Responses of pomegranate cultivars to severe water stress and recovery: changes on antioxidant enzyme activities, gene expression patterns and water stress responsive metabolites

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Abstract The biochemical and molecular responses of five commercially well-known pomegranate cultivars to severe water stress were studied. The cultivars were subjected to 14-day water stress by withholding irrigation, followed by re-watering for 7 days. Results showed clear differences in metabolites contents and activities of antioxidant enzymes among various pomegranate cultivars during severe water stress and recovery. According to our results, increased accumulation of proline in pomegranate was not related to osmotic adjustment during severe water stress. Except for ‘Ghojagh’, leaves grown under severe water stress conditions showed symptoms of oxidative stress such as reduced chlorophyll concentration. The improved performance of ‘Ghojagh’ under drought stress may be associated with an efficient osmotic adjustment. The up- or down regulated expression of cytosolic glutathione reductase (cytosolic GR) and glutathione peroxidase were observed under drought conditions. Moreover, the suppressed expression of cytosolic GR was also noted. Comparatively, ‘Rabab’ exhibited higher antioxidant capacity and an efficient ROS-scavenging mechanism under drought stress. Lower levels of membrane lipid peroxidation in ‘Ghojagh’ and ‘Rabab’ under drought stress and the marked reduction of

malondialdehyde concentration after re-watering represents that these cultivars have a good tolerance to drought stress. As a first step towards the study of the biochemical and molecular responses of pomegranate plants to water stress, this research provides new information into the mechanisms of drought tolerance in the plants.

Keywords Biochemical · Drought stress · Gene expression · Pomegranate · Tolerance

Abbreviations

SOD	Superoxide dismutase
CAT	Catalase
G-POD	Guaiacol peroxidase
GR	Glutathione reductase
GPX	Glutathione peroxidase
MDA	Malondialdehyde
Chl _{a+b}	Total chlorophyll
ROS	Reactive oxygen species

Introduction

Plants particularly horticulture section are raw material and used by human for food products and medicinal purposes (Sakar et al. 2016). Pomegranate is an important commercial fruit crop of the tropical and subtropical regions of the world which is valued for its nutritional and antioxidant characteristics (Parvizi and Sepaskhah 2015). Water stress is one of the most significant environmental factors restricting growth, performance, and distribution of plant species worldwide (Liu et al. 2011). Exposure of plants to such limiting environmental conditions induces a series of morphological, physiological, biochemical, and molecular responses to withstand stressful conditions and improve

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plant function in various ways (Reddy et al. 2004). During their life cycle, plants use some strategies to cope with drought stress. Two such strategies are drought escape (via completing plant life cycle before severe water deficit) and resistance mechanism. Resistance mechanism can be further classified into drought avoidance (via sustaining high tissue water potential by balancing of water uptake and loss, reducing stomata and leaf area, and increasing root/shoot ratios) and drought tolerance (via sustaining or conserving plant normal functions even at low tissue water potentials). This tolerance can be achieved through either dehydration avoidance (a mechanism that avoids leaf water loss at low tissue water potentials via solutes accumulation and cell wall hardening) or dehydration tolerance (a mechanism that avoids cellular damage caused by leaf water loss through accumulation of protective solutes and proteins, specific gene expression, metabolic changes and reactive oxygen species (ROS) detoxification) (Verslues et al. 2006; Wang et al. 2012). ROS including singlet oxygen ($^1\text{O}_2$), superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical ($\text{HO}^\cdot$) are unavoidable byproducts of various metabolic pathways in plants (Noctor et al. 2014). ROS formation is induced by a wide range of environmental stresses including chilling (Jung 2004), temperature stress (Sung et al. 2003), oxygen shortage (Blokina et al. 2003), salinity (Khan and Panda 2008), and drought (Bian and Jiang 2009). The generation of ROS in water deficit condition results from photosynthetic apparatus, photorespiration and respiration (Wang et al. 2012). Overproduction of ROS could destroy normal metabolism of plants and cause damage to cellular structures through destructive oxidative processes such as peroxidation of membrane lipids, protein oxidation, degradation of photosynthetic pigments, inactivation of photosynthetic enzymes, and damage to nucleic acids (Gholami et al. 2012; Silva et al. 2012). In order to remove ROS, plants have evolved a series of enzymatic and non-enzymatic antioxidant defense systems, which normally keep ROS balance in various compartments of cells. Therefore, oxidative damage occurs when the balance is disturbed (Menezes-Benavente et al. 2004). Enzymatic antioxidative defense system, including superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (G-POD), ascorbate peroxidase (APX), glutathione reductase (GR) and glutathione peroxidase (GPX) while the non-enzymatic antioxidants, including carotenoids, anthocyanins, ascorbate, glutathione, tocopherol, alkaloids, phenolic compounds and flavonoids (Bian and Jiang 2009; Boaretto et al. 2014; Chaves and Oliveira 2004). Accumulation of protective solutes include sugars (glucose, fructose, sucrose), sugar alcohols (mannitol, glycerol, sorbitol, pinitol, quercitol) proline, glycine betaine, and inorganic ions is a unique plant response to environmental stresses, specifically to water stress (Reddy et al. 2004; Slabbert and Krüger 2014; Wu and

