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The responses of enzymatic and non-enzymatic antioxidant systems of scion on different rootstocks under water stress deficit

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Abstract: Selecting of the specific type of rootstock is an appropriate and short-term method for increasing drought tolerance by improving the antioxidant systems in plants. In this research, the responses of the antioxidant systems of fig scion 'Sabz' were investigated on various rootstocks at different irrigation levels. Graft combinations were 'Sabz' on 'Sabz' (Sa/Sa), 'Siah' (Sa/Si) and 'Torch' (Sa/T) rootstocks, plus 'Sabz', 'Siah' and 'Torch' cultivars with no grafting, for a total of six groups. The plants were irrigated with 4 levels of 25, 50, 75 and 100% of water requirement (WR) for a duration of 12 weeks. The experiment was performed in a randomized complete design with 5 replications per treatment. The results showed that the 'Torch' rootstock induced the greatest amount of anthocyanin, glutathione and ascorbic acid in 'Sabz' (Sa/T) at 25% WR. Superoxide dismutase and catalase activities of 'Sabz' grafted on 'Siah' were more evidence compared to 'Sabz' grafted on 'Torch' rootstock at 25% of WR. The cv. Sabz grafted on both 'Siah' and 'Torch' rootstocks indicated higher chlorophyll content, chlorophyll stability index and shoot growth than cv. Sabz with no grafting. As a result, both rootstocks (T and Si) with the activation of enzymatic and non-enzymatic antioxidant systems caused the scion protects its integrity and be able to tolerate more water stress.

1. Introduction

Water scarcity is one of the most important environmental stresses, with its greatest impact on agriculture worldwide, especially in arid and semiarid regions (Knapp *et al.*, 2001; Alizadeh *et al.*, 2011). In recent years, the issue of climate change, combined with global warming, has been a major contributor to the increased water scarcity and plant losses in many parts of the world (Kramer and Boyer, 1995). Since most of a plant's processes are directly or indirectly affected by water, it is clear that most plants are affected by moderate to long-lasting drought throughout their life cycles (Bhattacharjee and Saha, 2014). The effects of drought stress on plants depend on genotype, rate and severity of the stress, age and stage of plants growth and development (Rostami and

Rahemi, 2013). Plant tolerance to water stress depends on their morphological, physiological and biochemical mechanisms that determine the responses of the plant under stress conditions (Penella *et al.*, 2014).

The fig (*Ficus carica* L.) belongs to the Moraceae family, a perennial fruit tree, which generally has a high tolerance to water deficit (Faghih and Sabet Sarvestani, 2001). This tree is managed in two ways, irrigated or rain-fed, and is cultivated in many parts of the world with diverse climatic conditions such as Iran, Turkey and other Mediterranean countries. The total area in the world cultivated with figs is reported to be about 388368 hectares with a production of 918813 tons per year (El-Shazly *et al.,* 2014). Iran, with an annual production of more than 76414 tons of fig, ranks fourth in the world (FAO, 2012). Most fig trees in Iran are cultivated in Estahban, a semi-arid region in the southeast of Fars province (Shirbani *et al.,* 2013).

Drought stress stimulates the accumulation of active oxygen species in plants. These reactive oxygen species are active forms of the oxygen molecule that are produced by the excitation of an oxygen molecule, or the transfer of one, two or three electrons to oxygen molecules, which ultimately lead to the formation of superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH) (Gill and Tuteja, 2003) These reactive oxygen species may be the first step in the degradation of oxidative processes, such as lipid peroxidation, chlorophyll breakdown, protein oxidation, and nucleic acid damage (Anjum *et al.,* 2011; Gu *et al.,* 2013). Plants have specific defense mechanisms that provide protection on the molecular level. These mechanisms comprise non-enzymatic antioxidants, such as ascorbic acid (ASA), glutathione (GSH) and anthocyanin and enzymatic antioxidants include catalase (CAT) and superoxide dismutase (SOD) (Guerfel *et al.,* 2009; Liu *et al.,* 2012 a).

Superoxide dismutase is a major scavenger that counters superoxide damage. Catalase catalyzes the decomposition of the excess of hydrogen peroxide to water and oxygen and protects cells from oxidative damage. These two enzymes play an important role in the degradation of reactive oxygen species (Mittler, 2002). With regard to the climate change models that predict an increase in drought and excessive groundwater depletion in the future, water scarcity appears to be the main limiting factor in agriculture (Alizadeh, 2005). In this regard, the use of drought tolerant rootstocks can play an important role in managing water absorption, water use efficiency, survival potential, growth capacity and the success of grafting in dry conditions.

Different rootstocks show different levels of tolerance in response to drought, and their ability to have high tolerance depend on improving the vegetative growth of the scion, the horizontal and vertical expansion of the roots, the ability to absorb water and minerals and antioxidant system activities for scavenging of active oxygen species (Ballesta *et al.,* 2010; Corso and Bonghi, 2014).

