The effect of ageing on antioxidant and biochemical changes in wheat (*Triticum aestivum* L.) seeds

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Abstract

This research was carried out to study the effect of ageing on antioxidant and biochemical changes of wheat (*Triticum aestivum* L.) seeds. The experiment laid out in completely randomized design (CRD) with four replications in Islamic Azad University, Boroujerd Branch, Boroujerd, Iran in 2015. The seeds of wheat (cv Sardari) were harvested at maturity and ageing treatments were done at 43° C with 100% relative humidity for 1, 2, 3, 4, and 5 days. After ageing treatments enzymatic antioxidants, non-enzymatic antioxidants, and biochemical characteristics were measured. The results showed that ageing treatment decreased germination percentage. However, electrical conductivity, malondialdehyde content, and H_2O_2 were increased as the ageing progressed. Soluble sugars and proteins were decreased with an increase in ageing levels, but soluble protein in control treatment was less than that in days 1 and 2 of ageing. Non-enzymatic antioxidants such as ascorbic acid and proline increased until days 1 and 2 of ageing treatments, respectively while they decreased afterwards. Moreover, enzymatic antioxidants such as catalase, peroxidase and ascorbate peroxidase increased until 1, 2 and 3 days of seed ageing decreasing afterwards. The results of this research showed that increasing of hydrogen peroxide under ageing led to increasing of seeds damage. Also, under ageing treatment, enzymatic antioxidants were more efficient than non-enzymatic antioxidants in removing reactive oxygen species.

Key words: catalase (CAT); germination; metabolism; proline; Reactive Oxygen Species

Abbreviations: MDA: Malondialdehyde; CAT: Catalase; POX: Peroxidase; APX: Ascorbate Peroxidase; ROS: Reactive Oxygen Species

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Introduction

Progress of technology and industrialization of agricultural production increased opportunities for long term seed storage. Seed deterioration and storage has marked effects on seed viability and vigor. Resolution of this problem must begin in the field during seed production, and it should be continued after the harvest. However, healthy seeds are able to produce new plants under favorite conditions. Seed deterioration depends on the temperature, seed moisture content (MC), and duration of storage (Priestley, 1986). Silva et al (2005) showed that during storage, many physiological and physicochemical changes occur

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which are generally termed ageing. The main external reasons causing seed damage during storage are the temperature, relative air humidity, and oxygen. In addition, water is the important factor for chemical reactions in seeds (Basra, 1984).

Some researchers suggested that ROS play physiological roles in seed germination and act as signaling molecules (Bailly, 2004). The organelles such as Chloroplasts, Mitochondria and Proxies zoom or ultra high-speed electron flow are the major sources of ROS in plant cells (Giannopolitis et al., 1977; Gill et al., 2010 and Grant et al., 2000). In seed physiology ROS are generally regarded as toxic molecules resulting in the accumulation of cell damage and impaired developmental processes of germination. However, important role of these compounds are also showed by many researchers (McDonald, 1999 and Moller, 2002). Recently, it has been found that plants actively produce ROS and there may be many different physiological processes such as biological stress response, non-biological defense against disease, and signal to control systemic formation (Gill et al., 2010). There are evidences that ROS play a key role in seed germination and cell wall loosing during seed growth (Liszkay et al., 2004 and Luck, 1962). Bailly (2004) showed that seed germination and post-germination seedling development are well-regulated process in plant physiology involving high metabolic activity and generation of reactive oxygen species in the plant cells. ROS play a dual role in seed physiology, displaying two major functions: as a kind of Cytotoxin and as a special role in seed development, dormancy breakage, and in defense against biotic and abiotic stresses (Apel and Hirt, 2004).

