RESEARCH ARTICLE

Antioxidant and carbohydrate changes of two pomegranate cultivars under deficit irrigation stress

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Abstract

The purpose of this study was to evaluate the biochemical responses to water stress tolerance of two pomegranate cultivars, 'Rabbab' and 'Shishehgap'. After the establishment of rooted stem cuttings of both cultivars under greenhouse conditions, they were treated with four levels of deficit irrigations (100%, 75%, 50% and 25% of field capacity) in a completely randomized design with four replications. The results showed a significant difference between the two cultivars regarding antioxidant enzymes activities. In both cultivars, the water stress increased the activity of superoxide dismutase, catalase and ascorbate peroxidase. However, at high water deficit (25% field capacity, FC), 'Rabbab' showed significantly higher enzyme activity than 'Shishehgap'. In each level of irrigation, there were not considerable differences in peroxidase activity between the two cultivars. An increment of 162% and 65.5% in soluble sugar was gained at 50% FC in 'Rabbab' and 'Shishehgap', respectively. 'Rabbab' showed better growth performance in each level of irrigation than 'Shishehgap'. Therefore, it can be concluded that 'Rabbab', with lesser decline in leaf relative water content (RWC), a strong antioxidant system and accumulation of more soluble carbohydrates, can resist higher water stress than 'Shishehgap'.

Additional key words: field capacity; enzyme activity; *Punica granatum* L.; water stress.

Abbreviation used: APX (ascorbate peroxidase); CAT (catalase); DW (dry weight); FC (field capacity); FW (fresh weight); NBT (nitro blue tetrazolium); POD (peroxidase); ROS (reactive oxygen species); RWC (leaf relative water content); SOD (super-oxide dismutase).

Introduction

Growth and development of plants are limited by water stress. Water stress is the main cause of farm production reduction (Blum, 2011). Iran is a dry country because the average of rain is only about 150 mm or less in dry and semidry zone that cover more than 65% of Iran areas (Eslamian & Soltani, 2002). Planting fruit trees that are low water consumers can be a suitable strategy for arid and semi arid regions in the world (Greenwood *et al.,* 2010; Jiménez *et al.,* 2010).

Pomegranate (*Punica granatum* L.) is an ancient fruit-bearing deciduous shrub or small tree which is native from Iran to the Himalayas in northern India and has been cultivated and naturalized over the whole Mediterranean region (Morton, 1987; Zamani *et al.,* 2007). The pomegranate tree grows in wide range of climates and soil conditions, because of its highly compatibility. This tree grows also in many different geographical areas such as Spain (Galindo *et al.,* 2014), Italy and California (Holland *et al.,* 2009). The optimal climate growth condition for pomegranate exists in Mediterranean-like climate as follows: high exposure to sunlight; mild winters with minimal temperature not lower than -12 °C; and hot summers without rain (Levin, 2006). Iran is the world's largest producer of pomegranate, with an annual production of 600,000 tons spread over in 65,000 ha of land under cultivation with ~10% exported to other parts of the world (Hol-

land & Bar-Ya'akov, 2008). Two cultivars of pomegranate, 'Rabab' (Rabab-e-Neiriz) and 'Shishehgap' (sheshe-Cap-Ferdows), are suitable for export due to their skin thickness, small aril, good taste, and longtime storage (Varasteh *et al.,* 2009).

Many studies have evidenced the promising health features of pomegranate fruit (Mena *et al.,* 2011). Consequently, an increase in cultivation of pomegranate in subtropical and tropical area of the world has been observed, leading to commercial orchard establishment (Al-Said *et al.,* 2009; Holland *et al.,* 2009; Fawole *et al.,* 2012).

