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Seasonal enzymatic and non-enzymatic antioxidant responses in seven Iranian pomegranate cultivars

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Key words: abiotic stress tolerance, antioxidant, *Punica granatum.*

Abbreviation: APX= Ascorbic peroxidase; CAT= Catalase; GSH= Glutathione; MDA= Malondialdehyde; MDG= Malas Daneh Ghermez; MMS= Malas Momtaz Yazd; SK= Shishe Kab; POD= Peroxidase; ZA= Zagh Aghda; SOD= Superoxide dismutase; SSF= Shirin Shahvar Fars; ZAA= Zard Anar Arsenjan; NB= Naderi Badrood.

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All relevant data are within the paper and its Supporting Information files.

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The authors declare no competing interests.

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Abstract: The present study was carried out as bifactorial in a completely randomized block design to compare seasonal changes of antioxidant response (enzymatic and non-enzymatic) in seven Iranian pomegranate cultivars ('Malas Mommtaz Saveh', 'Shishe Kab', 'Zagh Aghda', 'Naderi Badroud', 'Malas Daneh Ghermez', 'Shirin Shahvar Fars' and 'Zard Anar Arsenjan') for a deeper understanding of their physiological differences and selecting probable more tolerant and adaptable cultivars to environmental conditions. Uniform and healthy rooted (one-year) plants of seven Iranian commercial pomegranate cultivars were purchased from a commercial nursery and planted in an orchard site in Arsenjan region (one of the main hubs of pomegranate growing), Fars province, Iran. After full establishment of the trees, three rounds of sampling (fresh leaves) were conducted in spring, summer and fall. Results indicated that in summer, activity of enzymatic antioxidants and leaf content of non-enzymatic antioxidants (glutathione, α-tocopherol and total polyphenols) rose in comparison to the contents measured in the other seasons in all studied cultivars. Significant differences were observed among pomegranate cultivars for these parameters and also content of leaf pigments. 'Shishe Kab' was evaluated as a potential tolerant cultivar with high accumulation to changing environmental conditions, since this cultivar demonstrated the highest leaf content of non-enzymatic antioxidants, chlorophyll a/b ratio and lowest level of lipid peroxidation in warmest days of summer. Similarly, 'Zard Anar Arsenjan', 'Shirin Shahvar Fars' and 'Malas Daneh Ghermez' were evaluated as adaptable cultivars to regional conditions.

1. Introduction

Pomegranate (*Punica granatum* L.) is an ancient fruit-bearing decidu-

ous shrub or small tree native of Iran (Ebtedaie and Shekafandeh, 2016). Historical evidence reveals that the primary origin of pomegranate is Iran and that it has been spread from this region to other areas. A large number of pomegranate varieties can be found in Iran, more than 760 original, wild and decorative cultivars (Mousavinejad *et al*., 2009). With a production of 700,000 tons/year, Iran is the world's leading producer (Sarkhosh *et al*., 2009). Because of its high acclimation, pomegranate tree grows well in wide range of climates and soil conditions (Galindo *et al*., 2014).

Iran, with an annual precipitation of 200 mm, is considered as a dry country and due to global warming and climate change, abiotic stresses such as drought and salinity are predicted to intensify in near future (Ebtedaie and Shekafandeh, 2016). Planting fruit trees which are low water consumers such as pomegranate can be a suitable strategy for cultivating arid and semiarid regions (Greenwood *et al*., 2010; Jiménez *et al*., 2010).

One of the main steps in orchard establishment is selection of suitable cultivars. Previous investigations indicate varied levels of acclimation potential and tolerance to abiotic stress conditions among different pomegranate cultivars (Tabatabaei and Sarkhosh, 2006; Okhovatian-Ardakani *et al*., 2010; Ibrahim, 2016). That would be attributed to their varied enzymatic and non-enzymatic antioxidant potential in response to seasonal changes in environmental conditions (Jamali *et al*., 2016). Maintenance of a high antioxidant capacity to scavenge the toxic reactive oxygen species (ROSs) has been linked to increased acclimation of plants to environmental stresses (Sharma *et al*., 2012). ROSs are produced in plants as byproducts during many physiological and biochemical processes such as photosynthesis and respiration. Generation of ROSs causes rapid cell damage by triggering a chain reaction (Ahmad *et al*., 2010). Naturally occurring antioxidants in plant cells include: enzymatic and peptide defense mechanisms, non-enzymatic mechanisms, phenolic defense compounds, nitrogen compounds, carotenoids and chlorophyll derivatives. Both the enzymatic and non-enzymatic antioxidants play an important role as natural antioxidants (El-Missiry, 2012)

Previous literatures focused on comparison between differences in enzymatic and non-enzymatic antioxidants in various Iranian pomegranate cultivars are limited and more investigations seem necessary. The goal of present study was to compare seasonal changes in enzymatic and non-enzymatic antioxidant responses of seven Iranian commercial pomegranate cultivars for a deeper understanding of their acclimation to environmental and regional conditions.

2. Materials and Methods

Uniform and healthy rooted plants of seven Iranian cultivars of *Punica granatum* L. were purchased from a commercial nursery and planted in a completely randomized block design with 3 replications (each replication had 3 plants) with 3-meter distance in rows and 5-meter distance between rows in an orchard site in Arsenjan region (hub of pomegranate growing and production in Fars), Fars province, southern Iran. Average annual climate parameters in the experimental region were: precipitation (200 mm), relative humidity (Max: 55%, Min: 23%), temperature (Max: 42°C, Min: 4°C). All the cultivars were growing in same soil conditions and were irrigated in a similar way (Drip irrigation). Routine cultural practices suitable for commercial fruit production were carried out during the experimental period.

