging conditions compared to control. While it was determined that imbibition at alternating temperatures under control conditions increased the amount of leakage, it was determined that this situation reversed under 88h aging conditions. There was no statistically significant difference in 64h and 104h aging conditions (Figure 4D).

Principal component analysis (PCA) and heat map clustering

Due to high number of variables, we, herein, constructed relevant heat maps in order to visualize, clarify and then associate the findings corresponding to the experimental groups. Clustering of heat map suggested two major groups. The first cluster included 88h0, 104h2, 104h3, 104h0, and 104h1, being related to increases in MDA, H₂O₂ (88h1), CAT (except 88h0, 88h1), whilst second major cluster but with two subgroups included experimental groups of 88h1, C1, C3, 88h3, 64h2, 64h1, 64h3, C2, 88h2, C, and 64h0. The second cluster was correlated with increases in activities of APX and germination, in general but increases in SOD activities in 88h3, 64h2, 64h1, and 64h3, in particular were observed (Figure 5A).

Furthermore, the experimental data and groups were subjected to PCA analysis. Two principal components PC₁ (69.80%) and PC₂ (14.57%) with an eigenvalues of
greater than 1.0 explained 84.37% of the variability of the original data. According to the extracted Eigenvectors, CAT, MDA and H₂O₂ with coefficients of greater than 0.4 were included in PC1. Furthermore, a reverse correlation was observed between germination and variables of PC1.

We also constructed heat map and performed PCA for the metabolites and ions released from leakages (Figure 5B). Heat map clustering suggested two major groups. The first cluster was clearly based on experimental groups of 104h1, 104h3, 104h0, and 104h2, with substantial lower values of glycine and glutamic acid. In the second principal component, PC2, explaining 8.28% of the total variation, glycine had the highest eigenvector.

4. Discussion
Seed aging often results in reduced viability (Gupta and Aneja, 2004), delayed germination (Demir et al., 2019), increased ROS (Demidchik et al., 2014), and increased cellular metabolite leakage (Min and Hong, 2014). As expected, the current findings negatively affected these properties by the aging process in cress seeds. For example, high MDA and H₂O₂ content were coupled with low seed viability corresponding to the prolongation of aging. Furthermore, the aging triggered higher activities of antioxidant enzymes. This situation can be explained by the increase in lipid peroxidation in seed with the aging (Bailly et al., 1996; Goel and Sheoran, 2003). The relevant increases are manifested as reduced-seed viability. Similarly, higher antioxidant status alleviated to lipid peroxidation in the aged seeds of plants in general (Bailly, 2004) and in cress seeds, in particular (Demir et al., 2019). Antioxidant enzymes such as SOD, CAT and APX well-known for their roles in defense mechanism

Figure 4. Changes Proline (A), Phenylalanine (B), Glutamic acid (C) and Glysine (D) efflux measured in the solute leakage medium. Different letters indicate significant differences between treatments in the same aging period, according to Duncan’s multiple range test at p < 0.05. ns: non-significant.
Figure 5. PCA and heat maps for stress indicators and enzymes (A), metabolite and ions released from leakages (B) corresponding to the experimental groups in aged cress seeds

(Bailly, 2004; Apel and Hirt, 2004; Qi et al., 2017), which is consistent with the current findings. Those responses might be attributed to the aging period and the relevant temperature of the imbibition. Especially after 64h and 88h aging, imbibition had more effect on seed viability, and 5/25 °C – 1/1h imbibition treatment was the best treatment. In addition, it was observed that imbibition at alternating temperatures after those aging periods lowered lipid peroxidation and increased antioxidant activity. Following imbibition, an array of physiological alterations, especially enzymatic activity, is induced for germination (Simon, 1984). Corresponding to the induced-responses, the imbibition process helps the repair and restoration of the damaged structural integrity through contact between seed and water in its medium (Simon, 1984; Bewley and Black, 1986). The amount of water used during imbibition, imbibition time and plant species are of great importance (Marler, 2019). Similar to our study findings, Lin et al. (2019) stated that imbibition decreased in redox potential, H$_2$O$_2$ content, and MDA content, thus significantly decreasing the degree of membrane lipid peroxidation, and also decreasing the amount of EC leakage in *Brassica napus* L.