One of the main steps in orchard establishment is selection of suitable cultivars. Plant species have different tolerances to drought stress (Wang *et al.,* 2012). Adverse environmental conditions (such as drought) can cause oxidative stress when the over formation of free radicals such as superoxide anion ($O_2 \bullet -$), hydroxyl radical ($\bullet OH$), as well as non-radical molecules like hydrogen peroxide (H_2O_2), the so-called reactive oxygen species (ROS) occur in the plants (Gill &Tuteja, 2010). In stress condition, mal function of electron transport system leads to stepwise reduction of molecular oxygen (O_2) and production of the highly reactive ROS (Saraswathi & Paliwal, 2011). Despite signaling role, ROS are highly toxic. The enhanced production of ROS cause peroxidation of lipids, oxidation of proteins, damage to nucleic acids, enzyme inhibition, activation of programmed cell death (PCD) pathway and ultimately leading to death of the cells (Khalvati *et al.,* 2010; Miller *et al.,* 2010). Whether ROS will act as damaging or signaling molecule depends on the delicate equilibrium between ROS production and scavenging. The production of ROS occurrs in the cell at their production sites, including chloroplast, mitochondria and peroxisomes, where vital processes such as photosynthesis, respiration and photorespiration take place (Mittler, 2002).

Fortunately, plant species have specific defense mechanisms that make them enable to deal with drought stress. These defense mechanisms include antioxidant systems (Li *et al.,* 2009; Aghaleh *et al.,* 2010) and osmotic adjustment (by increasing of compatible metabolites) (Hongbo *et al.,* 2006). Plant antioxidants contain enzymatic and non-enzymatic systems. Enzymatic antioxidant system includes superoxide dismutase (SOD), peroxidase (POD), catalase (CAT) and ascorbate peroxidase (APX). Non-enzymatic low molecular metabolites comprise ascorbate (ASA), glutathione (GSH), α-tocopherol, carotenoids and phenolic compound (Chaves & Oliveria, 2004; Mittler *et al.,* 2004; Becana *et al.,* 2010). The maintenance of a high antioxidant capacity to scavenge the toxic ROS has been linked to increased tolerance of plants to environmental stresses (Sharma *et al.,* 2012). Zhang *et al.* (2010) have also shown that plants species can tolerate water stress by synthesis and accumulation of low molecular mass organic solutes such as soluble sugars, proline or other amino acids to regulate the osmotic potential of cells. The aim of this research was to investigate the role of antioxidant enzymes and carbohydrates in drought tolerance of two pomegranate cultivars.

Material and methods

The experiment was carried out from January 2012 to September 2013, in a greenhouse (52° 32' E, 29° 36' N) at day/night temperatures of 34º/16º ± 3° C and mean relative humidity of 50% under natural sun light. Ninety-six certificated disease-free one-year old rooted cuttings of two cultivars of pomegranate, 'Rabbab' and 'Shishehgap' (48 cuttings each cultivar) were supplied from Baharestan nursery, Neiriz, Fars province, Iran. They were disinfected by a fungicide, 3% Benomyl (Averstar Industrial Co., Ltd. Shenzhen, China) and then transferred to 10-L plastic pots containing 1/3 sand, 1/3 clay loam soil, 1/3 of peat moss, measured by volume, without drainage. The field capacity (FC) of the soil mixture used for potting was determined according to the protocol described by Richards (1949) using pressure plate apparatus. The amount of moisture of soil mixture at FC was calculated to be 20%, based on soil mixture dry weight (DW).

Potted plants were irrigated daily for four months to field capacity level, until the plants were established. Then, the experiment was conducted in complete randomized design with 4 replications and 3 plants in each replicate (12 plants in each treatment). Treatments were 4 levels of irrigation: 100% FC (control) 75%, 50% and 25% FC. The water supplies were maintained by weighing the pots every day. After three months of deficit irrigation treatments, the lengths of new shoots as a growth index were recorded using a ruler. The leaf relative water content (RWC) was determined as (FW − DW)/(TW − DW)×100, where FW is the fresh weight, DW is the dry weight after oven-drying the leaves at 80°C for 24 h, and TW is the turgid weight after re-hydrating the leaves at 4°C. For evaluation of biochemical responses, sampling and measurements were made, at least, on 10 fully expanded young leaves in each replicate.

For estimations of enzymes activities, leaves (0.5 g) were first homogenized in 50 mM potassium phosphate buffer (pH 7.8) containing 1 mM EDTA (ethylene diamine tetraaeetic acid), 3 mM 2-mercaptoethanol, and 2% (w/v) polyvinyl polypyrrolidone (PVPP) in a chilled mortar. The homogenate was then centrifuged

at 16000 g for 30 min at 4 °C and the supernatant was used for enzyme assays.