How the rootstock genotypes may be effective in response to water deficit stress has been investigated in many studies on fruit trees, such as apples and grapes (Alizadeh *et al.,* 2011; Liu *et al*., 2012 b; Corso and Bonghi, 2014). Most of these researches focus on the effects of rootstock on vegetative growth, fruit yield and quality, nutrition and hormones, and the scion's water content in response to drought. However, there are limited investigations on how rootstock affects the antioxidant systems of the scion in response to water deficit. The aim of this study was to investigate the effect of different fig rootstocks on the enzymatic and non-enzymatic antioxidant systems of the 'Sabz' scion in response to different levels of irrigation.

2. Materials and Methods

In early November, offshoots semi-hard woodcuttings of different cultivars ('Sabz', 'Siah' and 'Torsh') were disinfected with a commercial bleach (Clorax) solution containing 5% NaOCl and 2% benomyl for 20 min. Then, they were wrapped in a wet cotton cloth and placed at 4°C for 15 days to ensure that their chilling requirement was satisfied. After that, the bottoms of the cuttings treated with IBA solution (1000 mg/L) and cultured in cartonplast boxes containing perlite. They were put in a greenhouse with natural sunlight, relative humidity of 64% and an average temperature of 38/16±2°C day and night respectively.

After 2 weeks, rooted cuttings were transferred to 1 L plastic pots containing a mixture of soil: peat: sand (1:1:1, v/v/v). After 8 weeks, when the length of the new shoots reached 20 cm, cultivar Sabz as scion were grafted (cleft grafting) onto the three rootstocks ('Sabz', 'Siah' and 'Torsh'). The place of grafting was firmly enclosed with cellophane. To maintain moisture, grafted plants were covered with plastic bags to ensure grafting success. After 15 days, the plastic bags were removed and the cellophanes were

opened. Subsequently, all grafted and non-grafted plants (controls) were transferred to 10 L pots without drainage that contained the aforementioned ratio of the soil mixture. The field capacity of the soil used for potting was determined according to the protocol described by Richards (1949). The pots were irrigated daily at field capacity by the help of a balance. After complete deployment, the experiment was carried out by selecting 40 plants of each grafted combination ('Sabz'/ 'Sabz', 'Sabz'/ 'Siah', 'Sabz'/ 'Torsh') and 40 plants from cultivars with no grafting ('Sabz', 'Siah' and 'Torsh') in a randomized complete design with 4 levels of irrigation 100%, 75%, 50% and 25% of water requirement (WR). Three months after the start of treatments, various indices were measured as follows.

Anthocyanin

Extraction solution containing methanol, water and concentrated chloric acid (HCl) was prepared by a ratio of 80: 20: 1. Leaf samples were kept in the extract solution at 4°C in the dark for 48 h. Then, the extract passed through a Whatman filter paper (No. 1) and was read at 530 and 657 nm. Anthocyanin content was measured using the microplate reader spectrophotometer according to the method described by Alexieva *et al.* (2001).

The amount of anthocyanin was calculated as µg g^{-1} fresh weight.

Glutathione

The method used by Moron *et al.* (1979) was followed to measure glutathione concentration. An amount of 200 mg of fresh leaves was homogenized with 2 ml of cooled Trichloroacetic Acid (TCA) (5%), and they were centrifuged for 30 min at 15,000 rpm at 4°C. From supernatant extract, 75 µl was transferred to a vial containing 300 µl sodium phosphate buffer (0.2 M, pH 8) and 750 µl DTNB (5, 5-di-tiobis-2-nitrobenzoic acid) 0.6 mM. The extract was read at 412 nm by a spectrophotometer microplate reader. Glutathione concentration was calculated using standard curve.

Ascorbic acid

To determine the concentration of ascorbic acid, 1 g of fresh leaf tissue was thoroughly crushed in 5 ml of TCA (10% cold), and then centrifuged at 3500 rpm for 20 min. The supernatant was isolated and diluted to reach 10 ml. One ml of aqueous extract was mixed with 0.2 ml of DTC reagent (2, 4-di-nitrohydrazine, thiourea, copper sulfate) and was incubated at 37°C for 3 h. Then, 1.5 ml of sulfuric acid (65%) was added and mixed thoroughly. The extract was allowed to stand at room temperature for 20 min. The absorbance was read at 520 nm using a spectrophotometer. The ascorbic acid was calculated in µg g^{-1} F.W. (Elavarthi and Martin, 2010).

Antioxidant enzymes

To prepare leaf extract containing enzymatic antioxidant, 200 mg of fresh leaf tissue was ground with liquid nitrogen in a mortar. Then, 1.2 ml of K_2PO_4 buffer 0.2 M (pH 7.8) containing 0.1 mM EDTA (Ethylenediaminetetraacetic acid, $C_{10}H_{16}N_2O_8$) was added to the samples and homogenized. The samples were centrifuged at 15,000 *g* for 20 min at 4°C. The supernatants were separated and then the same operation was repeated on residual. The resulting extract was used for determining the activity of antioxidant enzymes (Elavarthi and Martin, 2010).