ROS defense network is composed of enzymatic and non-enzymatic antioxidants and ROS-producing enzymes are responsible for maintaining ROS levels under tight control. In plant cells, antioxidant enzymes such as superoxide dismutase (SOD), peroxidase (POX), and catalase (CAT) are considered to form a defensive team whose combined purpose is to protect cells from oxidative damage (Blokhina et al., 2003). Moreover, Goel and Sheoran (2003) stated that malondialdehyde (MDA) is considered as a sensitive marker commonly used for assessing membrane lipid peroxidation. However, during seed ageing some non-enzymatic antioxidants such as proline, ascorbic acid, and enzymatic antioxidants like CAT, POX, and APX have been demonstrated to scavenge ROS as H_2O_2 produced by interacting under oxidative stress (Yin et al., 2014). CAT reduces H_2O_2 to water and dioxygen, APX reduces H_2O_2 to water and generates MDA (Noctor and Foyer, 1998). Bailly et al. (1996) reported that activities of antioxidant enzymes have been observed to decrease in aged soybean seeds. Therefore, this work was undertaken to determine the changes in antioxidant system and biochemical traits of wheat seeds during accelerated ageing.

Material and Methods

This research was carried out in order to study the effect of seed ageing on antioxidant system and biochemical characteristics of wheat seeds in Young Researchers and Elite Club, Islamic Azad University, Boroujerd Branch, Boroujerd, Iran in 2015. The experiment was laid out in completely randomized design with four replications. Seeds were harvested at maturity stage and ageing treatments on wheat seeds (cv. Sardari) were done in 43° C at 100% relative humidity for 1, 2, 3, 4, and 5 days. After ageing treatments, the germination test and electrical conductivity were performed as standard (ISTA, 2012).

For electrical conductivity test, solute leakage of the seeds was estimated by soaking 10 g seeds in 250 mL of deionized water at 20° C in an incubator. The experiment was replicated 4 times. The electrical conductivity of seed leachates was measured by conductivity meter (Jenway-4010) after 24 h soaking.

Biochemical assay such as hydrogen peroxide, soluble sugar, soluble protein, MDA, proline, ascorbic acid, and three antioxidant enzymes such as catalase (CAT), proxidase (POX), and ascorbate peroxidase (APX) were carried out. For extract hydrogen peroxide 0.2 g of seed samples with 3 ml Trichloro acetic acid 0.1% were homogenized in a porcelain mortar and centrifuged at 15,000 rpm for 15 min at 4° C and the resulting extract was used to measure hydrogen peroxide. Active measurement was laid out by the method of Jiazdwska et al. (2010).

MDA content was determined by the Thiobarbituric acid (TBA) reaction (Bailly et al., 1996). Seed samples were homogenized with 0.1% Trichloroacetic acid (TCA) (m/v, 1/10) and the homogenates were centrifuged at 15000×g for 15 min. Then 1.0 mL of the supernatant, 3.0 mL of 0.5% TBA in 5% TCA were added. The mixture was heated at 95° C for 30 min and then cooled immediately in an ice bath. The reaction mixture was centrifuged at 15000×g for 10 min and the observance of the supernatant was recorded at 532 nm and 600 nm. Lipid peroxidation was expressed as MDA content in nM per gram fresh weight, by using an extinction coefficient of 155 mM^{-1} cm^{-1}.

Soluble sugars were extracted according to Omokolo et al. (1996). 60 mg of isolated cotyledon was ground at room temperature in a mortar with 5 ml 80% aqueous ethanol and kept at 70° C for 10 minutes then centrifuged for 10 min in 1000g for 5 times. This was maintained for 5 minutes at room temperature and centrifuged again. The five supernatants were combined and reduced to dryness. Absorption was measured at 620 nm.

Soluble proteins were estimated using the bovine serum albumin as standard. Extraction of protein from sample was carried out using Tris-HCl buffer (pH 7.8) and the supernatant was used for the estimation and the absorbance was measured at 660 nm (Sadasavim et al., 1966).

For measuring proline content, seeds (500 mg) were ground using a mortal and pistil, homogenized in 10 ml 3% Sulfosalicylic acid, and then centrifuged at 12000 rpm for 5 min. The supernatant (200 µl) was mixed with 200 µl glacial acetic acid and 200 µl Acidic Ninhydrin, and then mixed well. The mixture was incubated at 100° C for 60 min. The reaction was terminated by placing the mixture on ice, and then extracting the sample with 1.0 ml toluene. The absorbance of the mixture was measured at 520 nm. To prepare the standard curve, an L-proline stock solution (100 µg/ml) was diluted to 2, 4, 6, 8, 10, and 12 µg/ml.

