RESPONSES OF FIVE POMEGRANATE (*PUNICA GRANATUM* L.) CULTIVARS TO CONTRASTING WATER AVAILABILITY: LEAF MORPHOPHYSIOLOGICAL AND ANATOMICAL ADAPTATION

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Abstract. This investigation was carried out on five pomegranate cultivars (Wonderful, Manfalouty, Acco, Assuity and 116) to evaluate their performance under two irrigation levels 100% of field capacity (control) and 30% of field capacity (water stress) during two consecutive seasons 2019 and 2020. The results indicated that water stress at 30% of field capacity significantly reduced leaf area, stomatal conductance and transpiration rate in all studied pomegranate cultivars. Manfalouty cultivar recorded the least reduction in leaf area, transpiration rate and stomatal conductance compared to Acco cultivar. Also, Manfalouty cultivar showed the highest increase (32.30%) in the thickness of upper epidermis under 30% of field capacity. On the other hand, lower epidermis showed an increase in all studied cultivars except for 116 cultivar. Moreover, the leaves of both Wonderful and Manfalouty cultivars were characterized by a great thickness of palisade (81.9 and 73.5 µm) and spongy (89.1 and 65.9 µm) tissues under water stress, while the lowest palisade tissue was recorded in Acco cultivar (56.7 µm) and the lowest spongy tissue was recorded in Assuity cultivar (44.4 µm). Furthermore, the highest thickness of xylem tissue was recorded in Manfalouty cultivar (8.70%) and the lowest decrease (6.98%) in phloem tissue. Based on physiological and anatomical characteristics, it could be concluded that Manfalouty cultivar was the most drought tolerant, while Acco cultivar was the most sensitive.

Keywords: *water stress, leaf area, stomatal conductance, transpiration rate, leaf anatomy*

Introduction

Pomegranate (*Punica granatum* L.) belonging to the Punicaceae family, is considered one of the oldest edible fruits (Blumenfeld et al., 2000). It is valued for its nutritional, pharmacological and medicinal benefits and is being used as a herbal treatment for diabetes, cancer, inflammation, blood pressure and many other diseases (Akbarpour et al., 2010; Adhami et al., 2012). Pomegranate production has expanded greatly worldwide due to its hardy nature and xerophytic characteristics that enabled it to cope with adverse and unsuitable climatic and water conditions (Borochov-Neori et al., 2013; Singh et al., 2014), which means it can survive in arid and semi-arid areas even under harsh environmental conditions (Aseri et al., 2008). However, pomegranate tree needs a regular irrigation regime during the dry season to obtain an optimal growth and fruit quality (Holland et al., 2009). Increasing the production and expansion leads to lack of precise available data on pomegranate area and production, although it could be estimated that around 1.5 million tonnes of fruits are produced annually in the world (Holland and Bar-Ya'akov, 2008), and cultivated nearly in more than 302,000 ha, 76% of them found in India, Iran, China, Turkey and USA (Melgarejo et al., 2012). Egypt is one of the most important Mediterranean countries producing and exporting pomegranate fruits with a total cultivated area of about 44809.8 ha and an amount of exports about 122387.2 tonnes (Egyptian ministry of Agriculture and land reclamation, 2019). There are many local

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verities in Egypt like Manfalouty, Assuity, Araby and Nab El-Gamal and other foreign varieties such as Wonderful, 116, Acco, Sweet and Ever sweet.

Facing that agriculture sector consumes about 70% of world fresh water (FAOSTAT, 2016). Limitation of water resources is one of the most important critical factors influencing plant growth specifically in arid and semi-arid regions (Tatari et al., 2020). Worldwide, it is expected that areas of arable land could decrease due to climate change (Harrison et al., 2014; Sherwood and Fu, 2014). Water stress is a serious limiting factor restricting plant performance, growth, and distribution globally (Liu et al., 2011). Exposing plants to such restricting environmental conditions stimulate a series of morphological, biochemical, physiological, and anatomical responses to cope with stressful circumstances and ameliorate plant functions in different ways (Reddy et al., 2004). Mechanisms and strategies to adapt to drought stress vary between cultivars and species. Intrigliolo et al. (2011), Rodriguez et al. (2012) and Parvizi et al. (2016) have shown that in pomegranate, deficit irrigation decreased photosynthesis and transpiration rates due to reduced stomatal conductance. There are few studies assessed anatomical responses of pomegranate leaves to water stress. Besides biochemical and physiological changes, anatomical functions are stimulated in plants leaves enabling them to withstand drought conditions through reducing water loss and maintenance of photosynthetic rates (Hameed et al., 2012).