Superoxide dismutase

Total SOD (EC 1.15.1.1) activities were estimated by measuring its ability to inhibit the photochemical reduction of nitro blue tetrazolium complex (NBT) by the enzyme at 560 nm as described by Dhindsa *et al.* (1981) in a reaction mixture consisting of 0.1 mL enzyme extract, 50 mM sodium phosphate buffer (pH 7.5), 13 mM L-methionine, 75 μM NBT, 0.1 mM EDTA, and 75 μM riboflavin. The reaction mixture was irradiated for 12 min and absorbance was recorded at 560 nm (using Biochrom WPA Biowave II UV/Visible Spectrophotometer, England) against the non-irradiated blank. One unit of SOD activity is defined as the amount of enzyme, which caused 50% inhibition in NBT reduction.

Ascorbate peroxidase

APX (EC 1.11.1.11) activity was estimated by Nakano & Asada´s (1981) method. Briefly, each 3 mL of the reaction medium contained 50 mM K-phosphate buffer (pH 7.0), 0.1 mM H_2O_2 and 20 μL enzyme extract. APX activity was evaluated by the decrease in absorbance at 290 nm (using Biochrom WPA Biowave II UV/Visible Spectrophotometer, England) as ascorbate was oxidized.

Catalase

The determination of the activities of CAT (EC 1.11.1.6) was based on the rate of H_2O_2 decomposition as measured by decreasing the absorbance at 240 nm (Dhindsa *et al.,* 1981). The solution contained 50 mM potassium phosphate buffer (pH 7.0) and 15 mM H_2O_2. The reaction was started by the addition of 100 μL of enzyme extract to the reaction mixture and the change in absorbance was followed 1 min after the reaction started. One unit of activity is the amount of enzyme that decompose 1 mM of H_2O_2 in one minute.

Peroxidase

POD activity (EC 1.11.1.7) was measured according to the method of Chance & Maehly (1955). The tetra-guaiacol formed in the reaction has a maximum absorption at 470 nm and thus the reaction can be followed spectrophotometrically. The enzyme was determined in a solution including 50 mM phosphate buffer (pH 7.0), 5 mM H_2O_2 and 13 mM guaiacol. The reaction was initiated by adding 33 μL of POD extract at 25°C. One unit of enzyme was calculated on the basis of the formation of guaiacol to tetraguaiacol for 1 min.

Total sugars and starch

Total sugars were estimated by the method of Dubois *et al.* (1956). Cold anthrone reagent (14 mL) was added to 1 mL of ethanol extract sample. This mixture was shaken vigorously and boiled for 10 min in a boiling water-bath. After cooling in running tap water, the absorbance was read at 620 nm in a spectrophotometer. The amount of total sugar was estimated with reference to a glucose standard curve.

The amount of starch was estimated by the method of McCready *et al.* (1950). The residue left behind after alcoholic extraction of the leaf materials was dissolved in 5 mL of 52% perchloric acid (PCA) for 1 h. The mixture was filtered through Whatman No. 42 filter paper and the filtrate was made up to 100 mL with distilled water. To 1 mL of the PCA extract, 4 mL of distilled water and 10 mL of freshly prepared cold anthrone reagent were added carefully along the side of the tube. The contents of the tubes were shaken vigorously and heated in a boiling water bath for 7.5 min. The tubes were then cooled immediately in running tap water and shaken well before reading the color intensity at 630 nm. The starch content calculated with reference to glucose standard, and multiplied by 0.9 and expressed in mg/g DW.

Statistical analysis

The experiment was carried out as factorial in completely randomized design. The factors were two cultivars of pomegranate and four levels of water stress with four replications including three plants in each replication (so, 12 plants per treatment). Data were analyzed using SAS software (vers. 9.0; SAS Inst. Inc., Cary, NC, USA) and the means were compared at 5% probability using Tukey's multiple range test.