Ascorbic acid content was assayed according to Kampfenkel et al. (1995). Samples of ten seeds were taken for analyses. The extraction was performed in 6% TCA (w/v) in an ice bath. The assay is based on the reduction of Fe^{3+}–Fe^{2+} by ASA in an acidic solution. Fe^{2+} forms complexes with bipyridyl, giving a pink color with the maximum absorbance at 720 nm.

For the catalase (CAT) assay, soluble proteins were extracted by homogenizing 1 g (fresh weight) powdered sample in 3 ml of 100 mM mono sodium phosphate buffer (pH 7.5) containing 0.1% Triton X-100. Other materials for determination of CAT activity were 54 mM mono sodium phosphate buffer (pH 6.8) and 450 mM H_2O_2. CAT activity was measured following the change in the absorbance of the reaction mixture at 240 nm (Philippe et al., 2007). The assay was detected in 3 ml reaction mixture containing 2.85 mL 54 mM mono sodium phosphate buffer (pH 6.8), 100 µL H_2O_2 (450 mM), and 50 µl of crude extract.

POX activity was measured by Sakharov and Aridilla (1999) method with slight modifications. 3 ml mixture consisting of 100 µl of base solution guaiacol, 100 µl H_2O_2 (1.2 M), and 200 µl enzyme extract and the changes of absorbance at 470 nm were measured using a UV/vis spectrophotometer. One unit of POX activity was expressed as 1.0 change in absorbance per minute.

Total APX was measured based on the method of Nakano and Asada (1981) with slight modifications in 1 ml total volume. The reaction mixtures included 25 µl ascorbic acid (0.03%), 25 µl EDTA (0.1%), 50 µl H_2O_2 (0.015%), 800 µl , 312.5 mM sodium phosphate buffer (pH 7.0), and 100 µl protein extract. The changes of absorbance were read at 290 nm using a UV/vis spectrophotometer for 3 minutes.

The statistical analyses were conducted using JMP 5.0.1.2 (SAS Institute Inc., 2002). Statistical significance was declared at $P \leq 0.05$ and $P \leq 0.01$.

Results

The germination percentage of wheat seeds decreased with increasing of seed ageing duration. Differences were not significant between both control and 1, 2, and 3 days ageing treatments and germination percentage in 1day of ageing treatment (95%) was higher than control treatment (92%). Minimum germination percentage was recorded in day 5 of ageing treatment (11%) (Fig. I).

Results showed that there were significant differences between treatments for electrolyte leakage (EC). With increasing of ageing levels, EC was increased. Minimum (39 $ds.m^{-1}$) and maximum (72 $ds.m^{-1}$) EC were recorded for control and 5 days ageing treatments, respectively. However, differences between 2, 3, and 4 days of ageing treatments were not significant (Fig. II).

In the ageing treatments of wheat seeds, the MDA production rate increased significantly ($P < 0.05$) with increasing of ageing duration. The MDA production rate in control treatment was minimal (31 nM.g FW^{-1}). However, for 5 days aging treated seeds the MDA production rate was maximum (92 nM.g FW^{-1}) (F.g. III).

Reactive oxygen species such as hydrogen peroxide (H_2O_2) can increase during seed germination. In this study there was very intensive generation of hydrogen peroxide as ageing increased. The H_2O_2 content of untreated control seeds was 50 $nM.g^{-1}$ FW^{-1}. It remained close to this early value, ranging from 100 to 350 $nM.g^{-1}$ FW^{-1}, during ageing at 43° C and 100% RH (Fig. IV). When seeds aged at 43° C and 100% RH, the H_2O_2 content increased significantly. In day 1 of ageing treatment the minimum value of 110 $nM.g^{-1}$ FW^{-1} was observed which was then increased regularly up to 350 $nM.g^{-1}$ FW on day 5 of ageing (Fig. IV).