In this respect, one of the most important strategies to achieve water conserving is to use drought-tolerant species which is more economical, sustainable and eco-friendly. Therefore, in this study we aim to evaluate different responses of some pomegranate cultivars and to understand physiological and anatomical responses in relation to water stress.

Materials and methods

Plant material, growth conditions and treatments

A pot experiment was carried out during two consecutive seasons 2019 and 2020 at the experimental net greenhouse of pomology department Faculty of Agriculture Cairo University, Egypt (30°01'04"N31°12'30"E). Five pomegranate cultivars namely; Wonderful, Manfalouty, Acco, Assuity and 116 were used. All cultivars were propagated by hard wood stem cuttings under net greenhouse conditions, the length and diameter of the cuttings were in the ranges of 25–30 cm and 2–2.5 cm, respectively. Pomegranate cuttings were prepared in 15th February and stored in a moist peat moss for two weeks to promote callusing and root formation. On 1st of March selected cuttings were planted into 10 liter plastic pots filled with washed sandy soil and watered twice a week with tap water (EC: 0.48 ds/m). In the early June, 21 plants of each cultivar for each treatment with an average height of 35-40 cm pruned by lateral shoot removal to uniform plants with single main shoot were subjected to two water stress levels for three months, control (100% of field capacity) and water stress (30% of feild capacity). Field capacity (FC) was calculated using the following equation:

$$FC = \frac{(FW - DW)}{\mathrm{DW}} \text{ x } 100 \qquad \text{(Eq.1)}$$

where FW was the fresh weight of soil sample and DW was the dry weight of soil sample after oven drying at 85°C for 3 days (Coombs et al., 1987). *Table 1* shows the results of soil and water samples analysis. During the experimental period water levels were maintained by weighing three pots from each treatment every three days and adding proper water volumes. Every second week of the month, experimental plants were fertilized by 0.25 strength of Hoagland solution for macronutrients and full strength for micronutrients (Fozouni et al., 2012).

***Table 1.** Estimation of the physical and chemical properties of the experimental soil and irrigation water*

Characteristics	Soil	Water
Particle size distribution %		
Fine sand	44.2	
Coarse sand	41.6	
Silt	11.2	
Clay	3	
Texture	Sandy soil	
EC (dsm^{-1})	1.21	0.48
P^{H}	7.91	7.53
Soluble cations (meq/l)		
Ca^{+2}	3.8	2.3
Mg^{+2}	1.9	1.4
Na^{+}	60	0.84
K^{+}	0.3	0.18
Soluble anions (meq/l)		
CO_3^{-2}	--	--
HCO_3^{-}	0.37	1.8
Cl^{-}	10	2.5
SO_4^{-2}	1.63	0.24
Soil moisture constants		
Saturation percentage (SP)	24	
Field capacity (FC)	15%	
Wilting point (WP)	6.2	
Available water (AW)	8.8	

At the end of the experimental period (September) the following measurements were recorded.

Leaf area

Leaf area (cm^2): was calculated using the following equation:

$$LA = -0.0477 + 0.0282*L + 0.0842*W + 0.965*L*W \qquad \text{(Eq.2)}$$

where, LA is leaf area, W is leaf width and L is leaf length according to Meshram et al. (2012).

Gas exchange measurements

Transpiration rate (T_r) (µg $H_2O/cm^2.s$) and stomatal conductance (g_s) (cm/s) were measured in the 5th and 6th mature leaves from shoot apex using a portable steady-state porometer (LI-1600M, LI-COR, Nebraska, USA) between 10:00 am and 12:00 pm with an air temperature around 30°C, relative humidity ranged between 35:42% and an active photosynthetic radiation of 1200 mmol m^{-2} s^{-1} according to Surendar et al. (2013).