Results

The growth of two genotypes was differently influenced by water deficit. In 'Shishehgap', with reduction of water rate to 50% and 25% FC, the average lengths of new shoots decreased 21% and 50%, while the re-

duction for 'Rabbab' was 4% and 23% respectively (Fig. 1a). However, there was not significant difference between the two genotypes in 100% and 75% FC. In both cultivars, in high water deficit (25% FC) necrosis in fringe and apex of the leaves initiated after one month of starting treatments and gradually developed in all part of the leaves leading to leaf drop. Figure 2 shows that this was more severe for 'Shishehgap' than for 'Rabbab'. With increasing water stress to 25% FC, RWC significantly decreased in both cultivars; however, this reduction was more pronounced in 'Shishehgap' than in 'Rabab'. In 50% FC 'Shishehgap' had a significant 30% decrease in RWC, while this reduction was 12.5% in 'Rabab', non-significant in comparison to the control (Fig. 1b).

SOD enzyme activity

The analysis of variance (ANOVA) shows that there was a significant difference between irrigation regimes, cultivars and their interaction on SOD activity (Table 1). In both cultivars, when reducing the amount of irrigation water, the activity of SOD increased (Fig. 3a). However, at high irrigation deficit (25% FC), the rate of SOD activity was significantly higher (445 U/g FW) in 'Rabbab' than in 'Shishehgap' (313 U/g FW).

CAT activity

The analysis of variance (ANOVA) shows that there was a significant difference between irrigation regimes,

cultivars and their interaction on CAT activity (Table 1). In 'Rabbab', with increasing water stress, CAT activity significantly increased from 251 U/g FW at 100% to 662 U/g FW at 25% FC. However, in 'Shishehgap' there were not significant differences between CAT activities in the four levels of irrigation (Fig. 3b).

APX activity

The analysis of variance (ANOVA) shows that there was a significant difference between irrigation regimes, cultivars and their interaction on APX activity (Table 1). When the severity of water stress increased, the APX activity in leaves of both cultivars increased (Fig. 3c). In 'Rabbab', at 25% FC, APX activity was 2.5 times greater than APX activity at 100% FC. In 'Shishehgap', although this activity at 25% FC was 1.5 folds higher than APX activity at 100% FC, there was a not statistically significant difference between them.

POD activity

The analysis of variance (ANOVA) shows that there was a significant difference between irrigation regimes and interaction between irrigation and cultivar on APX activity (Table 1). In both cultivars, with reducing the amount of irrigation water, the activity of POD increased (Fig. 3d). At high water stress (25% FC), 'Rabbab' cultivar increased 4.4 times its POD activity in comparison with 100% FC.
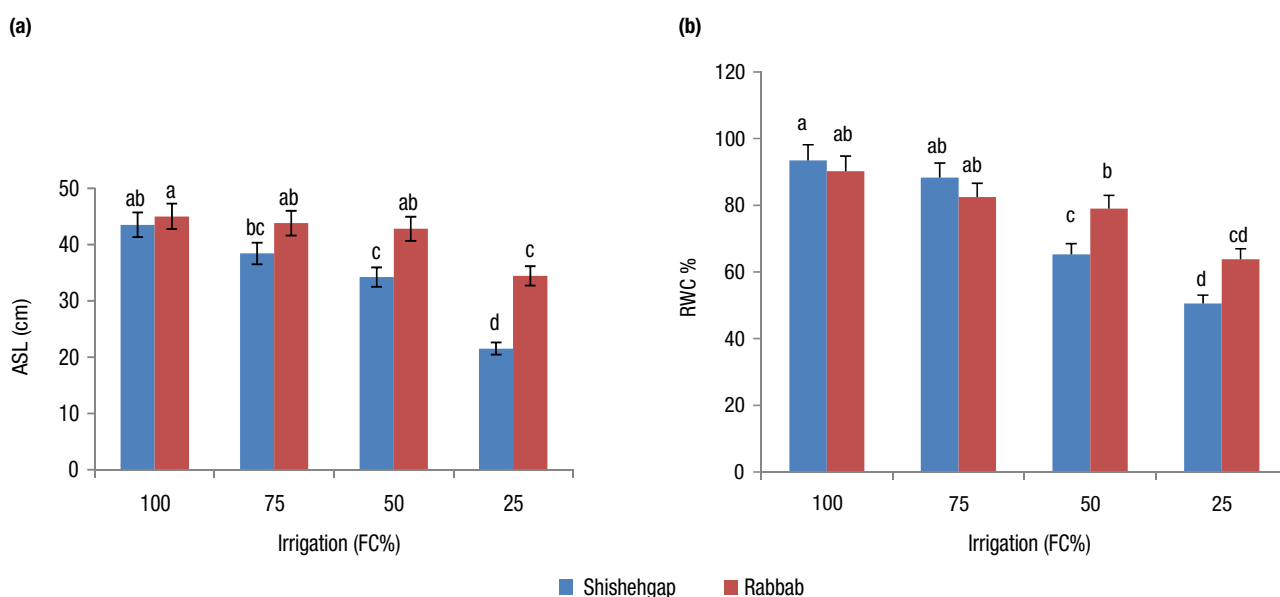