Figure (V) shows the changes in soluble sugar contents in wheat seeds excised from seeds aged for various treatments at 43° C and 100% RH. In untreated control seeds, soluble sugars were 190 $mg.g^{-1}$ FW^{-1}. During ageing, the soluble sugar content decreased, ranging from 180 to 90 $mg.g^{-1}$ FW^{-1}. Soluble sugar content decreased between 1 day and five days of ageing treatment. Minimum (90 $mg.g^{-1}$ FW^{-1}) and maximum (180 $mg.g^{-1}$ FW^{-1}) soluble sugar was recorded for 1 and 5 days ageing treatments, respectively (Fig. V).

In the present study in untreated control seeds, soluble protein was 42 $mg.g^{-1}$ FW^{-1}. Minimum (61 $mg.g^{-1}$ FW^{-1}) and maximum (24 $mg.g^{-1}$ FW^{-1}) soluble protein were recorded for 1 and 5 days ageing treatments, respectively. During ageing at 43° C and 100% RH, the soluble protein of the seeds decreased, ranging from around 61 to 24 $mg.g^{-1}$ FW^{-1}. (Fig. VI).

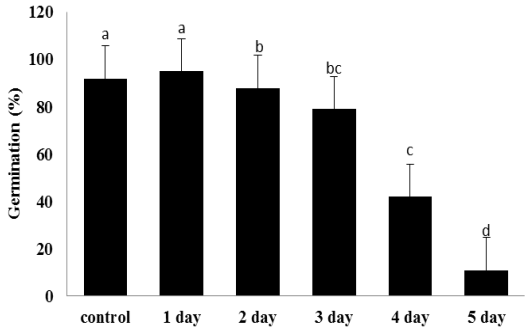

Fig. I. Effect of ageing on germination percentage in wheat seeds

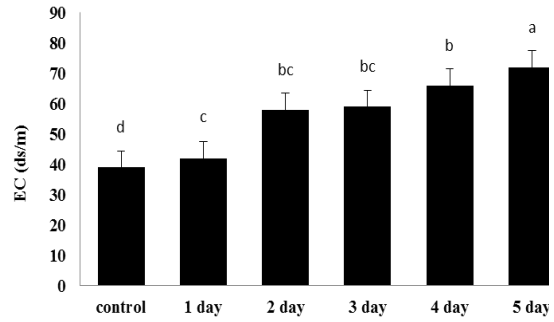

Fig. II. Effect of ageing on EC in wheat seeds

