Water Stress Effects on Winter and Spring Leaves Anatomy of Different Wheat (Triticum aestivum L.) Genotypes

Tayebe Jafarian1*, Aliakbar Maghsoudi Moud1 and Vahid Reaz Saffari2

Abstract
Water stress effects on winter and spring leaves anatomy were investigated in experiments conducted at the experimental field of SB University of Kerman using five wheat cultivars. Xylem and phloem elements diameter and mesophyll, bundle sheath and epidermal cells area were measured in transverse sections prepared from middle parts of the leaves. Results showed that significant difference exists among genotypes in terms of anatomical characteristic. Results also showed that water stress changes the diameter and the surface area of the cells. However, the changes were not the same in winter and spring leaves. The changes in some cases such as xylem vessels diameter were considerable. For example, in cultivars Azar2 and Azadi, winter leaves had bigger xylem vessels under water stress compared to the normal condition while in spring leaves the xylem diameter was smaller under the same condition.

Keywords: Epidermis; Mesophyll; Vascular tissue

Introduction
Wheat crop growth and productivity is under the strong effects of water stress in the world (Reynolds et al. 1994; Blum 1996). Selection of drought tolerant genotypes is considered as an alternative way to maintain high grain yield under low soil moisture content. Plant features that could be used as selection criteria in this regard are needed to be identified. Different plant features such as leaf anatomy have been considered as characters useful for increasing water stress tolerance (Jones et al. 1980; Venora and Caleagno 1991). It has been shown that anatomical changes in leaf may help plants to maintain high levels of photosynthetic rates and high transpiration efficiencies (Evans et al. 1994). Cuticle thickness (Rojas et al. 1983), stomatal frequency (Rebetzke et al., 2010), length (Bohnert and Jensen, 1996), movement and sensitivity (Drake et al., 2013) are among anatomical characteristics which are believed to be useful for breeding water stress tolerant genotypes. Leaf morphological characters including leaf area (Zagdanska an Kozdo, 1994), shape (Reddy et al., 2004), duration (Verma et al., 2004) and developing behavior (Hu et al., 2000) are also considered as effective characters in environmental stress tolerance.

Vascular tissue systems including xylem and phloem are involved in transportation of the different compounds. However, the diameter and size of the conducting elements are the key factors determining the flow rate (Martre, and Durand 2001). In wheat leaves the venation network consists of a series of large or lateral and small or intermediate longitudinal veins which are connected to each other by transverse veins (Altus
and Canny 1985a). The diameter of the lateral veins decreases toward the leaf tip while the number and the size of the intermediate veins do not change along the length of the leaf and their water conductivity remains constant (Altus et al. 1985). However, the xylem vessel diameter is considered as the main factor limiting flow rate (Altus and Canny 1985b) though the changes of the xylem conductivity along the leaf axis also depends on the different stages of xylem maturation (Martre et al. 2000) and apoplastic and symplastic movement of water to the evaporation sites inside the leaf at mesophyll cells surface (Cochard et al. 2004).

Anatomical changes of the leaf are used as indicators of stress symptoms (Niinemets and Sack 2006). In the developing leaves these changes have significant effects on photosynthesis. For example, palisade mesophyll cells length and number in leaves are shown to be correlated with photosynthetic capacity (Syvertsen et al. 1995). Morphological and anatomical modifications under water stress condition are associated with leaf structure (Niinemets and Sack 2006). Transpiration rate of plants growing in dry regions is under the control of leaf size (Dias et al. 2007), epidermal cells and cuticle thickness (Wenzel et al. 1997), density (Press 1999) and stomatal pore area (Drake et al., 2013). Dehydration tolerance also has been shown to improve by other characteristics such as increased mechanical resistance of the cell walls by increasing the level of lignification (Blum 1996), increased succulence, increased water storage capacity and accumulation of mucilage (Kriedmann 1986).