Cultivars included: Malas Momtaz Saveh (MMS), Naderi Badroud (NB), Malas Daneh Ghermez (MDG), Shirin Shahvar Fars (SSF), Zagh Aghda (ZA), Shishe Kab (SK) and Zard Anar Arsenjan (ZAA). After 4 years and full establishment of the plants, samples (fresh leaves) were taken from the trees. Leaf samples were taken from different orientations of the trees (north, south, west and east); 25 fully expanded mature leaves from each side of all trees (100 leaves per tree as bulk samples) were samples in liquid N_2 and transported to laboratory. Leaves were taken from shoots without terminal fruit. Leaves with abnormal symptoms such as chlorosis and mechanical lesions caused by pests or diseases were avoided. Leaf samples were taken at 3 times (June 2nd, August 10th and October 10th) during the growing season; temperature and humidity of experimental region at the time of leaf sampling are given in Table 1.

Table 1 - Average day and night temperature and relative humidity in the experiment region at the time of sampling

Date of sampling	Average day/night temperature (°C)	Relative humidity (%)
June 2nd	27/14	45
August 10th	38/25	23
October 10th	27/15	27

The following parameters were measured in studied cultivars for two consecutive years and an average was reported.

Enzymes extraction

For enzymes extraction, leaves (0.5 g) were ground to fine powder in liquid nitrogen with mortar and pestle and then homogenized in 2 mL extraction buffer (50 mM potassium-phosphate buffer, pH 8.0), 10% (w/v) polyvinylpyrrolidone (PVP), 0.1 mm ethylenediaminetetra acetic acid (EDTA), 1 mM dithiothreitol (DTT). The homogenate was centrifuged (15000 × *g*) at 4°C for 30 min. Then, the supernatants were collected.

Superoxide dismutase activity

The activity of SOD was determined by adding 0.1 mL of the enzymatic extract to a tube containing 13 mM l-methionine, 25 mM nitro-blue tetrazolium chloride (NBT), 0.1 mM EDTA, 50 mM sodium carbonate and 2 mM riboflavin in a 50 mM phosphate buffer in pH 7.8 (Dhindsa *et al.,* 1980). Tube was placed under two 15 W fluorescent lamps for 15 min. A complete reaction mixture without enzyme, which gave the maximal color, served as control. Reaction was stopped by switching off the lights and keeping the tubes in dark. A non-irradiated complete reaction mixture served as a blank. The absorbance was recorded at 560 nm, and one unit of enzyme activity was taken as that amount of enzyme that reduced the absorbance reading to 50% in comparison with tubes lacking enzyme. SOD activity was expressed as units per milligram of protein per minute.

Catalase activity

Catalase (CAT, EC 1.11.1.6) activity was measured spectrophotometrically according to the method of Chance and Maehly (1955), by monitoring the decline in absorbance at 240 nm due to H_2O_2 consumption. One milliliter of reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0) and 15 mM H_2O_2. The reaction was initiated by adding 50 µL of crude extract to this solution. CAT activity was expressed as units (µmol of H_2O_2 consumed per minute) per milligram of protein.

Peroxidase activity

The activity of guaiacol peroxidase (POX) was determined by adding 50 µl of the crude enzyme preparation to 2 ml of a solution containing 50 mM potassium phosphate buffer (pH 7.0)., 13 mM guaiacol and 5 mM H_2O_2.

Increase in absorbance due to oxidation of guaiacol (extinction coefficient: 26.6 $mM^{-1} \cdot cm^{-1}$) was monitored at 470 nm for a minute. Peroxidase activity was expressed as units (µmol guaiacol oxidised per minute) per milligram of protein.

Ascorbate peroxidase activity

Ascorbate peroxidase (APX, EC 1.11.1.11) activity was measured spectrophotometrically according to Nakano and Asada (1981) by following the decline in absorbance at 290 nm due to ascorbate oxidation. The oxidation rate of ascorbate was estimated between 1 and 60s after starting the reaction with the addition of H_2O_2. One milliliter of reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM ascorbate, 0.15 mM H_2O_2, 0.1 mM EDTA and 50 µL of enzyme extract. APX activity was expressed as units (µmol of ascorbate oxidized per minute) per milligram of protein.

Protein content

Protein content was determined according to Bradford (1976) by using bovine serum albumin as a standard. For preparation of Bradford reagent, 100 mg of Coomassie Brilliant Blue G-250 weight and then dissolved in 50 mL 0f 95% ethanol. Then, 100 ml of 85% orthophosphoric acid (H_3P0_4) added to aforesaid solution and volume reached into1000 mL volume with distilled water. Bradford reagent filtered using Whatman paper to remove precipitates before use. for assessment the protein content, 5 mL of Bradford reagent and 100 µL protein extraction added in the test tube and shaken vigorously for a few seconds. Reaction mixture remained at ambient temperature for 5 min and absorbance was read using spectrophotometer at 595 nm. Bovine serum albumin (BSA) was used to elaborate a standard curve. The protein content was calculated according to the obtained equation.

Proline content

Proline was extracted and its concentration determined by the method of Bates *et al*. (1973). Leaf segments were homogenized with 3% sulfosalicylic acid and the homogenate was centrifuged (3000 × *g*) for 20 min. The supernatant was treated with acetic acid and acid ninhydrin, boiled for one hour and then absorbance at 520 nm was determined. Contents of proline are expressed as $\mu mol \cdot g^{-1}$ fresh weight.

Leaf chlorophyll and carotenoids content

Leaf discs (0.5 g) were extracted in 5 mL of acetone (80%), then centrifuged (8000 × g) for 10 minutes. The supernatant was used to make a final volume of 100 mL of the leaf extract. Extraction of leaf tissue with the buffer continued until decoloration.