Xia 2006). These solutes play important roles in sustaining leaf relative water content (RWC) and cellular functions under water stress. Pomegranate, a deciduous fruit tree, is native to the area stretching from Iran to the Himalayas in India (Fawole and Opara 2013; Parvizi et al. 2016). It has been cultivated since ancient times and is mentioned in the Christian bibles and holy Quran. Iran has the first rank regarding the number and diversity of pomegranate cultivars, cultivated area, producing and exporting of the fruit in the world (Parvizi et al. 2016). In recent years, water stress has become a huge problem in pomegranate orchards caused reductions in quality, such as fruit cracking, and quantity of fruits. Therefore, identifying and introducing the most tolerant pomegranate cultivar to water deficit is very essential. The majority of studies on pomegranate trees under water stress have primarily investigated physiological responses such as transpiration, stomatal conductance, photosynthesis, intrinsic water use efficiency, stem and leaf water potential, RWC, and leaf osmotic potential (Galindo et al. 2013; Intrigliolo et al. 2011; Mellisho et al. 2012; Parvizi et al. 2016; Rodríguez et al. 2012). To the best of our knowledge, there is no information on biochemical and molecular responses of pomegranate under water stress. Hence, this study is the first report in this respect. Exploring differential antioxidant enzyme responses, gene expression levels associated with antioxidant defense systems and water stress responsive metabolites would provide valuable information for better understanding biochemical and molecular adaptation of pomegranate plants to drought stress conditions, thus enhancing the selection of drought tolerant cultivars. Therefore, the aim of present study was to investigate antioxidant enzyme activities, the changes in gene expression levels of antioxidant enzymes, proline accumulation, lipid peroxidation, and chlorophyll, carotenoid, and anthocyanin concentrations in the leaves of various pomegranate cultivars under severe water stress and recovery.

Materials and methods

Plant material and drought stress applications

The experiment was carried out during the 2014 growing season at the experimental greenhouse in the college of Agriculture, Shiraz University, Iran (latitude 29° 56'N; longitude 52° 02'E; 1810 m altitude). 2-year-old commercially pomegranate cultivars propagated by stem cuttings including 'Rabab-e-Neyriz' ('Rabab'), 'Shishe-cap-e-Ferdows' ('Shishecap'), 'Malas-e-Saveh' (M-Saveh), 'Malas-e-Yazdi' ('M-Yazdi'), and 'Ghojagh-e-Qom' ('Ghojagh') were used. They were grown under greenhouse conditions, in large containers (15 L) filled with a mixture of leaf mould, sand, and soil (1:1:1, by volume) and a gravel layer at the bottom. The

field capacity of the soil used for potting was determined according to the protocol described by (Richards 1949). Potted pomegranate trees were irrigated regularly for 4 months to field capacity level. The maximum and minimum temperatures during the experiment period were 36 and 16 °C, respectively, and mean relative humidity was approximately 55%. In order to simulate natural conditions, all of the pots were covered with jute bags. By the end of July, 32 plants of each cultivar were divided into two uniform groups: control plants and water-stressed plants. The first group (control plants) was watered daily to field capacity level during the whole experimental period, whereas the second group (water-stressed plants) subjected to a severe water stress period by withholding irrigation for 14 days until the plants showed a strong loss of turgescence and majority of leaves became withered and discolored. After the period of drought, stressed plants were re-watered to field capacity and followed by a recovery for 7 days. Leaf material was sampled at midday and immediately frozen in liquid nitrogen and stored at –80 °C until subsequent analyses.