Catalase activity

Catalase activity was determined by the method of Aebi (1984) as described by Elavarthi and Martin (2010) by measuring the amount of hydrogen peroxide (H_2O_2) degradation via reducing the absorbance at 240 nm with a spectrophotometer microplate reader. To this purpose, 3 ml of the reaction mixture was made up of 2 ml of leaf extract, diluted with 50 mM K_2SO_4 buffer at pH=7 and H_2O_2 10 mM, which reached 3 ml by distilled water, the reaction started and the absorbance of the samples were recorded for 1 min.

Superoxide dismutase

Superoxide dismutase activity (SOD) was evaluated using a modified nitro-blue tetrazolium (NBT) method (Elavarthi and Martin, 2010). Accordingly, 2 ml of the reaction mixture including 50 mM phosphate buffer (pH= 7.8), 2 mM EDTA, 9.9 mM methionine, 55 µM NBT (Nitro blue tetrazolium chloride, $C_{10}H3_{30}Cl_2N_{10}O_6$), 0.025% triton X-100 to was added to 40 µl of diluted sample (×2) and then, added 20 µl of Riboflavin (1 mM). The reaction began by exposing the samples under a fluorescent tube (15 Watts) for 10 min. The blank received the same chemical mixture but without leaf samples throughout the steps. The absorbance of the specimens was read at 560 nm in a microplate reader spectrophotometer and one unit of enzyme activity was taken as the quantity of enzyme, which reduced the absorbance reading of the sample to 50% in comparison to control. Finally, SOD was calculated as U g^{-1} fresh weight.

Chlorophyll stability index

Chlorophyll stability index (CSI) was measured

according to the method used by Murty and Majumber (1962). The fresh leaf samples were emerged in 20 ml of distilled water and placed in a warm water bath of 56±1°C for 30 min, and the chlorophyll contents of the samples were determined. The chlorophyll stability index was obtained from the following formula.

CSI (%)= 1-(chlorophyll content without heating/chlorophyll content after heating) × 100.

Chlorophyll content

Total chlorophyll, chlorophyll a and chlorophyll b content of leaves were measured using the Dimethyl sulfoxide (DMSO) method introduced by Hiscox and Israelstam (1979). Leaf samples (100 mg fresh leaf) were submerged in 7 ml of dimethyl sulfoxide solution and put in the dark for 17 h. After, they were incubated in an oven at 60°C. By adding 3 ml of dimethyl sulfoxide to the samples, the volume was adjusted to 10 ml. Then, 200 µl of the samples extract were transferred to a plate and read by microplate reader spectrophotometry at 633 and 645 nm wavelengths. The following formulas were used in order to determine the total chlorophyll concentration as mg g^{-1} fresh weight (FW).

$$Ch_a \text{ (mg g}^{-1}\text{ FW)} = \frac{12.7(A663) - 2.69(A645) \times \text{volume made}}{\text{w.t of the sample} \times 10}$$

$$Ch_b \text{ (mg g}^{-1}\text{ FW)} = \frac{22.9(A645) - 4.68(A663) \times \text{volume made}}{\text{w.t of the sample} \times 10}$$

$$TCh \text{ (mg g}^{-1}\text{ FW)} = \frac{20.2(A645) + 8.02(A663) \times \text{volume made}}{\text{w.t of the sample} \times 10}$$

In these formulas, Ch_a represents chlorophyll a, Ch_b = chlorophyll b, TCh = total chlorophyll; A = absorbance in the wavelength (nm).

Cell membrane injury

The cell membrane damage index, measured by electrolyte leakage, is an indicator of estimating the tolerance of cellular protoplasts and the ability of the membrane to maintain integrity under conditions of water scarcity (Bajji *et al.*, 2002). This index was measured using the method followed by Kocheva and Georgiev (2003). A punching machine was used in order to prepare leaf samples measuring 1 cm in diameter. The leaf sample discs of 1 cm in diameter were washed three times with distilled water to remove surface contamination. Then they were transferred to vials containing 20 ml of deionized water and kept at 10°C for 24 h. After measuring their electrical conductivity, they were placed in an autoclave of 120°C for 15 min, after cooling at 25°C, the electrical conductivity was re-measured. Cell membrane damage (CMI) was obtained from the following formula.

$$CMI\ (\%) = [1-(T1/T2)] / [1-(C1/C2)] \times 100$$

T and C are the stressed and control samples, respectively, while 1 and 2 are the primary and secondary EC measurements.

The experiment had a factorial layout, based on 4 irrigation levels and 6 combinations of grafted and non-grafted plants in a randomized complete design with 5 replications and 2 plants per replicate. Data analysis was performed using SAS software version 9.3 and Duncan's Multiple Range Test at 5% of probability was used for the means comparison.