Leaf anatomical measurements

A microscopic examination was performed on Leaf samples. Specimens (ca. 1 cm^2) from the middle section of the youngest fully grown leaves were killed and fixed for at least 48 hrs. in F.A.A. (10 ml formalin, 5 ml glacial acetic acid and 85 ml ethyl alcohol 70%). The plant samples were washed in 50% ethyl alcohol, dehydrated in a normal butyl alcohol series and embedded in paraffin wax of melting point 56°C then sectioned to a thickness of 20 microns, double stained with crystal violet-erythrosin and cleared in xylene and mounted in Canada balsam (Nassar and El-Sahhar, 1998). Measurements of the micrographs were detected using light compound microscope (LEICA DM750) and a LEICA ICC50 HD by the Leica Application Suite program.

Statistical analysis

Data were statistically analyzed following the analysis of variance (ANOVA) technique according to split plot design; water stress as the main plot in 2 levels (factor A) and 5 pomegranate cultivars as the sub-plot (factor B). 42 plants of each cultivar were selected and arranged in complete randomized block design. Each treatment was represented by three replicates. Means of the treatments were compared by least significant difference (L.S.D.) at significance level of 0.05 (Duncan, 1955).

Results and discussion

Leaf area

As shown in *Table 2* the results indicate that compared to stress treatment (30% of FC), full irrigated plants (100% of FC) considerably recorded the highest significant values of leaf area during both seasons. Manfalouty cultivar was the least affected by stress conditions among all cultivars and reduced leaf area by 21.20% and 10.55% during first and second seasons respectively, meanwhile Acco cv. was much affected and recorded a reduction by 34.74% and 33.61% in the first and second seasons respectively. Hamdy et al. (2016) found that maximum values of leaf surface area of two studied pomegranate cultivars (Early 116 and Wonderful) were obtained when the plants were irrigated with 100% of field capacity, while the minimum values were recorded at 40% of available water. Moreover, under different irrigation levels 100%, 75% and 50%, it was found that among four pomegranate cultivars Manfalouty cv. recorded the highest values of leaf area during two consecutive seasons (Haleem et al., 2020). Furthermore, it has been reported that reducing irrigation level up to 7 m^3/tree/year decreased leaf area of twenty-year-old pomegranate Manfalouty trees, meanwhile increasing irrigation level up to 15 m^3/tree/year rate recorded the highest leaf area values during two seasons (Khattab et al., 2011). Matthews (1986) found that the drop in leaf area produced by water stress in cantaloupe vines plants was attributable to a decrease in epidermal cell mitotic activity, which resulted in a reduction in the total number of cells. She added that leaves first developed during water stress conditions were the most affected and showed a sever reduction in cell division. While leaves first developed under irrigated conditions then exhibited to stress conditions showed a relative normal cell division and leaf size. Indicating that the majority of cell division took place during and shortly after leaf initiation.

Table 2. *Effect of water stress on leaf area (cm^2) of five pomegranate cultivars*

Cultivars	**1st season**		**Mean**	**2nd season**		**Mean**
	Water levels			**Water levels**		
	100%	**30%**		**100%**	**30%**	
Wonderful	11.130 d	8.100 f	9.615 B	14.213 c	10.450 f	12.332 D
Manfalouty	11.663 cd	9.190 e	10.427 A	14.873 bc	13.303 d	14.088 B
Acco	12.570 b	8.203 f	10.387 A	17.810 a	11.823 e	14.817 A
Assuity	12.173 bc	8.540 ef	10.357 A	15.260 b	12.300 e	13.280 C
116	13.570 a	8.233 f	10.902 A	12.963 d	10.350 f	11.657 E
Mean	12.221 A	8.453 B		15.024 A	11.445 B	

Means with the same letter within each season were significantly equal at L.S.D. 5% level