In the study, EC and specific ion amounts in the solute leakage medium decreased with the imbibition treatments after control, 64h and 88h aging. No notable changes in EC and specific ion amounts were observed after 104 h. Increases in the EC of solutes leaking from the seed during aging might be the indicator of deterioration of cell membrane structure (Coolbear, 1995), meaning that the more ion leakage results in high levels of cell membrane disintegrity. The imbibition after aging decreased K$^+$, P$^{3+}$, Ca$^{2+}$ and Na$^+$ ion effluxes. Of the treatments, 5/25 °C – 1/1h imbibition was the most effective in reducing the relevant ions. Potassium is the main ion leached from seeds, followed by P$^{3+}$, Na$^+$ and Ca$^{2+}$ ions can be used as a pointer of cell membrane integrity (Halloin, 1975; Dias et al., 1996). Min and Hong (2014) reported that in aged and
unaged radish and Chinese cabbage seeds the K\(^+\) efflux rate is much greater than other minerals, followed by P\(^{3+}\), N\(^{4+}\), Ca\(^{2+}\) leakage amounts in the seeds. Also, De Souza et al. (2020) reported that water loss in corn leaves with stress caused a change in membrane permeability and an increase in the amount of electrolyte leakage, and an increase in the activities of antioxidative enzymes. These responses have been regarded as protective strategies of the plants against stress. Ion loss in plant tissues can reveal the permeability level of the cell membrane and may be associated with the production of ROS, which can damage macromolecules and cell structures (Demidchik et al., 2014).

Viability of the seeds have been correlated with EC, in general (Thornton et al., 1990; Demir et al., 2019; Ozden et al., 2021) and K\(^+\) efflux, in particular (Marcos-Filho et al., 1982). ROS formation has been hypothesized as the consequence of higher level of K\(^+\) leakage in plants under stress conditions (Demidchik et al., 2014). Plants exposed to water efflux show higher production of O\(_2\)\(^-\) and H\(_2\)O\(_2\). These compounds cause high levels of lipid peroxidation, damage to cell membranes and increased electrolyte leakage (Pereira et al., 2019). In this study, it is thought that increased SOD, CAT and APX antioxidant enzyme activities suppressed free radicals, suggesting that imbibition can be used effectively at alternating temperatures in this respect.

Demirkaya and Sivritepe (2011) reported that the total amount of protein in onion seeds decreased with aging. The decline in total protein content in seeds results in increase in solute leakage. The findings of the current study revealed that imbibition decreased the content of leaked proteins. In the current report, an increase in the leakage of relevant sugars were observed with the aging. However, Rahoui et al. (2010) reported that increased duration of Cd and H\(_2\)O\(_2\) stress caused an increment in amount of total sugar, fructose and glucose efflux. Herewith the study, imbibition led to decline in the amount of total sugar, fructose and glucose leakages, especially under 64h and 88h aging conditions. The same effect was achieved in sucrose, a disaccharide, under 64h aging conditions. It is worth noting that imbibition exhibited similar effects on the total protein and the total sugar, fructose and glucose leakage rates in aged seeds.

With the increases in leakage of amino acids, the decline in germination was reported (Min et al., 2013). As the case of specific amino acids, proline efflux was buffered with imbibition but no significant changes were observed for phenylalanine, glutamic acid and glycine except imbibition treatments after 88 h. In particular, proline did not differ under control conditions as the aging period extended, the amount of efflux increased with the aging but imbibition decreased the leakage. As of the most abundant amino acids, proline protects the plants from various stresses and besides helps plants to recover faster from stress (Hayat et al., 2012), participating in the detoxification of free O\(_2\)\(^-\) radicals in plants (Matysik et al., 2002).

In accordance with findings of MDA and H\(_2\)O\(_2\) content and antioxidant enzyme activity in the aged seeds, proline content in leakage decreased. Solute leakage from nonviable and deteriorated seeds of different species include proteins, sugars, free amino acids and phenolics (Samad and Pearce, 1978; McKersie and Stinson, 1980; Duke et al., 1983; Min et al., 2013).

Integrity of the cell membrane is considered to be one of the fundamental physiological events of the seed degradation process (Matthews and Powell, 2006). As a result, lower viable or vigor seed lots lose higher density cellular components such as more inorganic ions from the cell membrane (Kim et al., 2011). Since seeds begin to lose their germination ability over time during storage, regular seed viability tests are mandatory for both seed companies and seed banks. For this reason, several tests such as AA test, EC test, determination of the amount of ions efflux are used. However, there are not enough alternative solutions (fast, cheap and easy to apply) to preserve viability in seeds that aged and started to lose their viability for this reason. The present findings revealed that short-term imbibition can be easily applied, especially on cress seeds with high and intermediate viability levels.

5. Conclusion
To the best of our knowledge, the current research is of the first report regarding physiological changes that occur along with seed aging and postaging solute leakage medium, and imbibition. It was clearly observed that imbibition after aging the seeds changed the efflux rate of many solutes in leakage. The imbibition increased the antioxidant capacity in aged seeds and decreased the lipid peroxidation level, and consequently, the amount of K\(^+\), P\(^{3+}\), Ca\(^{2+}\), Na\(^+\) ions, soluble sugar, total protein and amino acids in the solute leakage medium decreased. For that reason, the current findings suggest that this method might be considered as simple, fast, cost-efficient and nondestructive.

References