Growth responses of plants to water stress are the result of changes in cell division, enlargement and deposition of cell wall materials (Fricke and Flowers 1998). It has been shown that the suberised lamellae of the mestome sheath cells form an incomplete barrier near the xylem to keep separate the oppositely directed fluxes of water and assimilates through the sheath (Canny 1986). Water stress has shown to prevent cell division and growth (Zagdanska and Kozdoj 1994). Tissues exposed to environments with low water availability have generally shown reduction in cell size, and increase in vascular tissue and cell wall thickness (Guerfel et al. 2009). Mesophyll cells are more vulnerable to water stress damages compared to the bundle sheet cells (Mansoor et al. 2002). In water stress tolerant sugarcane genotypes cell wall thickness increased under stress condition (Rojas et al. 1983). Smaller epidermal cells were found in Lolium perenne under water stress condition (Jones et al. 1980). Epidermal and mesophyll cell sizes were shown to reduce under water stress condition (Arteimos et al. 2002).

Salinity also is shown to reduce thickness of the leaf, cross-sectional area, width, and radii of epidermal and mesophyll cells in wheat (Hu et al. 2005). Reduction in cross-sectional area was attributed to a decrease in the size of the vein segments and a reduced number of medium and small veins (Hu et al. 2005). Hu et al. (2005) stated that reduced number of small veins under salt stress condition may be responsible for limitation of the capacity of translocation of nutrients and assimilates. Reduced area of protoxylem and metaxylem in midrib and large
vein segments in growing tissues may be responsible for lower water deposition into the growth zone under saline conditions (Hu et al. 2005).

Wheat leaves can be classified as winter leaves which are usually narrow, small and thin with small sheaths growing very close to each other and spring leaves which are wide, long and thick with large sheath make them to be separated from each other. Winter leaves are cold stress tolerant and do their metabolism under low temperature conditions while spring leaves do their best performance under higher temperature levels. However, in the growing leaves there are three distinct regions. The first is up to 30mm from ligule in which cells are dividing. In the second region, 30-60mm from ligule, newly produced cells are enlarging and cell wall is developing. The last region which expands up to the leaf tip is photosynthetically active (Hu et al. 2000). Little information exists regarding the effects of environmental factors on the anatomy of these leaves. The aims of this study was therefore to determine the effects of water stress on the size of epidermal, mesophyll and bundle sheath cells and vascular tissues of wheat leaves at rosette and booting growth stages and also to investigate the relationships between the change in the size of these cells under water stress condition.

**Material and Methods**

Two separate experiments were conducted in pot and field conditions at the experimental field of Kerman University (30° 14”N, 57° 7” E, 1775 meters above sea level). The pot experiment was factorial based on randomized complete design with three replications in which half of the pots were water stressed under a rain shelter. Plants were sampled at fourth leaf stage in order to obtain winter leaves. Five wheat cultivars including Azar2 and Azadi as drought tolerant, Omid and Shahpasand as semi-tolerant and Shole as a sensitive genotype were used. Seeds were germinated on wet tissue papers and were then sown at Nov 20, 2011 in pots containing a mixture of vermiculite and peat (1:1). Soil water holding capacity (FC) was determined (Mckim et al. 1980) before planting and the amount of water applied to the pots was adjusted at 100% and 60% of FC for normal and water stressed pots, respectively. In each replication there were two pots for each genotype, one was water stress treatment and the other kept at normal condition and all were randomly arranged. During the growth period pots were weighted every day using an electronic balance (precision=1 gram) and irrigated up to their initial weight to maintain the growing condition constant. One week after full expansion of the fourth leaf, leaf blades of the same size from each pot were sampled. From each, small segments at 60-65mm from the blade base were again sampled.

The same genotypes were sown at the same time in 2 × 4 meters experimental plots in the field condition in order to obtain typical spring leaves. A randomly arranged series of five plots, each for one genotype, were considered as main plots and two main plots were grouped as a complete replication. In each replication, irrigation of one main plot withheld before booting stage so that when flag leaves emerged the soil water content was at about 55 percent of soil water holding
capacity. The other was irrigated normally every seven days. When plants reached at late stem elongation stage penultimate leaves were sampled and used for preparing microscopic slides.

Samples from both leaf types were immediately transferred into 10% formalin solution for 48 hours. To prevent leaf curling samples were fixed on small pieces of cardboards by nips. Samples were then processed as follows to be prepared for taking transverse sections. First they transferred to a 1:1 mixture of 96% ethanol and 10% formalin for 60 minutes. Then they were immersed step by step in 50%, 70%, 80%, 90% and 96% ethanol solutions each for 60 minutes. The samples were then immersed two times in 100% ethanol and two times in 100% xylol. Finally samples were submerged in melted paraffin inside the blocking cassettes. Paraffin blocks were then fixed in the microtome clump and were transversely sectioned while the blade was adjusted at 5 µm. Sections were then transferred on microscopic slides and incubated into an electric oven adjusted at 70°C for 20 minutes. Sections were then stained using hematoxylin and eosin.