Figure 1. Effects of deficit irrigation on average shoot length, ASL (a) and on leaf relative water content (RWC) (b) of two pomegranate cultivars. Means with the same letters are not significantly different (*p*<0.05) using Tukey's multiple rang test.
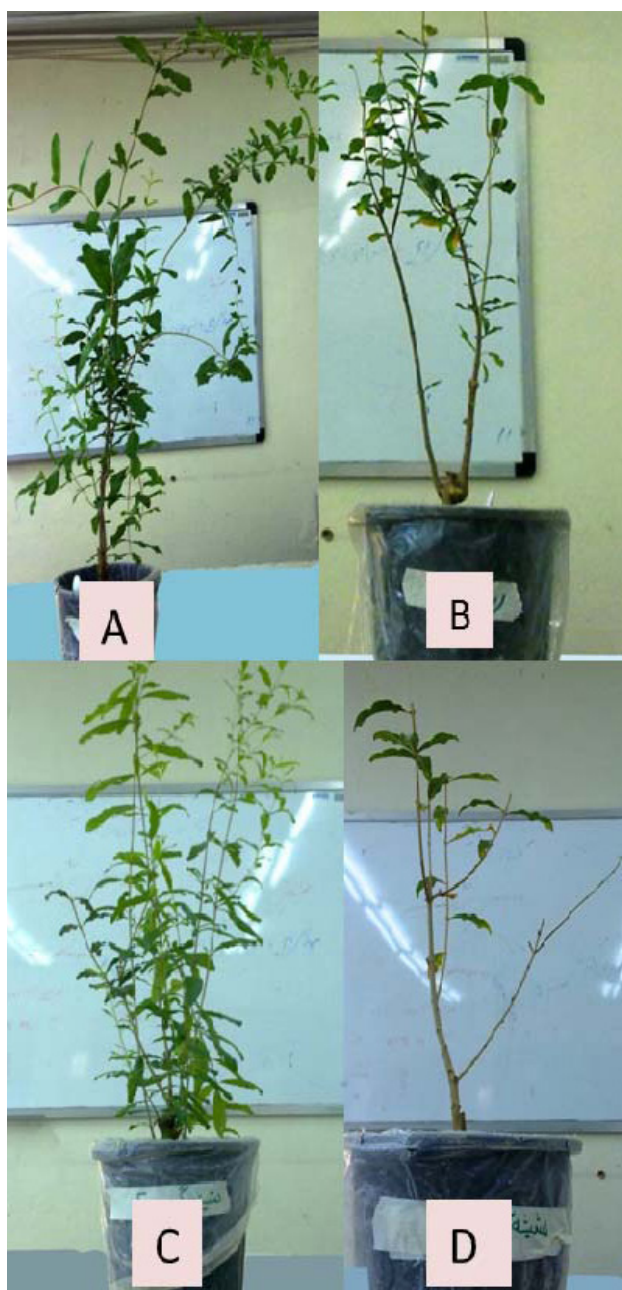
Sugar and starch

The results showed that the concentration of soluble carbohydrates increased in both cultivars in moderate water stress and then significantly decreased in high irrigation deficit (25% FC) (Table 2). Totally, the highest sugar rate (34.1 mg/g DW) was recorded in 50% FC, which was significantly higher than in the control (16.3 mg/g DW) and in 25% FC (23.3 mg/g DW). There was no significant difference between the two cultivars in each level of irrigation. Collapse of insoluble carbohydrate can result in an increase in the level of soluble sugar during drought stress. However, the concentration of sugar significantly decreased at high water stress (25% FC) (Figs. 4a and 4b). In relation to starch content, although with increasing water stress the amount of starch decreased, this decline was not significant except at 25% FC, in which the amount of starch significantly decreased (Table 2).