Pigments

Total Chlorophylls (Chl_{a+b}) and carotenoids were determined spectrophotometrically according to the method of Lichtenthaler (1987). Leaf pigments were extracted using pure methanol and samples were kept in darkness at 4 °C for 24 h. The absorbance readings were taken at 470, 652.4, and 665.2 nm. The content of chlorophylls and carotenoids was expressed as mg g FW⁻¹.

Anthocyanin was extracted with methanol: water: concentrated HCl (80:20:1). Samples were put on a shaker in the dark at 4 °C for 48 h. Anthocyanin was measured spectrophotometrically according the method of Alexieva et al. (2001). The absorbance was read at 530 and 657 nm. Anthocyanin concentration was determined by the formula $[A] = A_{530} - 1/3 A_{657}$ and was given as A/g fresh weight of leaf tissue.

Determination of antioxidant enzyme activities

For antioxidant enzyme assays pomegranate leaves (0.5 g) were ground to a fine powder in liquid nitrogen and extracted by homogenization in 1 mL ice-cold 50 mM potassium phosphate buffer (pH 7.0) containing 2 mM EDTA and 2% (w/v) polyvinyl pyrrolidone (PVP). The homogenate was centrifuged at 14,000 rpm for 15 min, at 4 °C. The supernatant was used to measure the activities of SOD, G-POD, and CAT. All steps of the extraction procedure were carried out at 4 °C.

SOD activity in leaves was estimated by monitoring the decrease in absorbance of nitroblue tetrazolium (NBT) by the enzyme according to the method described by Giannopolitis and Ries (1977). One unit of SOD was defined as

the amount of enzyme that produced 50% inhibition of NBT reduction at 560 nm. SOD activity was expressed as SOD units per g fresh weight (Unit g FW⁻¹).

G-POD (EC 1.11.1.7) activity in leaves was based upon the method as described by Chance and Maehly (1955) which measure the oxidation of guaiacol in the presence of H₂O₂ to tetraguaiacol. The increase in absorbance was recorded at 470 nm for 1 min. G-POD activity was calculated according to the molar extinction coefficient of tetraguaiacol (26.6 mM⁻¹ cm⁻¹) and expressed as $\mu\text{mol g FW}^{-1} \text{ min}^{-1}$.

CAT (EC 1.11.1.6) activity in leaves was determined according the method of Chance and Maehly (1955). The decomposition of H₂O₂ was measured by the decrease in absorbance at 240 nm for 1 min. CAT activity was calculated according to the molar extinction coefficient of H₂O₂ (40 mM⁻¹ cm⁻¹) and expressed as $\mu\text{mol g FW}^{-1} \text{ min}^{-1}$.

Gene expression analysis

Total RNA from pomegranate leaves were isolated using the RNeasy Plant Mini Kit (Qiagen) following the manufacturer's instructions. Traces of genomic DNA were removed by DNase treatment (DNase I, Fermentas). The quantity and quality of RNA were determined on a Nanodrop (Thermo Scientific NanoDropTM 2000) and confirmed by agarose gel electrophoresis (1%). cDNA synthesis was performed by cDNA Synthesis Kit (Fermentas) according to the manufacturer's instructions. Gene expression profiles were obtained by real-time quantitative PCR (qRT-PCR) performed on a PCR device (C1000TM Thermal Cycler, Bio-Rad, USA). qRT-PCR was performed in a 12.5 μL reaction containing SYBR Green I PCR Master Mix (Fermentas), 0.15 μmol of each forward and reverse gene-specific primers, and 0.8 μL of diluted cDNA (1:10). Primer sequences used for qRT-PCRs of GPX, cytosolic GR and actin genes are listed in Table 1. Degenerated primers were designed for cytosolic GR gene, according to conserved regions from apple, pear, eucalyptus, and Japanese apricot. To normalize the relative expression of selected genes, actin gene was used as reference. The level of gene expression was given by $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen 2001).

Determination of lipid peroxidation

The level of lipid peroxidation in samples was determined by estimating the malondialdehyde (MDA) content according to the method of Lu et al. (2008). MDA of extract was determined using the thiobarbituric acid reaction. MDA concentration was calculated from the absorbance at 532 nm and the value of the non-specific absorption at 600 nm was subtracted.