Anatomical examinations were performed on five images randomly taken from slides using an eye-piece digital camera fixed on a light microscope at 10×40 magnification. Scion-image analysis software (Scion-Image Corporation) was used to measure the area of epidermal, bundle sheath and mesophyll cells and the diameter of phloem sieve tubes and xylem vessels. In each case mean values of 10 random observations were used for data analysis.

All data were subjected to analysis of variance using the corresponding linear additive model (Steel and Torrie 1980). Mean values were compared using Duncan's multiple range test at 5% level of significance.

Results

Winter leaves

Xylem vessel diameter: The effect of genotype and water stress on xylem vessel diameter was significant (Table 1). The interaction of the two factors was significant too. Under water stress condition xylem vessels diameter was increased in Azadi and Azar2 while decreased in Shole (Figure 3).

Phloem sieve tubes diameter: Phloem sieve tubes diameter was not affected by water stress. Meanwhile, the interaction of the two factors and the effect of genotype were significant on phloem sieve tubes diameter (Table 1). Water stress decreased phloem sieve tubes diameter in Shole and Azadi while increased in Shahpasand, Azar2 and Omid (Figure 2).

Bundle sheath cells area: The effects of genotype and water stress were not significant on bundle sheath cells area. However, interaction of genotype by water stress was significant (Table 1). Highest and lowest bundle sheath cell areas were found in Shole and Omid under normal condition, respectively. Compared to the control condition bundle sheath cells area was decreased in Shole under water stress condition while it was increased in Omid, Shahpasand, Azadi and Azar2 under the same condition (Figure 1).
Water Stress Effects on Winter and Spring Leaves Anatomy

Table 1. Analysis of variance of the data regarding different anatomical characteristics of winter and spring leaves of wheat cultivars grown under normal and water stress conditions

<table>
<thead>
<tr>
<th>Diameter</th>
<th>Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaf type</td>
<td>df</td>
</tr>
<tr>
<td>Genotype</td>
<td>4</td>
</tr>
<tr>
<td>Winter</td>
<td>3.9**</td>
</tr>
<tr>
<td>Spring</td>
<td>1</td>
</tr>
<tr>
<td>Water stress</td>
<td>1</td>
</tr>
<tr>
<td>Interaction</td>
<td>4</td>
</tr>
<tr>
<td>Error</td>
<td>18</td>
</tr>
<tr>
<td>0.517</td>
<td>0.88</td>
</tr>
</tbody>
</table>

* **Significant at 5% and 1% probability level, respectively. ns Not significant

Figure 1. Means of bundle sheath cells area of winter leaves in different wheat cultivars grown under normal (black) and water stress (gray) conditions. Columns with the same alphabet letters are not significantly different at 5% level.

Figure 2. Means of phloem sieve diameter for winter leaves (left) and spring (right) leaves of wheat cultivars grown under normal (black) and water stress (gray) conditions. In each case, columns with the same letters are not significantly different at 5% level.
Mesophyll cells area: Mesophyll cells area was significantly affected by genotype and genotype by water stress interaction (Table 1). Under water stress condition mesophyll cells area was decreased in Shole while increased in Azar2. Highest and lowest mesophyll cells area was found in Shole under control and Omid under water stress condition, respectively (Figure 4).

Upper epidermal cells area: Upper epidermal cells area was also significantly affected by genotype and genotype by stress interaction (Table 1). Epidermal cells area was decreased in Shole, Omid and Azar2 under water stress condition while it was increased in Azadi and Shahpasand. Highest and lowest epidermal cell areas were found in Shole under control condition and Azar2 under stress condition (Fig 5).

Lower epidermal cells area: Genotype and genotype by water stress interaction effects on the lower epidermal cells area were significant (Table 1). In Shahpasand and Azadi lower epidermal cells area increased under water stress condition while in the others the changes were not significant (Figure 6).

Spring leaves

Bundle sheath cells area: Data analysis showed that water stress and genotype effects and their interaction were not significant on the bundle sheath cells area (Table 1).