Leaf gas exchange measurements

In our study, according to the data presented in *Tables 3 and 4*, it can be noticed that water stress at 30% of FC significantly declined transpiration rate (T_r) and stomatal conductance (g_s) in all tested pomegranate cultivars in comparison to full irrigated plants (100% of FC), Manfalouty cv. recorded the lowest reduction in transpiration rate and stomatal conductance by 20.94% and 12.59%, respectively. However, Acco cv. recorded the highest significant reduction in both transpiration rate and stomatal conductance by 69.10% and 68.05%. In line with our findings, Intrigliolo et al. (2011), Rodriguez et al. (2012) and Parvizi (2016) reported a decrease in stomatal conductance under deficit irrigation strategy compared to full irrigation in pomegranate. Also, Pourghayoumi et al. (2017) found that exhibiting five pomegranate cultivars to water stress for 14 days progressively declined net photosynthesis rate (A_n), transpiration rate (T_r) and stomatal conductance (g_s) so as to control water loss via transpiration and maintain leaf turgor. Reduction of leaf gas exchange, stomatal closure, and slowing down photosynthetic activity are the main consequences of drought stress in plants (Hu et al., 2010). This may be due to the reduction in premature leaf senescence, leaf expansion, damaged photosynthetic process, oxidative chloroplast lipids as well as changes in structure of proteins and pigments (Murtaza et al., 2016). On the other hand, it is well known that reducing transpiration rate under stress conditions due to stomatal closure could also decline photosynthetic rate as a consequence of deceasing levels of CO_2 inside stomatal chambers and cells as well as amounts of CO_2 reaching carboxylation sites inside chloroplasts (Xue et al., 2021).

Table 3. *Effect of water stress on transpiration rate (µg $H_2O/cm_2.s$) of five pomegranate cultivars*

Cultivars	**Water levels**		**Mean**
	100%	**30%**	
Wonderful	5.823 a	3.847 b	4.835 A
Manfalouty	5.047 a	3.990 b	4.518 A
Acco	5.447 a	1.683 c	3.565 B
Assuity	5.480 a	3.123 b	4.302 A
116	5.337 a	3.920 b	4.628 A
Mean	5.427 A	3.313 B	

Means with the same letter were significantly equal at L.S.D. 5% level

Table 4. *Effect of water stress on stomatal conductance (cm/s) of five pomegranate cultivars*

Cultivars	**Water levels**		**Mean**
	100%	**30%**	
Wonderful	0.326 a	0.270 bc	0.298 A
Manfalouty	0.270 bc	0.236 c	0.253 B
Acco	0.313 ab	0.100 e	0.206 C
Assuity	0.320 ab	0.173 d	0.246 B
116	0.306 ab	0.226 cd	0.266 AB
Mean	0.307 A	0.201 B	

Means with the same letter were significantly equal at L.S.D. 5% level

From the microscopic scrutinization of cross sections (*Figure 1*) and tabulated data (*Table 5*), it revealed anatomical differences of leaf pomegranate cultivars under study in response to water stress (30% of FC). There was a decrease in thickness of upper epidermis of the cultivars Wonderful, Acco and 116 by 22.81%, 25.80% and 3.77% respectively, due to low water irrigation. Meanwhile, Manfalouty cv. showed a maximum increase by 32.30% followed by Assuity cv. that increased by 22.63% compared to that at full irrigation (100% of FC). On the opposite, lower epidermis showed an increase at low water level in all studied cultivars except 116 cv. which recorded a decrease by 18.24%. Such increase in thickness of epidermis tissues could impact the loss of water through leaf surface. Similarly, the earlier studies Boughalleb et al. (2012) revealed that thicker epidermis tissues play an important role to minimize nonstomatal water loss through leaf surface under harsh environmental conditions. Palisade and spongy tissues showed an increase in thickness only in Wonderful cv. by 22.23% and 11.23%, respectively, beside spongy tissue of Assuity cv. that increased by 3.25% under water stress conditions. Meanwhile all the cultivars under study showed a reduction in the thickness of palisade and spongy tissues. However, it is worth to mention that Manfalouty cv. had a very low reduction (3.41%) in palisade thickness. Moreover, leaves of both Wonderful and Manfalouty cultivars characterized by great thickness of palisade (81.9 and 73.5 µm) and spongy (89.1 and 65.9 µm) tissues respectively under water stress conditions compared to other cultivars under study. Many studies have mentioned that increase the thickness of palisade tissue could have a role in increasing the number of carboxylation sites as well as a thicker spongy parenchyma may result in improving the diffusion of CO_2 to those sites leading to maintain photosynthesis rate of leaves under low water availability conditions (Ennajeh et al., 2008, 2010; Flexas et al., 2008, 2012). Lamina thickness reduced negatively in all pomegranate cultivars under 30% of FC except Wonderful cv. recorded an increase by 9.89%, this fits with Ennajeh et al. (2010) who observed a total lamina increase under drought conditions in two studied olive cultivars (Chemlali and Meski). Also it is known that succulence of plant leaf thickness is a substantial structural modification to adapt to dry habits (Kaleem and Hameed, 2021). Midvein tissue of Manfalouty cv. was more thickness (733.4 and 714.3 µm) either at full or low water availability conditions respectively, compared to other cultivars under study. However, there was a decrease in midvein thickness of all cultivars except Wonderful cv. which showed an increase by 2.93%, besides noticing that Manfalouty cv. recorded the least decrease by 2.60%. Thicker midvein or least reduction in its thickness may enhance transportation of substances and plant survival under unfavorable conditions (Song et al., 2021).