Table 1 Sequence, annealing temperature and product size of primers used in real time PCR

Primer	Sequence (5'–3')	Annealing temperature (°C)	Product size (bp)
Cytosolic GR-F	TTGTACTSTTTGCCACTGGTCG	51	362
Cytosolic GR-R	CATTGGRKTGAADGTHGATGTG		
GPX-F	ATGTGGATCTGAGCACCTAC	51.8	141
GPX-R	AATGCCAGAATCTCCAGACC		
Actin-F	ATACCAGGGAACATGGTGA	52.8	185
Actin-R	ATTACCATTGGTGCGGAGAG		

The content of MDA was determined using the extinction coefficient of $155 \text{ mM}^{-1} \text{ cm}^{-1}$ and expressed in nmol MDA per g FW (Heath and Packer 1968).

Proline content

Proline concentration was determined spectrophotometrically by the ninhydrin method according to Bates et al. (1973) with some modifications. The amount of 100 mg of frozen leaf sample was homogenized in 1.5 mL 3% sulfolalicylic acid and centrifuged at 12,000 rpm for 10 min. 1 mL of the supernatant was reacted with 1 mL acid ninhydrin (1.25 g ninhydrin warmed in 30 mL glacial acetic acid and 20 mL 6 M phosphoric acid until dissolved) and 1 mL glacial acetic acid for 1 h in a water bath at 100°C and the reaction was then terminated in an ice bath. After cooling, the reaction mixture was extracted with 2 mL toluene, mixed vigorously with a test tube stirrer for 15–20 s. The absorbance of fraction with toluene aspired from liquid phase was read at 520 nm using toluene as a blank. Proline concentration was calculated using calibration curve and expressed as $\mu\text{mol proline g FW}^{-1}$.

Statistical analysis

Experimental units were arranged in a completely randomized design. The values of different measured parameters were expressed as means of four replicates. The results were presented as mean $\pm$ SD. Analysis of variance was performed by ANOVA procedures (SAS 9.1 for Windows). Significant differences were calculated according to Duncan's multiple range tests (DMRT, $P < 0.05$). Pearson's correlation coefficients (r) were tested among selected parameters using combined data from all five cultivars.

Results

Pigments

Foliar chlorophyll contents of water-stressed and control plants are shown in Fig. 1. After 14 days of withholding irrigation, Chl_{a+b} significantly decreased in all cultivars,

except 'Ghojagh'. As compared to control, the reduction of Chl_{a+b} in water-stressed plants were 5.25, 11.98, 34.33, 34.38, and 36.16% for 'Ghojagh', 'M-Saveh', 'Rabab', 'Shishecap', and 'M-Yazdi', respectively. Except for 'Ghojagh' and 'M-Saveh', plants showed no recovery for Chl_{a+b} at the end of recovery period.

Under severe water stress conditions, leaf carotenoid concentrations increased in 'Ghojagh' by 14.48%, in 'M-Saveh' by 13.08%, and in 'M-Yazdi' by 4.22% above control levels, while decreased about 5.34% in 'Rabab' and 4.5% in 'Shishecap', as compared with well-watered control plants (Fig. 1). The differences were not significant in 'Rabab', 'Shishecap', and 'M-Yazdi'. The levels of carotenoids in 'M-Saveh', 'Ghojagh', and 'M-Yazdi' dropped under recovery period.

A reduction in the ratio of $\text{Chl}_{a+b}/\text{carotenoid}$ was observed in the stressed plants at day 14, but 'Ghojagh' had the lowest reduction in the ratio than the other cultivars (Fig. 1). The reduction being much pronounced in 'M-Yazdi' cultivar. Except for 'Ghojagh', plants showed no recovery at the end of recovery period.

After 14 days of withholding water, all stressed plants showed a significant increase in leaf anthocyanin content (Fig. 1). Anthocyanin production increased in water-stressed plants by 136.52, 119.48, 86.72, 86.1, and 46.8% in 'M-Yazdi', 'Rabab', 'Shishecap', 'M-Saveh' and 'Ghojagh', respectively, as compared to the well-watered control plants. Anthocyanin content in all cultivars except 'M-Saveh', remained at a high level as compared to that in well-watered control plants at 7 days after re-watering.