Phloem sieve tubes diameter: Genotype effect was significant on phloem sieve tubes diameter (Table 1). Generally, phloem sieve tubes diameter was significantly higher in Azadi compared to Omid and Azar2 while the differences between Azadi, Shole and Shahpasand were not significant (Figure 2).

Xylem vessel diameter: Xylem vessel diameter was significantly affected by genotype and water stress and their interaction (Table 1). Xylem vessel diameter in Shole and Shahpasand was significantly decreased under water stress condition. Meanwhile, there were no changes in the xylem diameter in Omid and Azar2 under water stress compared to the normal condition (Figure 3).

Mesophyll cells area: Genotype and its interaction with water stress effects on the mesophyll area were significant (Table 1). In cultivar Shole mesophyll cells area was significantly increased under water stress condition while it was decreased in Azadi.

Figure 3. Mean values of xylem vessel diameter for winter leaves (left) and spring (right) leaves of wheat cultivars grown under normal (black) and water stress (gray) conditions. In each case, columns with the same letters are not significantly different at 5% level.
Changes in the mesophyll cells area of genotypes Omid, Shahpasnd and Azar2 under water stress condition were not significant (Figure 4).

**Upper epidermal cells area:** Water stress, genotype and their interaction effects on the upper epidermal cells area were significant (Table 1). Results are showing that compared to the normal condition upper epidermal cells area was increased in cultivars Shole, Shahpasnd, Azar2 and Azadi under water stress condition while it was decreased in cultivar Omid (Figure 5).

**Lower epidermal cells area:** The same results were obtained in the case of lower epidermal cells area so that it was significantly increased in Shole and Shahpsand under water stress condition and decreased in Omid. Meanwhile there were no changes in the epidermal cells area of Azar2 (Figure 6).

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**Figure 4.** Mean values of mesophyll cells area of winter leaves (left) and spring (right) leaves of wheat cultivars grown under normal (black) and water stress (gray) conditions. In each case, columns with the same letters are not significantly different at 5% level.

**Figure 5.** Mean values of upper epidermal cells area of winter leaves (left) and spring (right) leaves of wheat cultivars grown under normal (black) and water stress (gray) conditions. In each case, columns with the same letters are not significantly different at 5% level.
This study showed that the xylem vessel diameter in wheat leaves is under the effect of genotype, water stress and their interaction indicating that small size vessels, if desired, could be selected for, depending on the water availability. Decreasing xylem diameter may play a role in adaptation of plants to water stress condition since smaller diameter decreases the hydraulic conductivity of the xylem (Martre et al. 2001). It was shown that in the water stress susceptible winter wheat cultivars xylem diameter is greater compared to the tolerant ones (Ridly and Todd 1966). In this experiment xylem vessel diameter was increased under water stress condition in the winter leaves except in the cultivar Shole. However, xylem vessel diameter in Shole and Shahpasand reduced significantly under water stress condition. Meanwhile, reductions in other cultivars were not considerable. Therefore, lower xylem conductivity in the spring leaves is expected to cause less water movement under higher evaporating demands of their growing period.

The theoretical xylem hydraulic conductivity computed from the diameter of individual vessels using the Hagen–Poiseuille equation has been shown to be proportional to the observed values (Altus et al. 1985). It has been shown in spring wheat leaves that the largest lateral vessels decrease in diameter with distance along the leaf towards the tip, resulting in the decreased hydraulic conduction (Altus et al. 1985) which in turn may decrease the rate of water movement. This may help plants to use the available water slowly and as a result for a longer period of time.

On the other hand increasing the xylem vessel diameter in some plant parts may also be beneficial under water stress condition as may provide plants with a water reservoir which can be used at daytimes of high water demand. A bottleneck is shown to exist at the basal region of the leaf which could limit water transport (Martre and Durand 2001). This, however, needs to be investigated using more sophisticated instruments. It has been shown that there is a regulatory mechanism of water use in wheat leaf vein system which is composed of different diameter pipelines.