3. Results

Analysis of variance showed that the interaction between cultivars and different levels of irrigation were significant on all measured traits except anthocyanin (Table 1).

Anthocyanin

The results showed that increasing drought stress from 100% to 25% of WR the anthocyanin level increased in all grafted and non-grafted plants (Fig.

Table 1 - The variance analysis of some traits of fig cultivars under water stress condition

Source of variation	df	Mean square				
		SOD	CAT	Ant	GSH	ASC
Cultivar (A)	5	0.0259 **	10.9 **	0.008 **	14.8 **	68.3 **
Water stress (B)	3	0.4569 **	5.5 **	0.014 **	3.8 **	42.2 **
A × B	15	0.0293 **	2.3 **	0.0001 ns	3.3 **	15.8 **
Error	96	0.0028	0.044	0.0001	0.55	1.29
C.V.	-	3.63	19.33	14.07	22.41	10.64

SOD= Superoxide dismutase; CAT= catalase; GSH= glutathione; ASC= ascorbic acid; Ant= Anthocyanin.
**significant at 1% probability, NS; non-significant.

1A). A comparison of cultivars with no grafting ('Sabz', 'Siah', and 'Torsh') showed that 'Torsh' and 'Siah' had anthocyanin levels higher than the amount found in the 'Sabz'. However, 'Torsh' rootstock increased the amount of the anthocyanins in the scion ('Sabz') compared to the non-grafted 'Sabz' cultivar (Fig. 1B).

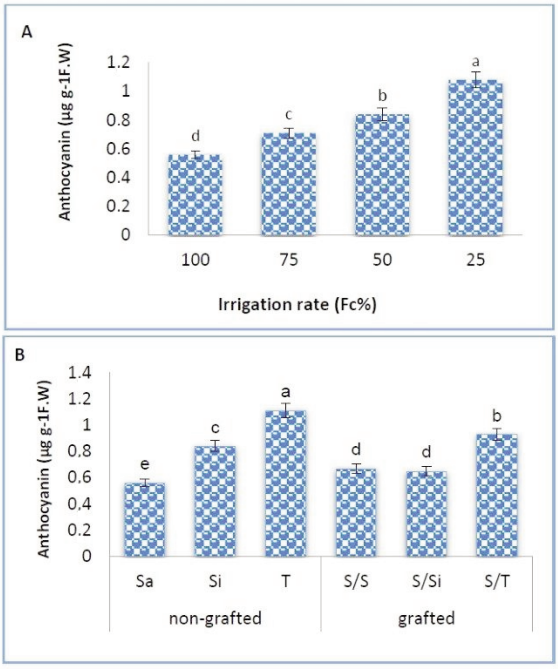

Fig. 1 - Main effects of irrigation levels (A) and grafted and non-grafted cultivars (B) on leaf anthocyanin rate. Sa= Sabz; Si= Siah; To= Torsh. Values are means ± SE (n=5).

Glutathione

The amount of glutathione in the leaves of non-grafted 'Torsh' increased with increasing the stress intensity from 100% to 25% of WR, while the 'Siah' cultivar reduced the glutathione significantly. The decrease in glutathione concentration in the leaf of the 'Sabz' non-grafted and the 'Sabz'/'Sabz' combination was not significant with increasing drought stress (Fig. 2A). Furthermore, both 'Siah' and 'Torsh' rootstocks significantly increased the glutathione level of the 'Sabz' scion at 75% of WR. However, there was no a significant difference in glutathione rate between 75%, 50% and 25% of WR in Sa/To combination.

Ascorbic acid

The results showed with increasing stress from 100% to 25% of WR, no increase in ascorbic acid content was observed in non-grafted 'Siah' and 'Sabz' cultivars. The non-grafted 'Torsh' showed an increase of 47.63% in the ascorbic acid content in the severe water stress compared with control. The 'Sabz' and 'Siah' rootstocks reduced the ascorbic acid content of the 'Sabz' scion at 50% and 25% of WR, compared to the non-grafted 'Sabz'. Nonetheless, 'Torsh' rootstock also reduced the ascorbic acid content of the 'Sabz' scion when 100% of WR was supplied, but this rootstock caused a significant increase in the ascorbic acid content (15.55%) of the 'Sabz' scion in the 25% of WR (Fig. 2B).