Antioxidant enzyme activities

Severe water stress significantly increased SOD activity in all cultivars (Fig. 2). At the end of drying cycle, its activity increased by 42.68, 29.96, 19.04, 18.91, and 12.2% in 'Rabab', 'Shishecap', 'M-Saveh', 'M-Yazdi', and 'Ghojagh', respectively, more than that in their control levels. After 14 days of withholding water, G-POD activity significantly was enhanced in all cultivars except 'Ghojagh' and 'M-Saveh' (Fig. 2). G-POD activity in drought-stressed plants of 'Ghojagh' and 'M-Saveh' were similar to that of well-watered control plants. It increased in water-

Fig. 1 Total chlorophyll (Chl_{a+b}), carotenoid, Chl_{a+b} /carotenoid ratio and anthocyanin contents of pomegranate plants subjected to severe water stress and re-watering. Bars represent the standard deviation (SD) of the mean

stressed plants by 63.98, 44.07, and 32.72% in 'M-Yazdi', 'Rabab' and 'Shishecap', respectively, as compared to well-watered control plants.

Fig. 2 Superoxide dismutase (SOD), Guaiacol peroxidase (G-POD) and Catalase (CAT) activities in pomegranate plants subjected to severe water stress and re-watering. Bars represent the standard deviation (SD) of the mean

After 7 days of re-watering, the SOD activity in all cultivars and G-POD activity in 'Rabab', 'Shishecap', and 'M-Yazdi' remained at a high level as compared to those of well-watered control plants. The G-POD activity in recovery period was elevated in 'M-Saveh' and 'Ghojagh' submitted to 14 days of stress. After 14 days of withholding irrigation, CAT activity was significantly increased in all cultivars, except 'M-Yazdi' (Fig. 2). Its increases were 110.75, 84.62, 56.18, and 25.56% for water-stressed plants of 'Rabab', 'Shishecap', 'M-Saveh', and 'Ghojagh', respectively, as compared to their controls. A significant increase in CAT activity (compared to water-stressed plants) was observed in 'M-Yazdi' at the end of recovery period. After 7 days of re-watering, CAT activity in

‘Shishecap’ was similar to that of water-stressed plants. CAT activity in ‘Rabab’, ‘M-Saveh’, and ‘Ghojagh’, remained at a high level as compared to that of well-watered control plants at 7 days after re-watering.

Gene expression

To understand molecular responses of different pomegranate cultivars at transcriptional level during severe water stress, transcript levels of two representative water stress-responsive genes, GPX and cytosolic GR, were detected by qRT-PCR. After 14 days of drought stress, the transcript levels of GPX clearly increased in all cultivars, except ‘M-Yazdi’ (Fig. 3). Expression of GPX was down-regulated in ‘M-Yazdi’ by severe drought stress. The transcript levels of the GPX increased in water-stressed plants by 46.0, 5.58, 3.66, and 1.81-Fold in ‘Rabab’, ‘Shishecap’, ‘M-Saveh’, and ‘Ghojagh’, respectively, as compared to the well-watered control plants.

Drought stress clearly increased cytosolic GR expression levels in ‘Rabab’ and ‘Shishecap’ while it down-regulated the gene in ‘M-Saveh’ and ‘Ghojagh’ (Fig. 3). It is interesting to note that the expression of cytosolic GR was suppressed in ‘M-Yazdi’ under severe water stress

Fig. 3 Effect of severe water stress on relative gene expression levels of glutathione peroxidase (GPX) and glutathione reductase (cytosolic GR) in pomegranate plants. Bars represent the standard deviation (SD) of the mean

conditions. After 14-day of severe water stress, the transcript levels of cytosolic GR decreased about 0.7-fold in ‘M-Saveh’ and 0.97-fold in ‘Ghojagh’, but increased about 41.68-fold in ‘Rabab’ and 6.37-fold in ‘Shishecap’, as compared with well-watered control plants.

Lipid peroxidation

After 14 days of severe drought, all stressed plants showed a significant increase in MDA concentration (Fig. 4). MDA levels increased in water-stressed plants by 34.82, 28.45, 20.97, 13.18 and 9.93% in ‘M-Yazdi’, ‘Shishecap’, ‘M-Saveh’, ‘Rabab’, and ‘Ghojagh’, respectively, as compared to the well-watered control plants. After 7 days of re-watering, MDA concentrations of stressed plants of ‘Rabab’ and ‘M-Saveh’ was restored to control levels, while it remained at a high level in ‘M-Yazdi’ and ‘Shishecap’ as compared to that in well-watered control plants. After re-watering, MDA levels dropped in ‘Ghojagh’ (14.66%) at a level below that of the well-watered control plants.