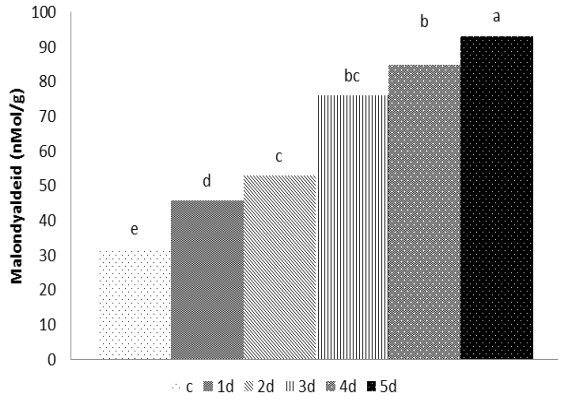

Fig. III. Effect of ageing on MDA content of wheat seeds

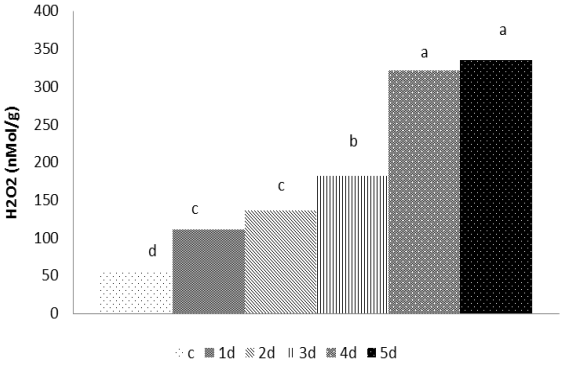

Fig. IV. Effect of ageing on H_2O_2 content of wheat seeds

The proline content in 1 and 2 days ageing

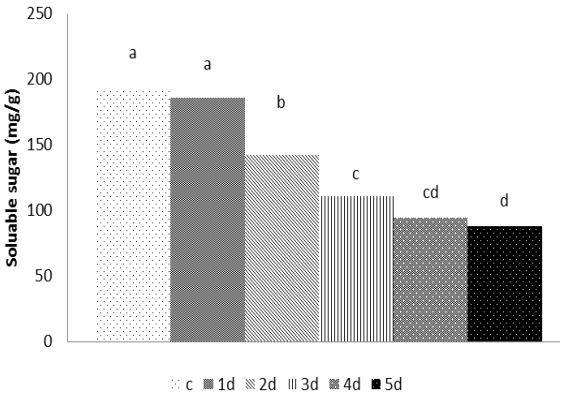

Fig. V. Effect of ageing on soluble sugar of wheat seeds

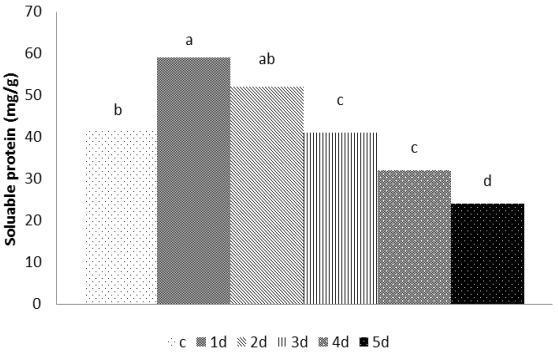

Fig. VI. Effect of ageing on soluble protein of wheat seeds

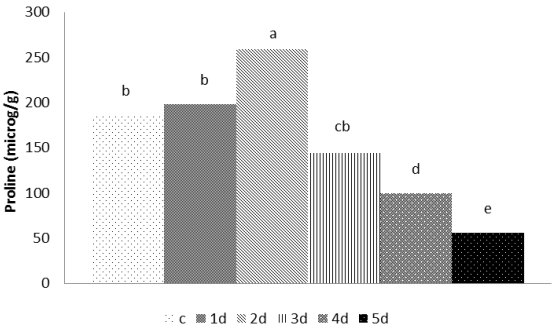

Fig. VII. Effect of ageing on proline of wheat seeds

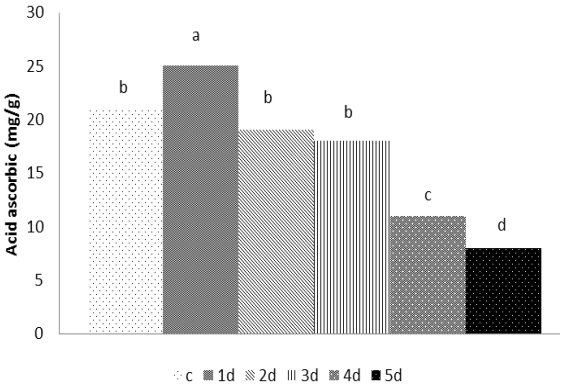

Fig. VIII. Effect of ageing on ascorbic acid in wheat seeds

treatments was higher than control. Maximum proline content was obtained in 2 days ageing treatment (258 µg.g^{-1} FW^{-1}). From 2 days until 5 days ageing treatments proline content decreased significantly so that minimum proline content was obtained in 5 days ageing treatment (50 µg.g^{-1} FW^{-1}). In control treatment, the proline contents of seeds were significantly ($P < 0.05$) lower than 2 days ageing treatment (Fig. VII).