Absorbance of the extract was read at 470, 645 and 663 nm with a spectrophotometer and 80% acetone was used as a blank. Finally, chlorophyll (a and b) and carotenoids contents were calculated according to the following equations (Lichtenthaler, 1987):

Chl a (mg. g^{-1} fresh weight): $[(12.25A_{663} - 2.79A_{645}) \times v / 1000 \times W]$
Chl b (mg. g^{-1} fresh weight): $[(21.50A_{645} - 5.10A_{663}) \times v / 1000 \times W]$
Chla + Chlb (mg. g^{-1} f.w.): $[(7.15A_{663} + 18.71A_{645}) \times v /1000 \times W]$
Carotenoids (mg. g^{-1} f.w.): $1000A_{470}$-1.82Chla- 85.02Chlb / 198

where Chla = chlorophyll a; Chlb = chlorophyll b; Chla+Chlb = total chlorophyll; A = absorbance at ƛ (nm).

Leaf anthocyanins content

Total leaf anthocyanins were measured spectrophotometrically by pH differential method (Lee *et al*., 2005) with two buffer systems: potassium chloride buffer (pH 1.0,0.025 M) and sodium acetate buffer (pH 4.5, 0.4 M). Leaf samples (0.5 g) were extracted with 2 mL methanol: water: concentrated HCl solution (80:20:1 v/v/v). 0.4 mL of leaf extract was mixed with 3.6 mL of corresponding buffers and read against water as blank at 510 and 700 nm. Absorbance (A) was calculated as

$$A = (A_{510} - A_{700})\ \text{pH 1.0} - (A_{510} - A_{700})\ \text{pH 4.5}$$

Then total anthocyanins content was calculated using the equation:

Anthocyanin (µg. g^{-1} fresh weight) = $(A \times Mw \times DF \times 1000) / \varepsilon$

Where *A* is the absorbance of the diluted sample and DF is the dilution factor (10), Mw is molecular weight of cyanidin-3-glucoside (449.2) and ε= 26,900 l/mol.cm, the molar extinction coefficient of cyanidin-3-glucoside.

Glutathione content

Glutathione (GSH) was estimated by the method of Moron *et al*. (1979). Two hundred mg of leaf tissue was homogenized in 2 mL of ice-cold 5% trichloroacetic acid. The homogenate was then centrifuged at 4°C at 17000 × *g* for 30 min. A volume of 75 µl of the clear supernatant was added to a cuvette containing 300 µl of phosphate buffer (0.2 M, pH 8.0) and 750 µL of 0.6 mM DTNB (5, 5-dithiobis-2-nitrobenzoic acid) in phosphate buffer. The absorbance at 412 nm was read and glutathione content was derived against a standard curve prepared with known amounts of GSH in 5% trichloroacetic acid.

Malondialdehyde content

Malondialdehyde (MDA) content was determined by the thiobarbituric acid (TBA) reaction as described by Ali *et al*. (2005), with slight modifications. Two hundred mg leaf samples were homogenized with 2 mL of 0.1% trichloroacetic acid and centrifuged at 10000 × *g* for 15 min. One mL of the supernatant was mixed with 2.5 mL 0.5% thiobarbituric acid in 20% trichloroacetic acid and incubated in hot water (95°C) for 30 min. Thereafter, it was cooled immediately on ice to stop the reaction and centrifuged at 10000 × *g* for 30 min. Absorbance at 532 and 600 nm was determined, and MDA concentration was estimated by subtracting the non-specific absorption at 600 nm from the absorption at 532 nm, using an absorbance coefficient of extinction (155 $mM^{-1} \cdot cm^{-1}$).

Leaf total polyphenols content

Leaf polyphenols content was determined with Folin-Ciocalteu reagent using gallic acid as a standard phenolic compound. In brief, 1 g of lyophilized leaf samples were placed in an Eppendorf tube, with 1 mL of methanol (80%), grinded at 4°C and centrifuged at 10000 × *g* for 15 min. The extract was mixed with 0.5 mL of Folin-Ciocalteu reagent (diluted 1:1 with water) then 1 mL of a 5% sodium carbonate solution was also added. After 30 min, absorbance was measured at 725 nm and expressed as mg · g^{-1} fresh weight.

Leaf α-tocopherol content

α-Tocopherol was extracted according to Chong *et al*. (2004). Two hundred mg lyophilized sample was homogenized in 1 mL acetone with a prechilled mortar and pestle at 4°C. Following the addition of 0.5 mL hexane, the homogenate was first vortexed for 30 s, then centrifuged at 1000 × *g* for 10 min. The upper hexane layer was removed while the acetone layer containing vitamin E remained in the vial. A second aliquot of 0.5 mL hexane was added, and the extraction process was repeated at least twice. α-Tocopherol was estimated by the method of Kanno and Yamauchi (1997). A 0.4-ml aliquot of 0.1% (w/v) 3-(2-pyridyl)-5, 6-diphenyl-1, 2, 4-triazine was added to 0.2 mL of pooled extract. The volume was made up to 3 mL with absolute ethanol, 0.4 mL 0.1% (w/v) ferric chloride ($FeCl_3 \cdot 6H_2O$) was added, and the content was gently mixed under dim light in a dark room to avoid photochemical reduction. After a 4 minutes reaction at room temperature, 0.2 mL 0.2 M orthophosphoric acid was added and the mixture left for another 30 min. Absorbance was determined at 554 nm spectrophotometrically and reported as µg. g^{-1} fresh weight. The blank was prepared in the same manner except that absolute ethanol was used instead of the sample. α-Tocopherol (Sigma Chemical) was used as a standard.