The diameter of phloem sieve tubes also is a key factor determining the flow rate of photo-assimilates from leaves to the sinks (Fitter and...
Hay 2002). Results of this experiment showed that the phloem sieve tubes diameter is affected in both winter and spring leaves while there was no effect by water stress. The existence of genetic variation in this case is shows that changing the capacity of the phloem sieve tubes can be targeted in breeding cultivars for higher assimilate translocation to the grains. Values of phloem cross-sectional area of different vein types along the wheat leaf blade supported the idea that lateral veins are responsible for translocation of assimilates while intermediate veins are working as collecting reservoirs (Altus and Canny 1982). Meanwhile, assimilated carbon is shown to be entered the intermediate and then the laterals veins (Altus and Canny 1985). It has been shown that phloem sieve tubes area depends on the sink with which they are related (Fitter and Hay 2002). However, the diameter of the phloem sieve tubes of the cultivars used in this study were not changed in accordance with their corresponding grain size as was reported by Ministry of Jihad-e-Agriculture and also our data (not reported here). This is in contrast with what is expected from the phloem sievetubes diameters implying that there may be other limiting factors affecting grain size in these cultivars.

Bundle sheath cells area of both leaf types were, on the other hand, not affected by genotype and water stress condition. The interaction effect of the two factors was however significant only in the case of winter leaves. Since bundle sheath cells are not photosynthetically active, increasing their size in cost of reducing mesophyll cell numbers may decrease the photosynthetic capacity of the leaf (McClendon 1992). It has been shown that increasing the number of the bundle sheath cells extensions reduces leaf photosynthetic capacity per unit area (McClendon 1992). Bundle sheath cells extensions can prevent effective lateral diffusion among surrounding mesophyll cells (Terashima 1992). On the other hand they can work as a light penetration system helping the light to penetrate into the deep cell layers (Nikopoulos et al. 2002) and increase photosynthetic capacity. There is however no report on the effect of the size of the bundle sheath cells on the rate of photoassimilates and solutes movement through plasmodesmata connecting xylem and phloem to them. The area of the bundle sheath cells was significantly reduced by water stress in the winter leaves of cultivar Sholeh while in the other cultivars there were no significant differences between water stress and control plants in terms of bundle sheath cells area.

It has been reported that reduction in the size is the major response of the cells to water stress condition. It was shown that mesophyll cells size decreased in olive plants leaves under water stress condition while the number of mesophyll cells and as a result the number of chloroplasts and CO2 fixation increased (Culter et al. 1977). Water stress decreased the size of the mesophyll cells and their intercellular spaces (Bongi et al. 1987; Mediavilla et al. 2001).

Our results showed that mesophyll cells area in both leaf types was affected by water stress condition (Figure 4) suggesting that selection for small mesophyll cells size is possible. This may help plants to have higher photosynthesis rates.

There is no report on the effect of water stress on wheat leaf mesophyll cells size. Environmental
stresses, however, were shown to change mesophyll cells dimensions in other crop plants. For example, high temperature stress increased the thickness of palisade and spongy cell layers and lower epidermal cells in soybean leaves. Decreased levels of photosynthesis are shown to be mediated through anatomical changes in soybean leaves under high temperature stress (Djanaguiramana et al. 2011). It has been found in wild and transgenic tobacco plants that irrespective of genotype or environment, CO$_2$ transfer conductance varied in proportion to the surface area of chloroplasts exposed to intercellular airspaces (Evans et al. 1994). In avocado changes in mesophyll cell structure was shown to be the cause of low photosynthetic rate (Chaves 1991; Chartzoulakis et al. 2002). Our results showed that mesophyll cell size was not reduced by water stress condition.

Changes in the size and shape of the epidermal cells in both winter and spring leaf types can facilitate the penetration of light into the mesophyll cells (Karabourniotic et al. 1994). Under water stress condition changes of the area of the epidermal cells in both leaf types were the same in Omid and Shahppasand while in the case of Azar2 and Azadi water stress effect was only significant on the epidermal cells of the upper surface of the spring leaves. Reductions of the epidermal cell size in the winter and spring leaves in cultivar Omid and also in the winter leaves of Sholeh are in accordance with the findings of Artemios and Bosabalidis (2002) in olive and Jones et al. (1980) in ryegrass.

**General conclusion**

Results obtained from this experiment showed that anatomical changes of the winter and spring leaves are not the same under water stress condition. Different responses of the two types of leaves are not unexpected since they grow under different environmental conditions. However, in some cases such as xylem vessels diameter responses are considerably different. For example, in cultivars Azar2 and Azadi winter leaves had larger xylem vessel diameter under water stress compared to the normal condition while in spring leaves they had smaller vessels under water stress condition.

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