Among low-molecular antioxidants ascorbic acid (ASA) and proline were assayed. ASA as compound directly involved in the Halliwell–Asada pathway plays an important role in the removal of ROS from cells (Asada, 1992). The changes of ascorbic acid (ASA) during wheat seed ageing under above conditions are presented in Fig. (VIII). In the wheat seeds, after 1 day ageing treatment, a slight increase was seen in the ASA content and this treatment had the highest rate of ASA accumulation (25 mg.g^{-1} FW^{-1}). ASA level returned to the initial level after 2 days ageing and even slightly decreased with increasing of ageing days and had significantly lower values in 5 days ageing treatment (8 mg.g^{-1} FW^{-1}) (Fig. VIII).

Enzymes activity for removing ROS from cells was studied after ageing treatment. Among the enzymes of Halliwell–Asada pathway, the activity of APX was assayed. Also the activities of other enzymatic scavengers of ROS, like POX and CAT were assayed. The activities of all enzymes were measured 3 days after incubation at 43° C and 100% RH. This was because it was assumed that humidity level of seeds returned to initial humidity after ageing treatment.

Accelerated ageing at 43° C and 100% RH induced an increase from 1900 to 4300 nM.mn^{-1}.g^{-1} FW^{-1} in control and 3 days ageing treatment, respectively.

Maximum CAT activity was found in 3 days ageing treatment. From 4 to 5 ageing day treatment CAT activity strongly decreased, so that minimum CAT activity was recorded for 5 ageing days treatment (260 nM.mn^{-1}.g^{-1} FW^{-1}). However, 3 and 2 ageing days treatment had the higher CAT activity than others (3600 nM.mn^{-1}.g^{-1} FW^{-1}) (Fig. IX).

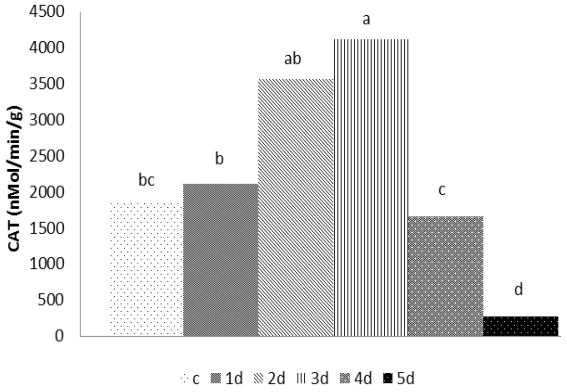

Fig. IX. Effect of ageing on CAT activity of wheat seeds

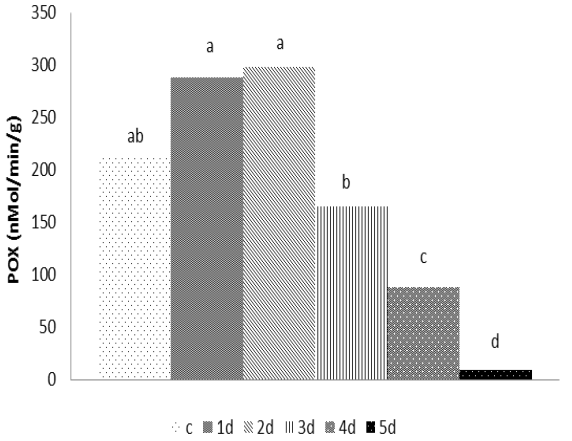

Fig. X. Effect of ageing on POX activity of wheat seeds

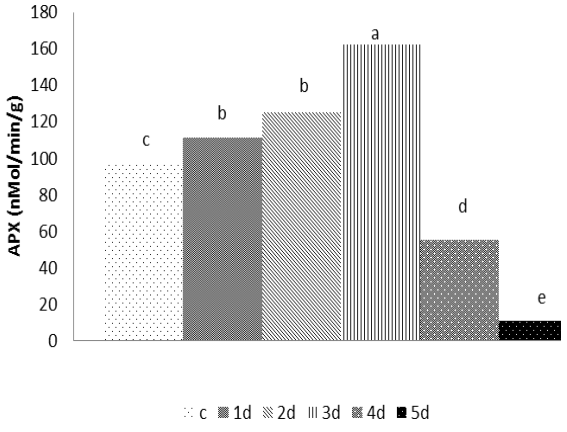

Fig. XI. Effect of ageing on APX activity ofwheat seeds

In untreated control seeds, the POX activity was 210 nM.min^{-1}.g^{-1} FW^{-1}. Accelerated ageing at 43° C and 100% RH induced an increase from 210 to 300 nM.min^{-1}.g^{-1} FW^{-1} in POX activity during 1 and 2 days of ageing and then decreased to 170, 105 and 11 nM.min^{-1}.g^{-1} FW^{-1} in 3, 4, and 5 days ageing, respectively. Maximum POX activity