Proline

Severe water stress significantly increased proline production in all five cultivars (Fig. 5). After 14 days without water supply, percentage increase of proline production compared with well-watered control plants were about 32.03, 133.08, 166.97, 182.47, and 204.45 for Ghojagh’, ‘M-Saveh’, ‘Shishecap’, ‘Rabab’, and ‘M-Yazdi’, respectively. Proline concentration in all five cultivar dropped significantly under recovery period.

Relationship among pigments, MDA, proline, RWC and antioxidant enzymes

The relationships among pigments, MDA, proline, RWC and antioxidant enzymes are shown in Table 2. Chl_{a+b}

Fig. 4 Effect of severe water stress and re-watering on lipid peroxidation (expressed as malondialdehyde (MDA) content) in pomegranate plants. Bars represent the standard deviation (SD) of the mean

Fig. 5 Effect of severe water stress and re-watering on proline accumulation in pomegranate plants. Bars represent the standard deviation (SD) of the mean

content at 14 (severe water stress) and 21 (recovery period) days after beginning the experiment was negatively correlated with MDA, anthocyanin, antioxidant enzymes (SOD, G-POD and CAT), and proline, whereas it was only positively correlated with RWC at the end of drying cycle. MDA was positively correlated with anthocyanin, proline, SOD, G-POD and CAT activities at the end of drying cycle and recovery, but was negatively correlated with RWC at the end of drying cycle. Proline was positively correlated with anthocyanin content and antioxidant enzyme activities at the end of drought stress, but was negatively correlated with RWC. There were negative correlation between RWC and anthocyanin and antioxidant enzymes at 14 and 21 days after start the experiment.

Discussion

A significant decrease in Chl_{a+b} concentration, as a result of fast degradation of their molecules, has been considered as a typical symptom of oxidative stress (Liu et al. 2011). Except for 'Ghojagh', Chl_{a+b} concentration in all cultivars declined at the end of the drying cycle. The reduction being much pronounced in 'M-Yazdi' cultivar. There was a significant decrease in leaf relative water content (RWC) in all cultivars except 'Ghojagh' (data not shown). The better leaf water status in 'Ghojagh' under severe drought stress allowed the plant to continue metabolic activities and maintain light dissipation systems. Therefore, it prevented degradation of chlorophyll molecules under severe water stress. The positive correlation between Chl_{a+b} and RWC in the studied cultivars suggested that the staying healthy of chlorophyll could be mainly attributed to the maintenance of RWC. 'Ghojagh' and 'M-Saveh' showed a good recovery for Chl_{a+b} after re-watering. The ability of rapid recovery of chlorophyll and photosynthesis after drought stress may play an important role in plant adaptation to drought environments (Chartzoulakis et al. 2002).

Increased carotenoid in drought-stressed leaves of 'Ghojagh' and 'M-Saveh' could confirm its possible roles in photosynthesis as a structural component of photosynthetic systems and as an antioxidant that scavenge $^1\text{O}_2$ to protect the photosynthetic complexes. Lower values for $\text{Chl}_{a+b}/\text{carotenoid}$ ratio are an indicator of stress and damage to the photosynthetic complex, which is explained by a faster degradation of chlorophylls than carotenoids (Lichtenthaler and Buschmann 2001). 'Ghojagh' had the lowest reduction in the ratio $\text{Chl}_{a+b}/\text{carotenoid}$ than those of other cultivars at end of severe water stress. The decline being much pronounced in 'M-Yazdi' cultivar. In addition to serving in osmotic adjustment (Hughes et al. 2013), anthocyanins play roles as antioxidant that scavenge H_2O_2 to protect plants from oxidative damage (Hatier and Gould 2008). In our research, 'Ghojagh' with the highest chlorophyll content had the lowest amount of anthocyanin, whereas 'M-Yazdi' with the lowest chlorophyll content had the highest one. It is interesting to note that the RWC of 'Ghojagh' at the end of drying cycle was high which shows this cultivar was less affected by drought stress. Therefore, it seems that increased production of anthocyanin in pomegranate is the result of severe water stress and ROS accumulation, rather than osmotic adjustment. Anthocyanins may play a role in scavenging ROS in pomegranate under drought stress. According to our results, SOD, POD and CAT activities at 14 days after withholding water were negatively correlated with Chl_{a+b} and RWC. Therefore, a reduction in plant water status and chlorophyll content, as a result of drought stress, could induce enzyme activities to protect plants from ROS injury. Increased SOD, G-POD and CAT activities, key ROS scavenging enzymes, in the pomegranate stressed plants showed that these enzymes play positive roles in controlling the cellular level of ROS under water deficit conditions. Lower SOD activity in 'Ghojagh' might translate a lower generation of O_2^- by the water–water cycle by maintaining slight stomatal opening, thus avoiding complete inhibition of CO_2 fixation, as also documented by Cruz de Carvalho (2008). Except for 'M-Yazdi', CAT activity in all cultivars increased markedly at the end of the drying cycle. It is interesting to note that the increase in G-POD activity in 'M-Yazdi' under severe water stress was larger than that in the other cultivars. It seems that in 'M-Yazdi', G-POD would be involved in the detoxifying of H_2O_2 that has not been scavenged by CAT. Compared to the well-watered control plants, higher activities of SOD, CAT, and G-POD in all pomegranate cultivars after re-irrigation indicate that the enzyme activities would be needed for scavenging of ROS when plants were re-watered. The increased activities of antioxidant enzymes such as APX, MDHAR, and DHAR in re-watering phase were also found in the leaves of Kentucky bluegrass that subjected to the severe water stress (Bian