Leaf ascorbic acid content

Ascorbic acid was estimated by the method of Omaye *et al*. (1979). Briefly, to 1 g of lyophilized leaf sample, 10% ice-cold trichloroacetic acid was added and centrifuged for 20 min at 3500 × g in room temperature. One mL of the supernatant was mixed with 0.2 mL of DTC reagent and incubated for 3 hours at 37°C. Then 1.5 mL of ice-cold 65% H_2SO_4 was added, mixed well and the solutions were allowed to stand at room temperature for an additional 30 minutes. The color developed was read at 520 nm spectrophotometrically and Leaf ascorbic acid content reported as µg. g^{-1} fresh weight.

Statistical analysis

The experiment was carried out as a 7×3 bifactorial in a completely randomized block design (seven pomegranate cultivars as first factor and three sampling dates as second factor). Data were analyzed by SAS and means were compared using Duncan's multiple range tests at 5% probability level.

3. Results

The activity of SOD, CAT, POD and APX in the studied pomegranate cultivars is reported in Table 2. SK and MMS showed the highest SOD activity. No significant difference was observed among NB, SSF and ZAA. The maximum POD activity (50.41 units mg^{-1} protein) was found in NB which was significantly higher than other cultivars. Activity of this enzymatic antioxidant was not different in MMS, SK and MDG. The highest CAT activity was detected in SSF (27.18 units mg^{-1} protein), however ZAA, NB and MMS were not statistically different. Activity of CAT was not significantly different in SK and ZA compared to MDG. APX activity in ZAA was higher in comparison to the other studied cultivars. Activity of APX was not statistically different in SK and MDG.

Leaf contents of some non-enzymatic antioxidants in studied pomegranate cultivars are presented in Table 3. The highest leaf glutathione content (158.57

Table 2 - SOD, CAT, APX and POD activity in the leaves of different pomegranate cultivars

Cultivars	SOD (mg^{-1} protein min^{-1})	CAT (mg^{-1} protein)	APX (mg^{-1} protein)	POD (mg^{-1} protein)
MMS	76.72± 6.08 a	23.37± 2.54 ab	7.02± 0.66 d	38.56± 6.74b
NB	67.25± 8.25 bc	26.15± 1.34 ab	7.25± 0.66 d	50.41± 1.52a
MDG	63.02± 7.96 cd	19.07± 1 c	11.29± 1.20 b	37.83± 5.78b
SK	76.17± 3.48 a	22.76± 2.02 bc	11.19± 1.45 b	36.60±2.02 b
SSF	68.55± 6.56 bc	27.18± 2.90 a	9.50± 1.45 c	28.38± 4.58d
ZA	58.82± 8.76 d	19.98± 2.72 c	7.28± 0.66 d	24.30± 2.72e
ZAA	70.21 ± 7.02 ab	24.04± 2.40 ab	13.90± 0.88a	32.58± 1.15 c

Means followed by the same letters within columns are not different at 5% probability using Duncan's test. All data indicated mean ± standard error (n = 3).

Table 3 - Leaf concentration of some non-enzymatic antioxidants and Malondialdehyde (MDA) in seven Iranian cultivars

Cultivars	Glutathione (µg g^{-1} f.w.)	Proline (µg g^{-1} f.w.)	Ascorbic acid (µg g^{-1} f.w.)	α-tocopherol (µg g^{-1} f.w.)	Polyphenol (mg g^{-1}f.w.)	MDA (µg g^{-1} f.w.)
MMS	142.2±2.8 c	6.04±0.5 b	3.07±0.84 b	210.66±14.8 d	26.84±0.5 ab	32.99±1.15 a
NB	147.5±4.61 b	3.26±0.1 d	2.55±0.2 c	199.3±8.3 f	18.77±0.77 e	29.01±1.63 b
MDG	138.74±10.1 d	4±0.1 cd	2.48±0.13 c	172.6±19.47 g	22.42±1.0 d	26.01±0.87 c
SK	158.57±4.0 a	7.35±0.3 a	3.58±6.26 a	269.66±7.8 a	27.98±0.66 a	23.23±0.82 d
SSF	140.19±11.5 cd	5.92±0.08 b	2.48±0.13 c	172.6±19.47 g	23.55±1.11 cd	23.99±0.72 cd
ZA	128.36±5.7 f	4.62±0.3 c	3.01±0.2 b	204±10.12 e	18.77±0.77 e	30.32±0.54 ab
ZAA	134.28±4.5 e	6.98±0.32 ab	2.92±0.3 b	229.33±12.34 b	25.31±0.5 bc	24.42±2.08 cd

Means followed by the same letters within columns are not different at 5% probability using Duncan's test. All data indicated mean ± standard error (n = 3).

µg g^{-1} fresh weight) was observed in SK which was significantly higher than other cultivars. No significant difference was detected between MDG and SSF. Leaf total polyphenols in SK was 32% higher compared to NB, MMS was not significantly different in comparison to SK. Also, ZA, SSF and MDG were not different. SK demonstrated the highest leaf proline content (7.35 µmol g^{-1} fresh weight), however ZAA was not statistically different in comparison to this cultivar. Leaf ascorbic acid content was significantly higher in SK compared to other studied cultivars. This parameter was not statistically different in MMS, ZA, SSF and ZAA. Leaf α-tocopherol content in SK (269.66 µg g^{-1} fresh weight) was the highest among the studied cultivars. The highest MDA content was found in MMS (32.99 µmol g^{-1} fresh weight), however, ZA was not statistically different.

Seasonal changes of SOD, CAT, POD and APX in studied pomegranate cultivars are presented in figure 1 (a-d). SOD activity rose in summer in MMS, ZA, NB, SSF and ZAA. In fall, activity of this antioxidant enzyme decreased significantly in ZAA, SSF and ZA compared to summer. Higher activity of POD was observed in MMS, SK, ZA, NB, MDG and ZAA in summer in comparison to spring. This characteristic was not statistically different in summer compared to spring in SSF. A significant decline in POD activity in fall compared to summer was detected in MMS, SK, ZA and NB. CAT activity did not change significantly during spring, summer and fall in MMS, ZA, NB, MDG, SSF and ZAA. Activity of this enzyme decreased significantly in SK (about 37%) in summer. Rise in activity of APX was observed in MMS, SK and ZAA in summer. This parameter was not different in MMS, ZA, NB, MDG, SSF and ZAA in fall compared to summer.