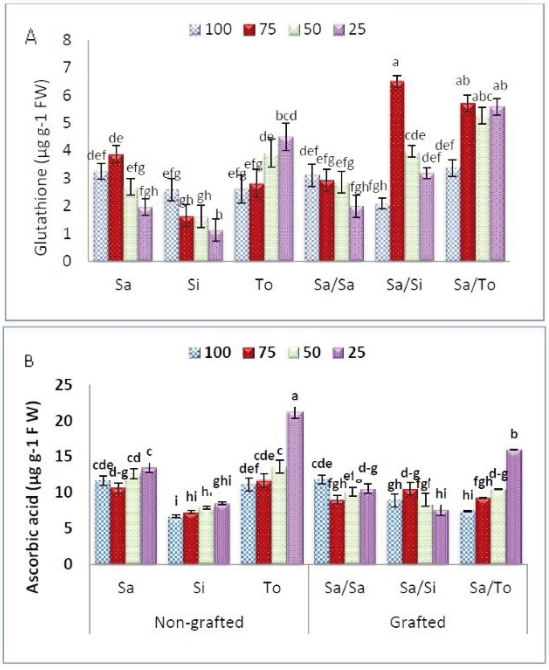

Fig. 2 - Interaction of fig grafted and non-grafted combinations and different levels of irrigation (100, 75, 50, 25% WR) on: Glutathione (A) and Ascorbic acid (B). Sa= Sabz; Si= Siah; To= Torsh. Values are means ± SE (n=5).

Superoxide dismutase

Superoxide dismutase activity increased with increasing drought stress in all non-grafted and grafted cultivars (Fig. 3A). Comparison of cultivars with no grafting showed that 'Siah' at 50% of WR had the highest enzyme activity compared with 'Torsh' and 'Sabz' cultivars. At 25% of WR, the SOD activity in the 'Sabz' rootstock was 1.78 times higher than the 'Siah' rootstock, and in the 'Siah' rootstock it was 1.63 times higher than in the 'Torsh' rootstock. Each of the three rootstocks reduced the activity of the enzyme compared to the non-grafted 'Sabz' cultivar when supplied with 50% and 25% of WR, although the differences in these values were not statistically significant (Fig. 3A).

Catalase activity

The activity of catalase enzyme increased significantly in all grafted and non-grafted cultivars in

response to intense drought stress (Fig. 3B). Comparison of non-grafted cultivars showed that catalase enzyme activity in 'Torsh' at 75%, 50% and 25% of WR was significantly higher than the two other cultivars ('Sabz' and 'Siah'), which did not differ significantly. However, only 'Siah' rootstock increased the activity of catalase enzymes (by 31.13%) in the 'Sabz' scion when 25% of WR was applied compared to the non-grafted 'Sabz' cultivar.

Analysis of variance showed that the interaction between cultivars and different levels of irrigation were significant on all measured traits (Table 2).

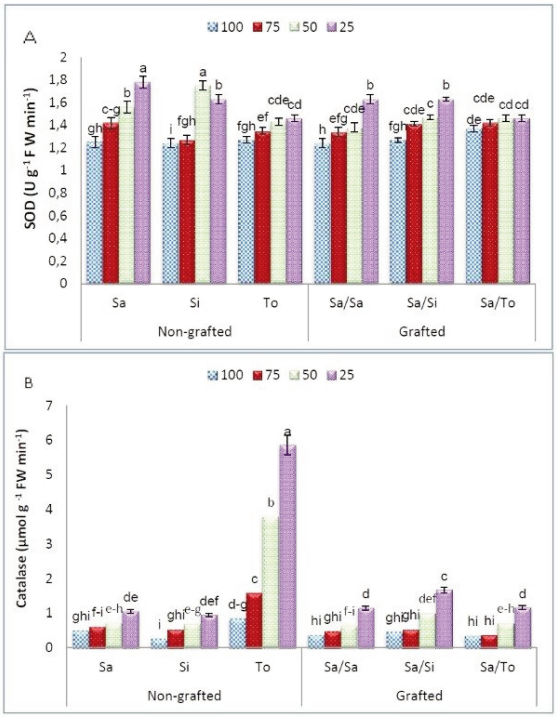

Fig. 3 - Interaction of fig grafted and non-grafted combinations and different levels of irrigation (100, 75, 50, 25% water requirement: WR) on Superoxide dismutase (SOD) activity (A) and Catalase activity (B). Sa= Sabz; Si= Siah; To= Torsh. Values are means ± SE (n=5).

Total leaf chlorophyll

The results showed that in non-grafted cultivars, 'Torsh' at 100%, 75% and 50% of WR showed higher chlorophyll contents than the other two cultivars. In grafted rootstocks, 'Siah' and 'Torsh' significantly increased the chlorophyll content in the leaves of the scion 'Sabz' in 100% and 75% of WR. 'Siah' rootstock also increased the chlorophyll content of the 'Sabz' scion by 42.52% in 50% of WR compared with non-grated 'Sabz' (Fig. 4A).