Table 2 Pearson's correlation coefficients (r) among pigments, MDA, proline, RWC and antioxidant enzymes in various pomegranate cultivars at 14 (severe water stress) and 21 (recovery period) days after start the experiment

	Chl _{a+b}	MDA	Proline	RWC	Anthocyanin
<i>Day 14 (severe water stress)</i>					
Carotenoid	0.22 ns	0.24 ns	0.05 ns	0.02 ns	0.19 ns
Chl _{a+b}		−0.79***	−0.89***	0.62***	−0.87***
MDA			0.92***	−0.64***	0.94***
Proline				−0.76***	0.97***
RWC					−0.70***
CAT	−0.45*	0.46**	0.65***	−0.76***	0.55**
SOD	−0.76***	0.80***	0.92***	−0.78***	0.88***
G-POD	−0.77***	0.76***	0.82***	−0.46**	0.81***
<i>Day 21 (recovery period)</i>					
Carotenoid	0.38*	−0.26 ns	−0.12 ns	0.05 ns	−0.14 ns
Chl _{a+b}		−0.60***	−0.38*	0.31 ns	−0.67***
MDA			0.59***	−0.35 ns	0.21 ns
Proline				−0.09 ns	−0.027 ns
RWC					−0.36*
CAT	−0.79***	0.58***	0.22 ns	−0.32 ns	0.63***
SOD	−0.74***	0.56**	0.038 ns	−0.50**	0.72***
G-POD	−0.66***	0.52**	0.23 ns	−0.53**	0.53**

Units: total chlorophyll (Chl_{a+b}), mg g FW^{−1}; carotenoid, mg g FW^{−1}; malondialdehyde (MDA), nmol g FW^{−1}; proline, μmol g FW^{−1}; leaf relative water content (RWC), %; anthocyanin, A g FW^{−1}; catalase (CAT), μmol g FW^{−1} min^{−1}; superoxide dismutase (SOD), U g FW^{−1}; guaiacol peroxidase (G-POD), μmol g FW^{−1} min^{−1}

n.s not significant

* $P < 0.05$

** $P < 0.01$

*** $P < 0.001$

and Jiang 2009). Reports on antioxidant enzyme activities under water deficit conditions are heterogeneous. Levels of these antioxidants have been shown to differentially alter, that is, they may be increased, remain unchanged or even decreased (Abedi and Pakniyat 2010; Chakraborty and Pradhan 2012; Gholami et al. 2012; Liu et al. 2011; Slabbert and Krüger 2014; Sun et al. 2013; Türkan et al. 2005). The study of expression patterns of genes encoding antioxidant enzymes could be valuable for understanding molecular adaptation of plant to drought stress conditions. Increased expression of cytosolic GR in 'Rabab' and 'Shishecap' during drought stress could have been responsible for the reduction of oxidized glutathione for the chain reactions of detoxifying H₂O₂ by GPX and APX, as also documented by Gratao et al. (2005). Since the increased expression or activity of antioxidant enzymes reflect the need for scavenging of the ROS (Jung 2004), the increase in expression of cytosolic GR and GPX could indicate elevated generation of the deleterious products. The expression levels of GPX and cytosolic GR in 'Rabab', in comparison to the other cultivars, were markedly up-regulated by severe water stress. This response in 'Rabab' leaves may indicate that its cellular organelles have an