Changes in content of some non-enzymatic antioxidants in studied pomegranate cultivars in spring, summer and fall are demonstrated in Fig. 2 (a-e). In all studied pomegranate cultivars leaf content of glutathione rose significantly in summer, and then declined in fall. The highest content of this non-enzymatic antioxidant (182.21 µg g^{-1} fresh weight) was detected in SK in summer. Leaf total polyphenols increased significantly in all studied cultivars in summer, whereas the content decreased in fall in MMS, SK, ZA, MDG, SSF and ZAA this characteristic decreased in fall. Proline in MMS, SK and ZA was higher in summer compared to spring and fall in NB, MDG, SSF and ZAA did not change significantly during spring, summer and fall. Ascorbic acid content increased (about 56%) in MDG in summer compared to spring then decreased significantly in fall. In contrary in MMS ascorbate content declined (34%) in summer then rose in fall. Other cultivars showed no significantly changes during these seasons. In all cultivars leaf α-tocopherol increased in summer then

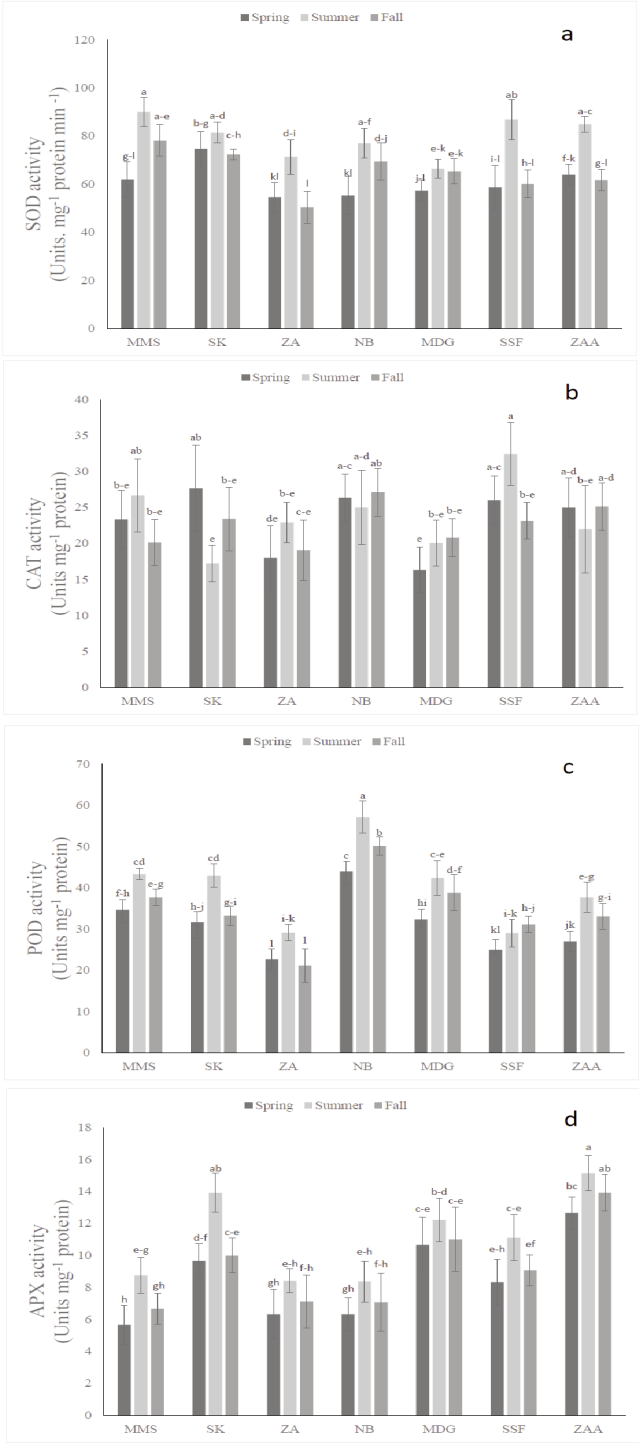

Fig. 1 - Seasonal activity changes of SOD (a), CAT (b), POD (c) and APX (d) in studied cultivars. Columns with different letters represent significant differences at 5% probability using Duncan's multiple range test, those are valid for all columns.

decreased in fall. This characteristic in MMS, ZA, NB, MDG and SSF was higher in fall in comparison to spring. ZAA and SK leaf α-tocopherol content was not statistically different in spring and fall.