Discussion

Water deficit affected the average length of new shoots and necrosis and abscission of leaves occurred under severe water stress. This abscission may be due to hormonal imbalance and enhanced synthesis of the endogenous plant hormones ethylene (Gomez-Cadenas et al., 1996). In water stress conditions (50% and 25% FC) 'Rabab' cultivar had higher RWC than 'Shi Medrano shehgap'. Thakur (2004) reported that under water deficit conditions, cultivars with lower decline in RWC were capable of retaining higher internal water status, which enables the plants to maintain the hydration of protoplasm for longer duration.

Abiotic stresses such as drought disrupt the balance between production and elimination of reactive oxygen species, and this lead to a sharp increase in intracellular levels of reactive oxygen species that cause significant damage to the cell structure (Gill & Tuteja, 2010).

Water deficit tolerance is often related with a more impressive antioxidative system (Tahi et al., 2008;

Figure 2. Growth of two pomegranate genotypes in control (100% FC) and severe water deficit (25% FC) during three months from May to July 2013: 'Rabab' in 100% FC (A), 'Rabab' in 25% FC (B), 'Shishehgap' in 100% FC (C), and 'Shishehgap' in 25% FC (D).

Table 1. Analysis of variance for different enzymes (CAT, catalase; SOD, superoxide dismutase; POD, peroxidase; APX, ascorbate peroxidase) activities of two pomegranate cultivars under water stress.

Source of variation	Df	CAT	SOD	POD	APX
Irrigation	3	109424**	135237**	1540**	9563023**
Cultivar	1	42411*	37339**	105ns	3606626*
Irrigation × Cultivar	3	28674*	6199*	106*	1751766*
Error	24	8452	1863.4	32.4	558702
CV		23.9	20.8	23.3	20.7