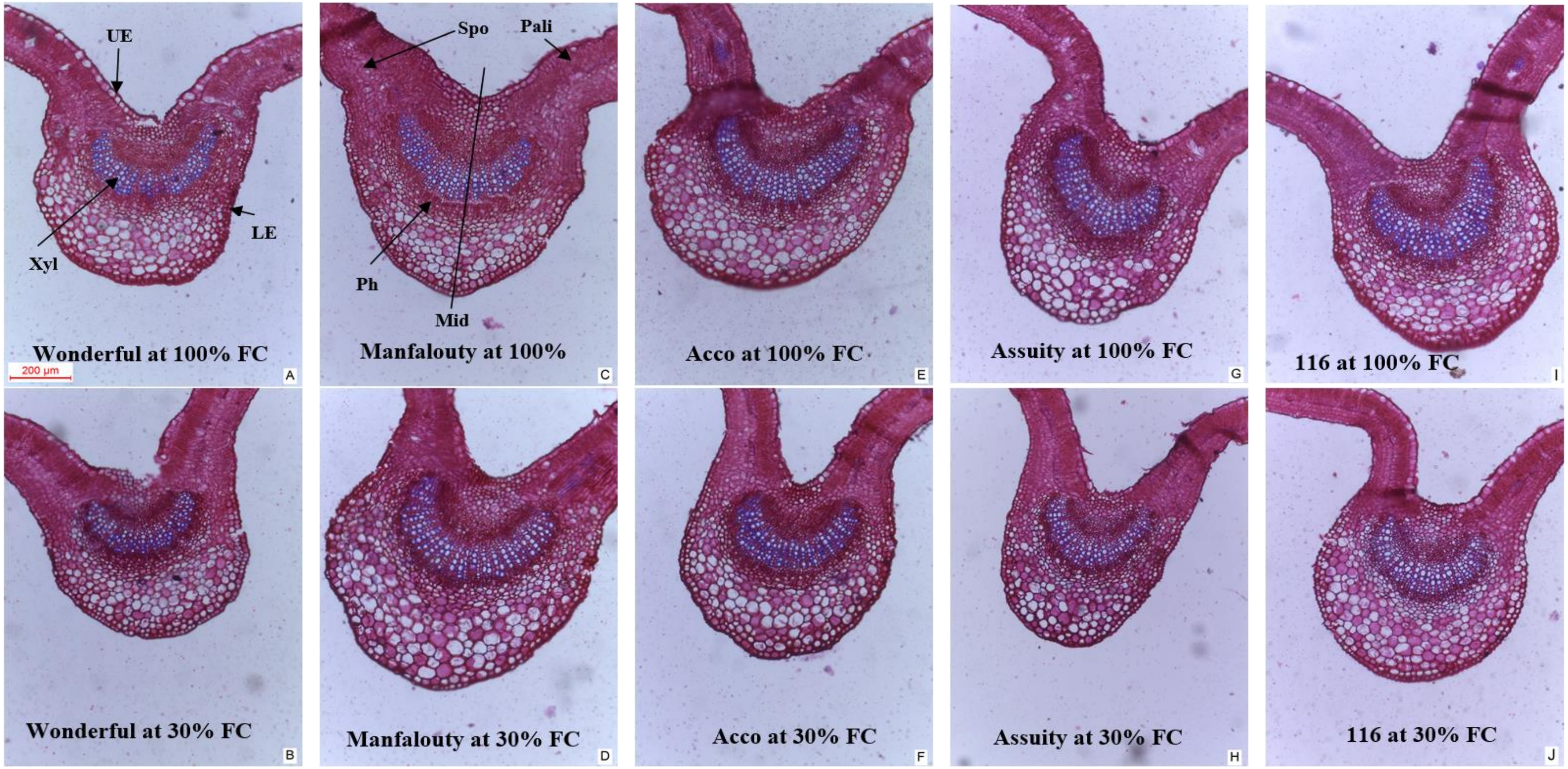

Figure 1. *Comparative leaf anatomy in blade cross-sections (100X) of five pomegranate cultivars Wonderful, Manfalouty, Acco, Assuity and 116.Uper epiderms (UE), Lower epidermis (LE), Midvein thickness (Mid), Palisade thickness (Pali), Spongy thickness (Spo), Xylem thickness (Xyl), Phloem thickness (Ph)*

Table 5. *Anatomical changes in cross sections of leaves of five pomegranate cultivars under water stress conditions*

Measurements (µm)	**Water levels**									
	100% of FC					**30% of FC**				
	Wonderful	**Manfalouty**	**Acco**	**Assuity**	**116**	**Wonderful**	**Manfalouty**	**Acco**	**Assuity**	**116**
Upper epidermis thickness	37.7	22.6	34.1	24.3	34.4	29.1	29.9	25.3	29.8	33.1
Lower epidermis thickness	22.3	29.1	18.6	22.5	30.7	27.5	32.6	28.7	28.0	25.1
Midvein thickness	548.1	733.4	622.1	678.5	658.6	564.2	714.3	587.6	527.7	607.6
Lamina thickness	207.1	216.3	199.0	176.8	210.0	227.6	201.8	165.0	160.0	178.1
Palisade thickness	67.0	76.1	84.9	87.0	65.2	81.9	73.5	56.7	57.8	64.2
Spongy thickness	80.1	88.5	61.4	43.0	79.6	89.1	65.9	54.3	44.4	55.7
Xylem thickness	84.5	117.2	104.5	111.4	135.2	88.2	127.4	97.8	97.3	117.2
Phloem thickness	84.1	73.0	88.4	106.2	62.7	70.7	67.9	61.9	68.3	72.9