was found in 2 days ageing treatment (300 $nMol.min^{-1}.g^{-1}$ FW^{-1} at 43C and 100% RH) (Fig. X).

Enzyme of the Halliwell–Asada pathway (APX) (Fig. XI) is clearly involved in scavenging of the excess of ROS from wheat seeds after their ageing. This is reflected in their increased activity in relation to the control very soon and after only 1-3 days of incubation, and a further decrease after the next 4-5 days of ageing treatment. However, APX activity increased with an increase in ageing levels until 3 days. Maximum APX was recorded on day 3 of ageing treatment (65 $nM.mn^{-1}.g^{-1}$ FW^{-1}) and minimum APX activity was recorded for day 5 of ageing treatment (9 $nM.mn^{-1}.g^{-1}$ FW^{-1}) (Fig. XI).

Discussion

Results of the present study showed that germination percentage of wheat seeds during ageing treatments decreased significantly (Fig. I). In 1 day ageing treatment the germination percentage was higher than control but the difference was not significant. In 2, 3, 4, and 5 days ageing treatments germination percentage strongly decreased. These findings suggested that ageing treatments decreased longevity and germinability of wheat seeds. Increasing of seed humidity content during accelerated ageing treatment affects seed longevity (Mira et al., 2015). Increasing seed germination in 1day ageing treatment compared to control is because of activation of early seed germination enzymes at high temperature and humidity. Similar results have been reported for seeds of many species under hermetic conditions (Ellis et al., 1988; Zhang et al., 2010).

The result showed that electrical conductivity increased with increasing of ageing days. The electrical conductivity was valid to assess seed vigor of pea and soybean (Association of Official Seed Analysts, 2002). Aged seeds had a higher EC values compared to the unaged seeds, which is consistent with earlier observations with *Brassica* species presented by Hampton et al. (2009).

ROS production such as H_2O_2 increased during seed germination but uncontrolled increasing of H_2O_2 content decreased seed germination. In the present study, the reduction of seed vigor was related to higher levels of ROS, such as H_2O_2 (Fig. IV). Wojtyla et al. (2006) explained that the loss of seed germinability has been attributed to the accumulation of reactive oxygen species. The results of the present study suggests that loss of seed viability is positively correlated with increasing ageing days in seeds of wheat. Applying higher ageing treatments such as 4 and 5 days in seeds led to greater oxidative damage and higher ROS generation. McDonald (1999) reported that oxidative stress imposed by ROS is an important cause of seed deterioration during ageing. In this study, with increasing ageing duration from 1 to 5 days, H_2O_2 production rate increased significantly. The lowest H_2O_2 rate was produced in control treatment. The differences in H_2O_2 rate might be related to higher level of moisture content and temperature during accelerated ageing.

The increase in MDA contents is often used as an indicator of oxidative damage (Sung, 1996). In the present study MDA contents increased as did the ageing period. Maximum MDA content was recorded in 5 days ageing treatment. However, Schopfer et al. (2001) stated that elevated MDA contents mediated by free radicals are considered to be one of the likely explanations for lipid peroxidation.

On the other hand, soluble sugar decreased during ageing progress. Amuti and Pollard (1977) found that ageing in angiospermous seeds led to the decreasing of soluble sugars. These solutes are known to contribute to development of tolerance to desiccation and to longevity itself (Bernal-Lugo and Leopold, 1995). The protective effects of soluble sugars are thought to occur through maintaining the structural integrity of membranes, and providing stability for macromolecules such as proteins (Crowe and Crowe, 1986). Moreover, control treatment had the highest soluble sugar (90 $mg.g^{-1}$ FW) (Fig. V). In control treatment, because of the extremely high viscosity that can be obtained in the glassy state, deteriorative reactions should be suppressed. Different responses of wheat seeds to accelerated ageing treatments mybe explained by their capacity for sugar consumption during ageing treatments.