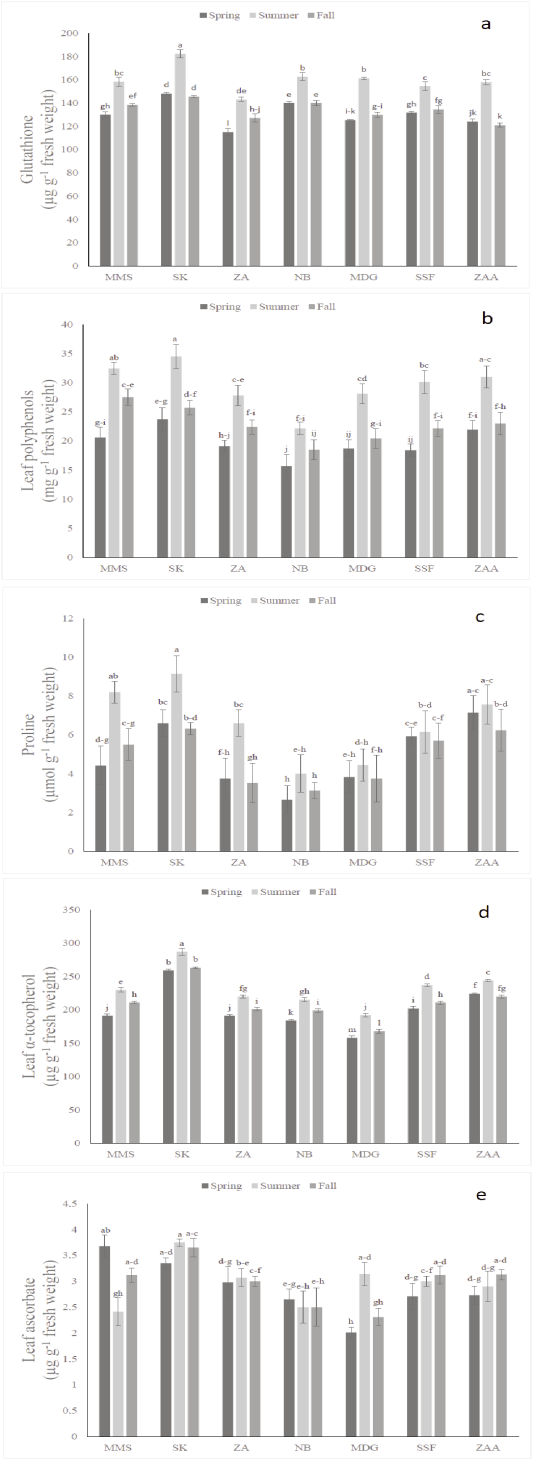

Fig. 2 - Seasonal changes in content of glutathione (a), polyphenols (b), proline (c), α-tocopherol (d) and ascorbic acid (e) in studied cultivars. Columns with different letters represent significant differences at 5% probability using Duncan's multiple range test, those are valid for all columns.

The highest MDA content was detected in summer, this parameter was higher in fall compared to spring. Leaf total proteins decreased significantly in summer (Fig. 3). Leaf MDA content increased in summer in all studied cultivars, and the rise was more pronounced in ZA, NB and MMS compared to SK, SSF and ZAA. In the former three cultivars MDA was higher than spring (Fig. 3).

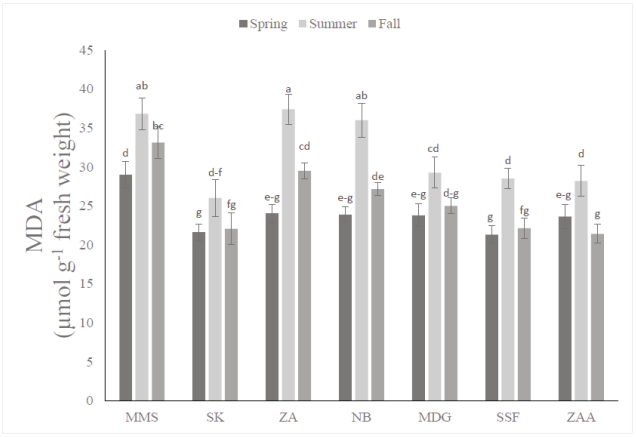

Fig. 3 - Seasonal changes in MDA content in studied cultivars. Columns with different letters represent significant differences at 5% probability using Duncan's multiple range test, those are valid for all columns.

Leaf pigments content in studied cultivars are indicated in Table 4. The highest total chlorophyll content was observed in SSF, but SK was not statistically different. Also no significant difference was observed between MDG and ZAA. The highest leaf anthocyanin (0.31 μg g^{-1} fresh weight) and carotenoid (0.34 mg g^{-1} fresh weight) were obtained from SK. No significant difference was observed between ZAA and SSF for these two parameters. Chlorophyll a/b ratio was significantly higher in SK and ZAA compared to other cultivars. NB and ZAA had significantly higher total leaf proteins in comparison to other cultivars. No significant difference was observed between SK, ZA, MDG and SSF.

Leaf total chlorophyll content was higher in spring in comparison to summer and fall. In summer the highest levels of anthocyanin and carotenoid contents were detected. Chlorophyll a/b ratio was significantly higher in summer or fall compared to spring (Fig. 4 a-d).

Changes in leaf pigments in spring, summer and fall in studied cultivars are shown in Fig. 4 (a-d). Leaf total chlorophyll content decreased in summer then rose in fall in all cultivars. In SK, ZA, NB, MDG, SSF and ZAA Leaf total chlorophyll was not statistically different in fall compared to spring. Leaf anthocyanin

and carotenoid content rose in all studied cultivars in summer. In fall leaf anthocyanin decreased in SK and ZA, similar decline in leaf carotenoid was observed in MMS, SK, ZA, NB and ZAA. Chlorophyll a/b ratio increased in MM, ZA and NB, decreased in ZAA and did not change in SK, MDG and SSF in summer. This parameter was not different in fall compared to spring in all cultivars.