CV: coefficient of variation. Df: degree of freedom. **,*, ns: significant at level of 0.1, 0.05 and non-significant, respectively.
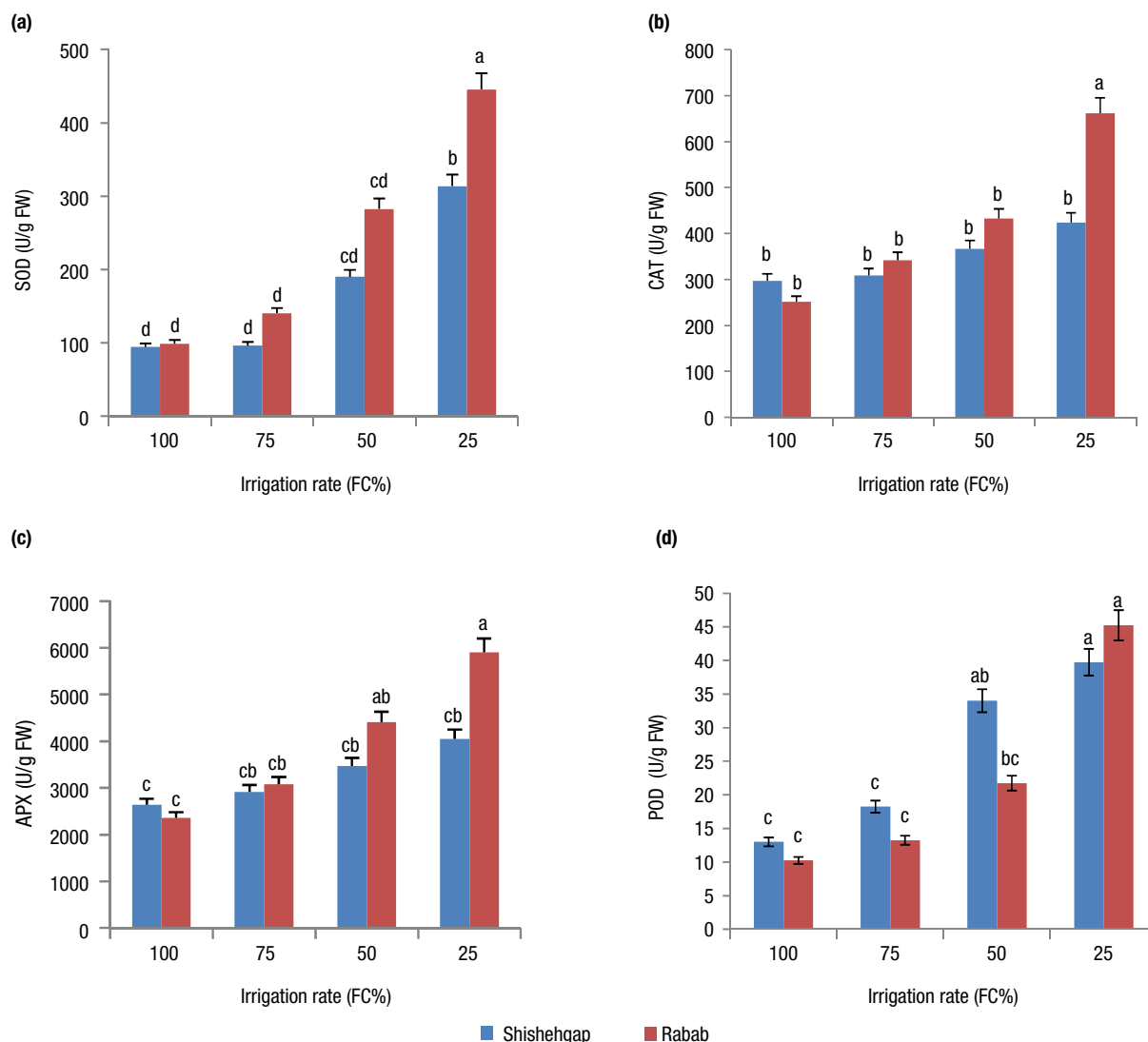
Figure 3. Effect of deficit irrigation on superoxide dismutase (SOD) (a), catalase (CAT) (b), ascorbate peroxidase (APX) (c) and peroxidase (POD) (d) activities in the leaves of two pomegranate cultivars. Means with the same letters are not significantly different ($p<0.05$) using Tukey's multiple rang test.

Hojati *et al.,* 2011). During water stress, especially in 25% FC, both pomegranate cultivars ('Rabbab' and 'Shishehgap') showed high activity of SOD, APX and CAT enzymes. These increments were more pronounced in 'Rabbab' than in 'Shishehgap'. The SOD catalyzes the dismutation of $\cdot O_2$ into H_2O_2 and O_2, accompanied by the uptake of two protons and hence decreases the risk of OH formation via the metal catalyzed Habere-Weiss type reaction (Gill & Tuteja, 2010; Heldt & Piechulla, 2011). Produced H_2O_2 is also catalyzed and converts to water by APX enzyme (Shigeoka *et al.*, 2002).

In relation to POD, it was at high water deficit (25% FC) that POD showed a considerable activity in 'Rabbab', but in 'Shishehgap' a sharp increase was obtained at 50% FC, where the RWC significantly

decreased in this cultivar. Peroxidase is located in both cytosol and chloroplast acts as a H_2O_2 scavenging enzyme. This enzyme can eliminate H_2O_2 properly due to its existence in chloroplast where CAT is not present (Huseynova, 2012). Our finding is in accordance with previous Manivannan *et al.*'s (2007) study. CAT and POD are two of the most important enzymes in removing toxic H_2O_2 from plant cell. These enzymes (Sofo *et al.,* 2005) conduct detoxification and conversion of H_2O_2 to water and oxygen. APX is the first antioxidant enzyme that reacts directly with H_2O_2, hydroxyl radicals and superoxide and protects chloroplast against oxidative damages (Hoekstra *et al.,* 2001). Increase in CAT activity has also been reported in other plants (Anjum *et al.,* 2011; Huseynova, 2012).