The biochemical analysis of the aged wheat seeds at 43° C and 100% RH conditions

showed differences in seed proteins. Soluble protein in 1 and 2 days ageing treatments was higher than control but in 3, 4, and 5 days ageing treatment soluble protein decreased (Fig. VI). Decline in protein content during ageing may be because of degrading total proteins by proteinase. Similar results were reported in *Arachis hypogaea* and *Zea mays* (Basavarajappa et al., 1991; Rao et al., 1970). Seed ageing changes protein metabolisms by carbonylation. Protein degradation was mainly due to condensation, rearrangement, fragmentation, and polymerization. However, condensation is the process of reaction between a reducing sugar and a primary amino acid (Radha et al., 2013). Also, they suggested that protein metabolism regroups several biological functions such as protein folding, protein translocation, thermotolerance, oligomeric assembly, and switching between active and inactive protein conformations. Impairment of these functions is closely linked with the loss of seed vigor.

Ascorbic acid and proline protect cellular functions by scavenging of ROS (Dange, 1990). In this study, proline content tended to increase with seed ageing until 2 days and ascorbic acid increased in 1 day ageing decreasing afterwards. Hoekstra et al. (2001) found that the accumulation of low-molecular weight metabolites acting as osmoprotectants such as ascorbic acid and proline are part of the adaptive response to oxidative stress in plants. The results of the present study suggests that increasing of ageing until 1 and 2 days led to increasing of ascorbic acid and proline content, respectively and decreasing afterwards. Non-enzyme antioxidants such as proline and ascorbic acid were increased as scavengers of ROS in low moisture content condition but enzymatic antioxidant activity were increased in high moisture content condition and with increasing of seeds MC their activities were increased. However, proline accumulation in seeds could confer some adaptive advantages under oxidative stress. Lei and Chang (2012) found that an increasing of ascorbic acid and proline content could also be considered as a stress-induced marker for oxidative damage during ageing. However, the results showed that, when seeds were aged for 1 to 5 days, the proline contents increased until 2 days and then decreased at 43° C and 100% RH (Fig. VII). This finding suggests that ascorbic acid and proline play an important role in adapting to oxidative stress in aged wheat seeds.

It is generally recognized that plants can protect themselves by inhibiting lipid peroxidation by activated antioxidant enzymes after imbibition (Bailly, 2004). The results of the present study showed that antioxidant system components such as ascorbic acid, proline, CAT, POX, and APX, contribute to reducing the concentration of ROS. Some researchers obtained similar results (Mittal et al., 2012; Oliveira et al., 2012). In this study, the change of CAT activity was related to the ageing duration. There are significant changes in CAT and APX activity with ageing days increase. CAT and APX could respond rapidly to scavenge H_2O_2 under oxidative stress. The highest POX activity in seeds under 100% RH at 43° C condition occurred after 3 days of accelerated ageing then their activities were decreased but, the APX activity was increased for 2 days and then decreased. These results showed that CAT and APX antioxidant enzymes activities were permanent until 3 days of ageing but POX activity increased until 2 days of ageing. Furthermore, variations in CAT, POX, and APX activities under 100% RH at 43° C indicated that CAT and APX were more tolerant than POX to oxidative damage as the ageing duration extended. These three enzymes may have complementary or interacting roles with non-enzymatic antioxidants such as proline and ascorbic acid. The activities of CAT, POX, and APX in wheat seeds showed similar trends. Kong et al. (2014) found that the reductions in CAT, POX, and APX activities may be due to ROS accumulation to toxic levels or intolerant of the higher ageing levels. Results of the present study showed that wheat seed ageing damaged seed germination but enzymatic and non-enzymatic antioxidants systems limited these damages and increased seed viability and vigor leading to higher wheat seed germination.

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