Chlorophyll stability index

The results showed that the chlorophyll stability index decreased as the drought stress intensified

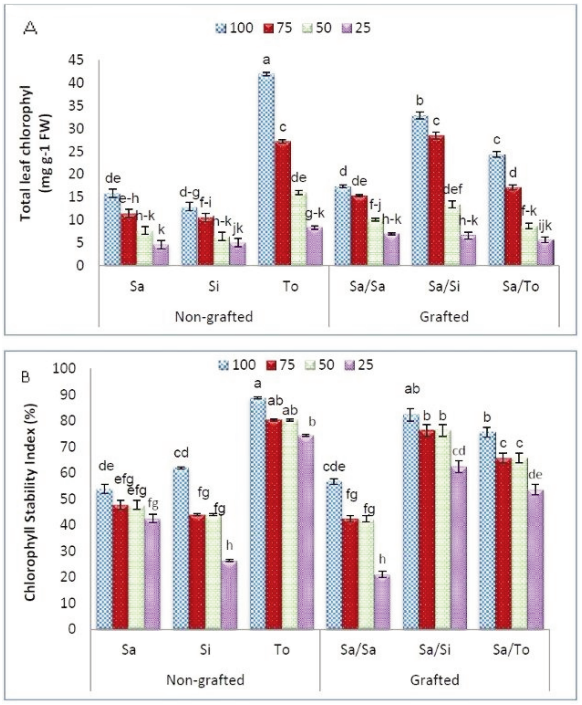

Fig. 4 - Interaction of fig grafted and non-grafted combinations and different levels of irrigation (100, 75, 50 and 25% WR) on leaf chlorophyll content (A) and chlorophyll stability index (B). Sa= Sabz; Si= Siah; To= Torsh. Values are means ± SE (n=5).

Table 2 - The variance analysis of total Chl, CSI, CMI, shoot FW and Shoot DW of fig cultivars under water stress condition

Source of variation	df	Mean Square				
		Total Chl	CSI	CMI	Shoot FW	Shoot DW
Cultivar (A)	5	685.6 **	5449.9 **	469.7 **	13355.4 **	1895.5 **
Water stress (B)	3	1963.6 **	8539.5 **	11707.8 **	22405.1 **	2749.35 **
A × B	15	108.2 **	134.7 **	180.4 **	691.71 **	83.24 **
Error	96	12.45	41.75	26.47	119.36	19.38
C.V.	-	23.83	12.45	5.59	13.03	12.77

Chl= chlorophyll; CSI= chlorophyll stability index; CMI= cell membrane injure; FW= fresh weight; DW= dry weight. **significant at 1% probability

(Fig. 4B). Among the non-grafted cultivars, 'Torsh' showed more stability in the leaf chlorophyll content than the other two cultivars at different levels of WR. 'Sabz' scion on the 'Siah' and 'Torsh' rootstocks showed the higher chlorophyll stability than non-grafted 'Sabz' and 'Sabz'/'Sabz' graft combination at all levels of WR (Fig. 4B).

Cell membrane injury

The results showed that high levels of water stress increased leaf cell membrane damage in all grafted and non-grafted plants (Fig. 5). The differences in the damage to the cell membranes of the 'Siah', 'Torsh' and 'Sabz' were not significant at 100% of WR, but the 75% and 50% of WR induced significantly greater damages to the leaf cell membrane of 'Siah' than the other two cultivars. At 25% of WR, the non-grafted 'Sabz' cultivar showed lower levels of membrane damage in its leaf cells, compared to 'Siah' and 'Torsh'. The results also showed that the effect of the 'Sabz', 'Torsh', and 'Siah' rootstocks on the 'Sabz' scion was not significant at all levels of water requirement, compared to the non-grafted 'Sabz' cultivar (Fig. 5).

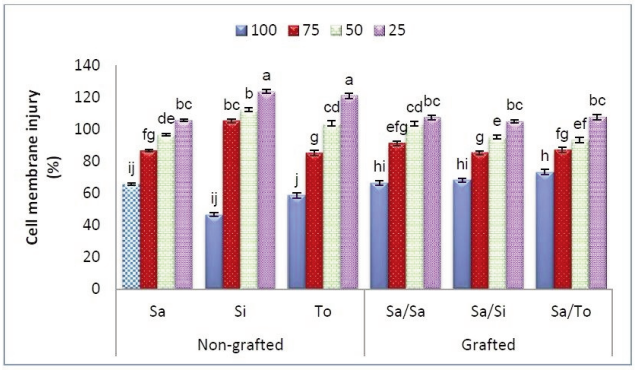

Fig. 5 - Interaction of fig grafted and non-grafted combinations and different levels of irrigation (100, 75, 50 and 25% of WR) on membrane injury index. Sa= Sabz; Si= Siah; To= Torsh. Values are means ± SE (n=5).

Shoot fresh and dry weight

The results showed that with increasing water stress deficit the shoot growth decreased in all non-grafted and grafted combinations of plants. However, 'Siah' and 'Torsh' rootstocks are associated with higher scion shoot fresh and dry weight compared to non-grafted (Sa) and self-grafted (Sa/Sa) Sabz cultivar (Fig 6. A and B).