4. Discussion and Conclusions

In present study mean activity of SOD, POD and APX rose significantly in summer similar to the concentration of non-enzymatic antioxidants. During summer months solar irradiance intensity and regional mean day temperature rise. Drought, salinity, high temperatures and UV-B radiation lead to enhanced generation of ROSs in plants due to disruption of cellular homeostasis (Mittler, 2002). Scavenging or detoxification of excess ROSs is achieved by an efficient antioxidative system comprising of the non-enzymatic as well as enzymatic antioxidants (Noctor and Foyer, 1998). Rise in activity of SOD, POD and APX as well as increase in concentration of non-enzymatic antioxidants in summer were a part of protective responses of studied plants. However, significant differences were observed

Table 4 - Leaf pigments and protein concentration in seven Iranian cultivars

Cultivars	Anthocyanin (μg g^{-1} f.w.)	Chlorophyll (mg g^{-1} f.w.)	Chlorophyll a/b ratio	Carotenoid (mg g^{-1} f.w.)	Proteins (mg g^{-1} f.w.)
MMS	0.29±0.01 b	1.17±0.59 c	1±0.02 d	0.24±0.006 d	19.62±0.95 c
NB	0.26±0.007 c	1.09±0.07 d	1.10±0.03 c	0.23±0.007 de	21.35±0.25 a
MDG	0.26±0.002 c	1.21±0.17 bc	1.17±0.01 b	0.27±0.009 c	20.32±1.25 b
SK	0.31± 0.002 a	1.34±0.06 a	1.23±0.01 a	0.34±0.008 a	20.48±0.98 b
SSF	0.29±0.01 b	1.36±0.04 a	1.15±0.008 b	0.31±0.004 b	20.62±1.59 b
ZA	0.25±0.006 c	1.03±0.06 e	1.07±0.03 c	0.22±0.005 e	20.48±0.40 b
ZAA	0.29±0.01 b	1.27±0.1 b	1.22±0.038 a	0.30±0.006 b	21.27±0.68 a

Means followed by the same letters within columns are not different at 5% probability using Duncan's test. All data indicated mean ± standard error (n = 3).

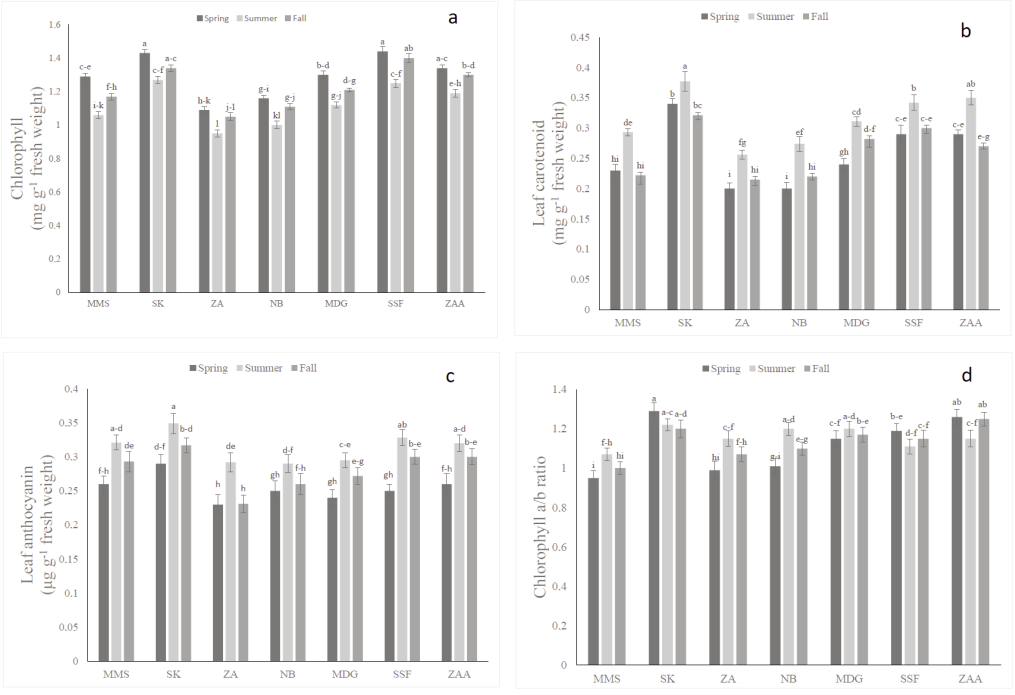

Fig. 4 - Seasonal changes in content of chlorophyll (a), carotenoid (b), anthocyanin (c), chlorophyll a/b ratio (d) in studied cultivars. Columns with different letters represent significant differences at 5% probability using Duncan's multiple range test, those are valid for all columns.

between cultivars.

Increased activity of SOD is often correlated with increased adaptability of the plants to environmental conditions (Zaefyzadeh *et al*., 2009; Sharma *et al*., 2012). High temperatures or drought cause either enhancement or depletion of CAT activity depending on the plant species (Sharma and Dubey, 2005; Han *et al*., 2009). CAT activity decreased in SK and did not change significantly in other cultivars in summer; similar results have been reported by Gholami *et al*. (2012). APX scavenges the H_2O_2 produced by SOD using ascorbate as the electron donor (Noctor and Foyer, 1998). Decline in ascorbate level in MMS in summer can be contributed to this process. However, this parameter did not change in SK in summer which can be due to higher tolerance of this cultivar to high temperature. Overexpression of a cytosolic APX-gene derived from pea in transgenic tomato plants ameliorated oxidative injury induced by chilling and salt stress (Wang *et al*., 2005). Similarly, over expression of the tApx gene in either tobacco or in *Arabidopsis* increased tolerance to oxidative stress (Yabuta, 2002).

Most of non-enzymatic antioxidants such as polyphenols, glutathione, proline and α-tocopherol rose in summer in our study concomitant with increase in mean day temperature and solar irradiance. This was in agreement with previous works. (Ghorbanali *et al*., 2012; Sivaci and Duman, 2014)

Glutathione is one of the most important cellular antioxidants (Sharma *et al*., 2012). It also plays an indirect role in protecting membranes by maintaining α-tocopherol and zeaxanthin in the reduced state (Hasanuzzaman *et al*., 2013). Glutathione accumulates to high concentrations, especially under stress conditions. Increase in glutathione concentrations during stress offsets stress initiated oxidation of glutathione and causes changes in gene expression directly or through interaction with regulatory proteins and/or transcription factors. This increase is equally important in signal transduction and defense against ROSs (Hasanuzzaman *et al*., 2013). Glutathione is a potential scavenger of $^1O_2$, H_2O_2 and $^{\bullet}OH$ (Gholami *et al*., 2012). Additionally, this tripeptide plays a key role in the antioxidative defense system by regenerating another potential water-soluble antioxidant like ascorbic acid (Foyer and Halliwell, 1976). Endogenous glutathione concentration has been reported to be associated with salt stress tolerance (Sumithra *et al*., 2006). Kattab (2007) reported that *Brassica napus* seed priming with glutathione improved seedling resistance probably by enhancing the activities of antioxidant enzymes.