Furthermore, the thickness of xylem tissue of the cultivars Wonderful and Manfalouty with its maximum in Manfalouty cv. (8.70%) could enable maintaining efficient water and nutrients uptake to the leaves, compared to other cultivars that showed a decrease under low water irrigation level. Phloem tissue thickness was decreased under water stress conditions, except 116 cv. that showed an increase by 16.26% and with the lowest decrease in Manfalouty cv. by 6.98%. The thickening of vascular bundles enables for greater flow of water and mineral salts in dry habits, giving the plants proper adaptive characteristics under water restriction conditions (Queiroz-Voltan et al., 2014). Previous results indicating that leaf morpho-anatomical adaptations are spices-specific, Wonderful followed by Manfalouty cultivars were the most adaptable to water stress conditions compared to other studied cultivars.

Conclusion

Water stress reduced leaf area, stomatal conductance and transpiration rate of all studied pomegranate cultivars. Under water stress conditions, Manfalouty cv. recorded a slight decrease in leaf area as a morphological mechanism for drought tolerance. Also, Manfalouty cv. reduced transpiration rate and stomatal conductance as a physiological mechanism for drought tolerance. In addition, Manfalouty cv. showed an increase in anatomical structures of the leaf such as the thickness of upper epidermis, palisade, spongy, xylem tissues as an anatomical mechanism for drought tolerance.

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