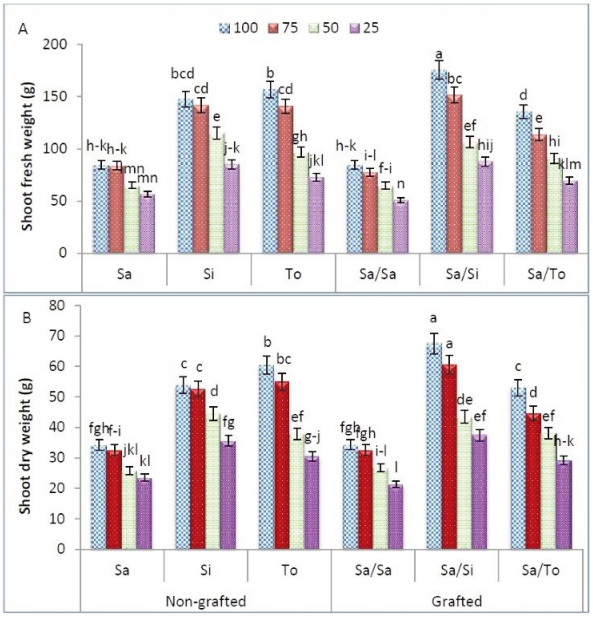

Fig. 6 - Interaction of fig grafted and non-grafted combinations and different levels of irrigation (100, 75, 50 and 25% WR) on shoot fresh weight (A) and shoot dry weight (B). Sa= Sabz; Si= Siah; To= Torsh.

4. Discussion and Conclusions

The increase in anthocyanin content in stressed leaves in this study confirms the protective role of anthocyanin against sunlight and active oxygen species in stressed plants (Gholami *et al.,* 2012 a). Stimulation of anthocyanin production has been proven by osmotic pressure and it is believed (in most cases) that tissues that contain more anthocyanins are more tolerant to drought (Chalker-Scott, 1999). Anthocyanins are able to protect cells against environmental damage through protection of cell membranes, organelles and nucleic acids (Neil *et al.,* 2002).

In this study, 'Torsh' rootstock increased the leaf anthocyanin in the 'Sabz' scion. Accordingly, the type of rootstock can increase the production of anthocyanins and thus counteract the reactive species of oxygen, increasing the ability of the plant to tolerate conditions of water stress.

In the green tissues of plants, ascorbic acid is a major antioxidant soluble in water. Leaf ascorbic acid changes seasonally, but as the leaf ages it remains at a constant level. However, exposure to stress can significantly change this situation and increase it (Sircelj and Batic, 2007). Ascorbic acid, like carotenoids, plays an important role in protecting the photosynthetic system against the harmful effects of reactive

oxygen species (ROS). In this research, the amount of glutathione and ascorbic acid increased in 'Torsh' rootstock, and this effect was transmitted to the 'Sabz' scion, which may be due to the rapid reaction of this rootstock to severe drought stress and the need for glutathione and ascorbic acid, which can confront ROS more efficiently. Even though the changes in glutathione and ascorbic acid were not significant at different levels of irrigation in the non-grafted 'Sabz' cultivar, by grafting the 'Sabz' scion on the 'Siah' rootstock, the glutathione level in the 'Sabz' scion increased significantly in 75% of water requirement and did not show any changes in ascorbic acid. In this regard, Tausz *et al*., (2004) also reported that drought stress leads to a decrease in glutathione concentration, and the oxidation and reduction of compounds ultimately leads to the system's degradation. An increase in the amount of glutathione in conditions of water shortage may be necessary to adjust the level of ascorbic acid in the plant (Gholami *et al*., 2012 b).

It seems that increased levels of glutathione in non-grafted 'Torsh' cultivar and 'Sabz' grafted on 'Torsh' rootstock have been effective in regulating ascorbic acid levels at mild and severe stress levels. Sircelj *et al.* (2005) also reported an increase in the level of glutathione and ascorbic acid at moderate levels, indicating compatibility with oxidative stress in apple trees, and stated that the decrease in glutathione levels at severe stress levels indicates severe oxidative stress.

Many researchers have shown that drought stress leads to oxidative stress in the plant (Mittler, 2002; Gill and Tuteja, 2010; Gholami *et al*., 2012 b; Shirbani *et al*., 2013). Excessive forms of stress can damage the plant by producing reactive oxygen species. The antioxidant defense system and the breakdown of active oxygen species are known to be under dry conditions (Zarafshar *et al.,* 2014). Under mild and moderate water deficit conditions, a number of compatible plant species increase the activity of enzymatic antioxidants such as superoxide dismutase and catalase, although severe drought stress may cause damage to cells by stronger stimulations or impairments via reactive oxygen species that suppress enzymatic antioxidant activity (Guerfel *et al.*, 2008).