Polyphenols have strong antioxidant properties and their presence at an elevated level is associated with increased abiotic stress tolerance. Increase in concentration of polyphenolic compounds following abiotic stress conditions such as high temperature has been reported by various authors in different species (Jamali *et al*., 2016). Bautista *et al*. (2016) compared the levels of total phenolic compounds and antioxidant flavonoids in a relatively large number of plant species from different families growing under varied environmental conditions. Their data strongly support a general and relevant role of these compounds in mechanisms of acclimation to environmental and regional conditions.

Proline accumulation can serve as a selection criterion for the tolerance and adaptability of most species (Parida and Das, 2005; Ashraf and Foolad, 2007; Ahmad *et al*., 2009). In addition to its role as a compatible osmolyte and osmoprotectant, several studies have attributed an antioxidant feature to this amino acid, suggesting ROSs scavenging activity and proline acting as a $^1O_2$ quencher (Smirnoff and Cumbes, 1989; Matysik *et al*. 2002). It has been reported that proline protects higher plants against osmotic stresses not only by adjusting osmotic pressure but also by stabilizing many functional units such as complex II electron transport, membranes, and proteins and enzymes such as rubisco (Hamilton and Heckathorn, 2001).

Several lines of evidence indicate that α-tocopherol plays a major role in plant stress tolerance, keeping an adequate redox state in chloroplasts (Munne-Bosch, 2005). Deficiency of this antioxidant leads to a slightly increased susceptibility to photo oxidative stress (Kanwischer *et al*., 2005). Other mechanisms such as increases in abscisic acid concentration under stress conditions are also known to enhance α-tocopherol (Singh *et al*., 2011).

In our study, ascorbic acid did not change significantly during spring, summer and fall. The increase of glutathione pool during summer could be necessary to regulate the levels of ascorbate (Foyer and Theodoulou, 2001).

Significant difference in concentration of leaf pigments was observed between cultivars and seasons in the present study. A decrease in the chlorophyll concentration in summer is a typical symptom of oxidative stress (Egert and Tevini, 2002). In addition to harvesting solar energy, carotenoids play protection roles keeping integrity of photosynthesis apparatus against photo oxidative damages by scavenging

free radicals (Andrade-Souza *et al*., 2011). Carotenoids are precursor of ABA which is an important phyto-hormone regulating plant responses to stresses. Presence of higher carotenoid concentration leads to lower photo-oxidative damage and higher potential for regulating plant growth under stress conditions (Han *et al*., 2008). Our results were in accordance with previous works (Christie *et al*., 1994; Chalker-Scott, 1999; Parida and Das, 2005). Plant tissues with a higher content of anthocyanins usually have a higher resistance to drought. The purple cultivar of pepper is more tolerant to water stress than the green cultivar (Bahler *et al*., 1991). The higher ratio of chlorophyll a/b which was observed in some of cultivars was considered to be the result of a decreased emphasis on light collection in relation to the rates of PSII photochemistry (Demmig-Adams and Adams, 1996).

It was suggested that SOD can be used as an indirect selection criterion for screening drought-resistant plant materials (Zaefyzadeh *et al*., 2009). Significant differences were observed for the measured parameters between studied pomegranate cultivars. SK cultivar is well adapted to changing environmental conditions since this cultivar had higher activity of SOD and elevated levels of non-enzymatic antioxidants compared to other cultivars. The presence of these compounds at higher concentration probably mitigated overproduction of ROSs in summer. This was in accordance with previous studies. Khayyat *et al*. (2014) compared salt tolerance in MMS and SK. They evaluated SK as more salt tolerant cultivar.

In various studies the chlorophyll concentration was used as a sensitive indicator of the cellular metabolic state (Chutipaijit *et al*., 2011). Higher chlorophyll concentration is related to elevated tolerance against abiotic stresses such as drought and salinity (Hasanuzzamn *et al*., 2013). Chlorophyll a/b ratio did not change in SK in summer which could be explained as no decrease of peripheral light-harvesting complexes and a higher stress tolerance in this cultivar (Liu *et al*., 2011). MDA concentration which is an index for lipid peroxidation was the lowest in SK. ZAA, SSF and MDG could be considered as cultivars with high tolerance capacity to abiotic stresses as well, and MYS, ZA and NB as cultivars with intermediate abiotic stress tolerance. Other important characteristics such as macro and micronutrients absorption (data not shown), endogenous plant growth regulators such as auxins, cytokinins and ABA (data not shown) and trees productivity and fruits quality (data not shown) were considered for this evaluation. However, as it was mentioned before, pomegranate cultivars have high compatibility capacity and they grow well in wide range of climates and soil conditions. This is the reason why Ghasemi Soloklui *et al*. (2012) did not find remarkable differences in freezing tolerance between seven pomegranate cultivars in midwinter.

Significant differences were observed between studied pomegranate cultivars for activity of enzymatic antioxidants, leaf content of non-enzymatic antioxidants and leaf pigment contents. SK was possibly evaluated as a cultivar that can acclimate against environmental condition changes extremely. ZAA, SSF and MDG cultivars were ranked following the SK, those may still responses against abiotic stress in a high level. However, MYS, ZA and NB were known as cultivars show stress tolerance moderately.

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