effective mechanism of ROS scavenging. If water deficit is continued over to a certain extent, ROS generation will affect the detoxifying role of antioxidant system and lead to imbalance between antioxidant defense systems and the amount of ROS resulting in extensive cellular injury and death (Cruz de Carvalho 2008). Therefore, it seems that suppressed and decreased expression of cytosolic GR and GPX in 'M-Yazdi' are the results of ROS accumulation and antioxidant defenses depletion. After 14 days of severe water stress the leaves of 'Rabab', 'Shishecap', and 'M-Yazdi' showed evident symptoms of stress (withering and discoloring). The symptoms in 'M-Saveh' was fewer than above cultivars. 'Ghojagh', in comparison to the other cultivars, presented no symptoms of stress, which could indicate better tolerance against water stress. Moreover, the values of Chl_{a+b} and Chl_{a+b}/carotenoid in 'Ghojagh' and 'M-Saveh' were higher than the other cultivars. Therefore, it seems that decreased expression of cytosolic GR in 'Ghojagh' and 'M-Saveh' is not the result of ROS accumulation and antioxidant defenses depletion. Unlike cytosolic GR, expression of GPX in 'Ghojagh' and 'M-Saveh' was up-regulated by severe drought stress. This response suggests a relatively important role of GPX in the

antioxidative defense. Under severe water stress, lipid peroxidation of cell membranes might lead to structural alterations in their integrity and stability (Reddy et al. 2004). Chakraborty and Pradhan (2012) found that MDA content was lower in the leaves of drought tolerant *Triticum aestivum* L. than that in susceptible varieties. Our data indicate that the levels of membrane lipid peroxidation were relatively less in ‘Ghojagh’ and ‘Rabab’ cultivars under severe drought stress. The increase in membrane lipid peroxidation in ‘M-Yazdi’ was accompanied by decline in CAT activity. The increased H₂O₂ levels under drought stress conditions would be reduced through the combined action of CAT and G-POD (Ali et al. 2008). The marked reduction of MDA concentration in ‘Ghojagh’, ‘Rabab’, and ‘M-Saveh’ after re-watering represents that these cultivars have a good adaptation to drought environments. Proline is one of the most important compatible solutes, and its concentration has been suggested as a general marker for screening drought tolerant cultivars (Ahmed et al. 2009; Liu et al. 2011). Sun et al. (2013) reported that the accumulation of proline in chrysanthemum under drought stress was the result of damage and was not a suitable marker for chrysanthemum drought tolerance. The highest proline accumulation founded in ‘Rabab’, while only a slight increase in it occurred in ‘Ghojagh’. ‘Ghojagh’ had the highest value of RWC under drought stress. The improved performance of ‘Ghojagh’ under drought stress may be associated with an efficient osmotic adjustment. We found a negative correlation between proline accumulation and RWC, indicating that proline does not play a role in osmotic adjustment in pomegranate. Proline concentration in pomegranate plants dropped under recovery period. It seems that the increased accumulation of proline in pomegranate during severe drought stress could be an indication of plant water status, rather than drought tolerance as it was also found by Slabbert and Krüger (2014) in *Amaranthus*. Proline content was positively correlated with SOD, G-POD and CAT activities at the end of drying cycle. It seems that proline may play an important role in reducing the injury caused by severe water stress by activating antioxidative defense mechanism (Ahmed et al. 2009; Slabbert and Krüger 2014).

Conclusions

To the best of our knowledge, this is the first research studying biochemical and molecular responses of pomegranate plants under drought stress. Increased SOD, GPOD and CAT activities in the pomegranate stressed plants showed that these enzymes play positive roles in controlling the cellular level of ROS under drought conditions.

The increased activities of SOD, G-POD, and CAT were observed after re-watering as compared to the control. The expression levels of cytosolic GR and GPX genes in ‘Rabab’, in comparison to the other cultivars, were markedly up-regulated by severe water stress. ‘Ghojagh’ had the highest Chl_{a+b} content and Chl_{a+b}/carotenoid ratio at the end of drying cycle. Lower levels of MDA were noticed in the leaves of ‘Ghojagh’ and ‘Rabab’ under drought stress. High membrane lipid peroxidation in ‘M-Yazdi’ was associated with higher oxidative stress and lower antioxidant defense system. However, it can be concluded that the most tolerant cultivars were ‘Ghojagh’ and ‘Rabab’, and ‘M-Yazdi’ was the most susceptible cultivar studied. This research will provide information for developing selection and breeding in pomegranate plants.

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