Superoxide dismutase and catalase are active enzymes for the elimination and degradation of harmful oxygen species in plants and maintain oxidative equilibrium during oxidative stress (Gill and Tuteja, 2010). Superoxide dismutase enzymes have been reported to play an important role in the antioxidant metabolism of plants under environmental stress conditions, such as water deficit, via regulating their gene expression or activities (Xu *et al.*, 2010). High SOD activity in 'Siah' and 'Sabz' non-grafted cultivars were achieved in 50% and 25% of WR respectively, and the activity of catalase in the non-grafted 'Torsh' cultivar and in the 'Sabz'/'Siah' graft combination (at 25% of WR) were also comparatively high. These results depend on the plant's better protection against oxidative damage caused by water stress. It seems that the scion of the 'Sabz' cultivar on the 'Siah' rootstock led to greater improvements in the antioxidant system.

Chlorophyll content has a positive correlation with the rate of photosynthesis. Therefore, the decrease in chlorophyll content under drought stress condition is a common symptom of oxidative stress, which may be due to photo-oxidation of pigments and chlorophyll degradation (Anjum *et al.,* 2011; Shirbani *et al*., 2013). The reduction of chlorophyll content was observed in all cultivars under stress in this study. According to Guerfel *et al.* (2009) the reduction of chlorophyll content can be attributed to its susceptibility to environmental stresses, especially drought. Chlorophyll levels tend to decrease or otherwise remain unchanged during the period of drought stress in many species. This, however, depends on the duration and severity of the drought (Anjum *et al.*, 2011).

In this study, chlorophyll contents at 75% and 50% of WR decreased less sharply in 'Sabz'/Siah graft combination. This indicates that the light dissipation and antioxidant systems may prevent the degradation of chlorophyll molecules (Niu *et al*., 2008). Sircelj *et al.* (2005) also reported no reduction in the chlorophyll content of apple leaves 'Elastar' under water stress is due to a strong antioxidant system and an efficient light dissipation system. Anjum *et al.* (2011) attributed the decrease in the content of chlorophyll to the chloroplast membrane damage, swelling, lamella distortion and the occurrence of small droplets of lipids under drought stress conditions.

The chlorophyll stability index is an indicator of measuring the membrane's integrity and the pigments stability under stress conditions (Ananthi *et al.*, 2013). Surendar *et al.* (2013) reported that a decrease in chlorophyll content under stress was due to the destruction of the chloroplast membrane with increasing phosphatase activity, which is located on the membrane. In this study, intense water stress (25% of WR) reduced the chlorophyll stability in all grafted and non-grafted rootstocks compared to

100% of WR. This could be due to the degradation of chlorophyll when proteolytic enzymes, such as the chlorophyllase enzymes, are produced. The values of the high chlorophyll stability index in the non-grafted 'Torsh' cultivar and the 'Sabz'/'Siah' or 'Sabz'/'Torsh' graft combinations indicate a higher chlorophyll content in the leaves, which leads to an increase in the rate of photosynthesis and the production of more dry weight which helps the plant with stand dehydration (Babu *et al.,* 2008; Ananthi *et al.,* 2013; Surendar *et al.,* 2013).

Under conditions of water scarcity, the cell membrane of the leaves are subject to changes such as increased permeability and reduced selectivity, which can be observed through an increase in electrolyte leakage (Zarafshar *et al.,* 2014). In this study, the overall increase for leakage under stressful conditions was observed in all grafted and non-grafted rootstocks as compared to fully irrigated plants.

The non-grafted 'Siah' cultivar experienced a greater damage to the cell membrane of its leaf, compared to the non-grafted 'Sabz' and 'Torsh' cultivars. This indicates that the rootstock's ability to maintain the integrity of its cell membrane in severe stress conditions is considered an important factor in determining tolerance to drought (Bolat *et al.,* 2014). Undesirable performance of the cell's metabolism during periods of drought stress leads to the stimulation of reactive oxygen species or the disruption of systems that prevent or reduce the activity of reactive oxygen species, which would damage the cell membrane and increase electrolyte leakage (Guerfel *et al.,* 2008; Karimi *et al.,* 2013). Therefore, the fact that damage to the cell membranes of the non-grafted 'Sabz' cultivar occurred less than in the non-grafted 'Siah' rootstock may indicate a more tolerance to drought in the 'Sabz' cultivar. However, Shahidi-Rad *et al.* (2015) reported that leaf abscission occurred more rapidly in 'Siah' than 'Sabz' cultivar in 16 days of water deficit stress, but after rewatering, 'Siah' recovered more efficiently and more rapidly than 'Sabz'.

The growth of grafted plants (Sa/Si and Sa/T) showed that by activating their enzymatic and non-enzymatic antioxidant systems, they could maintain the growth of the scion much higher than control (Sa).

In this study, both rootstocks affected the scion (Sabz cultivar) antioxidant systems and increased SOD and catalase (enzymatic), ascorbic acid and glutathione (non-enzymatic) in high water deficit. Both combinations of Sa/T and Sa/Si indicated higher chlorophyll content, chlorophyll stability index and shoot fresh and dry weight than non-grafted 'Sabz' cultivar. Consequently, they tolerated higher water deficit.

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