Table 2. Interaction of water stress (irrigation rates at 100%, 75%, 50% and 25% field capacity) and cultivar on the rate of sugar and starch (mg/g DW).

Cultivar	100% FC	75% FC	50% FC	25% FC	Mean
			Sugar (mg/g DW)		
Rabbab	14.5 d	17.0 cd	38.0 a	21.0 bcd	22.6 A
Shishehgap	18.3 cd	22.8 bcd	30.3 ab	25.5 bc	24.2 A
Means	16.3 C	19.8 CB	34.1 A	23.3 B	
			Starch (mg/g DW)		
Rabbab	184.3 a	170.0 a	137.0 ab	40.8 c	133.3 A
Shishehgap	153.4 ab	140.5 ab	128.4 ab	87.4 bc	127.4 A
Means	168.8 A	155.7 A	132.7 A	64.0 B	

FC: field capacity, DW: dry weight. In each treatment, means with the same letters (small letters for interaction and capital letters for main effect) are not significantly different using Tukey's multiple rang test $p \geq 0.05$.
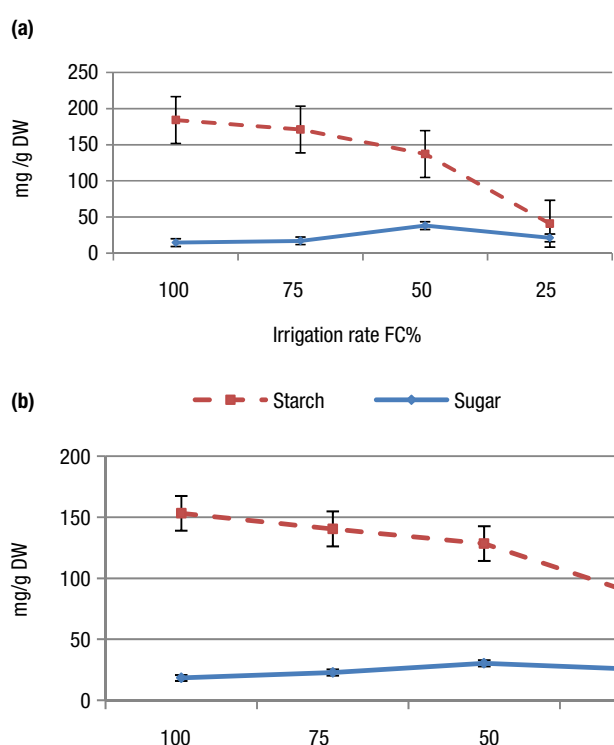
Figure 4. Changes in leaf sugar and starch contents in two pomegranate cultivars: 'Rabbab' (a) and 'Shishehgap' (b).

Our study indicated that the concentrations of soluble sugars in leaves of both cultivars increased during mild water deficit (at 75% and 50% FC), while the concentration of starch decreased. In order to tolerate deficit water stress, plants accumulate a high concentration of low molecular-mass organic solutes such as soluble sugars, proline or other amino acids to regulate the osmotic potential of cells aiming at improving absorption of water under drought stress (Zhang *et al.,* 2010). It seems that the treatment 25% FC, due to photosynthesis system damage, resulted in reduction of carbohydrates. Damage to the photosynthetic system may be due to dehydration of mesophyll cells, disorder in mesophyll metabolism, functional and structural changes in chloroplast, and loss of chlorophyll content, which is considered a main cause of inactivation of photosynthesis and impaired production of carbohydrates (Flexas & Medrano, 2002; Anjum *et al.,* 2011). In addition, elevated respiration consumes storage carbohydrate (Taiz & Zeiger, 2010). Therefore, the concentration of soluble sugar decreased (Hessini *et al.,* 2009).

In summary, diverse cultivars of pomegranate tolerate differently drought stresses. Our results showed that 'Rabbab' and 'Shishehgap' cultivars can tolerate adverse effects of water stress by increasing the synthesis and accumulation of osmotic such as sugar and maintain the hydration of protoplasm or the activity of antioxidant enzymes. In this regard, 'Rabbab' cultivar with lower decline in leaf relative water content indicated a better growth performance and more antioxidant enzyme activity than 'Shishegap'.

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