Effects of Drought Stress on Some Anatomical Characteristics of Barley Leaves

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Abstract
In this research, effect of drought stress on leaf characteristics was investigated in the experimental field of Miandoab Azad University, Iran using four facultative barley cultivars. Two separate experiments were conducted in pot and field conditions. In both experiments a factorial arrangement was used and the treatments were completely randomized in four replications. In each experiment half of the experimental units were drought stressed and the other half were irrigated normally. For the pot experiment, plants were sampled at fourth leaf stage in order to obtain winter leaves. For the field experiment, when plants reached at late stem elongation stage, penultimate leaves were sampled and used for preparing microscopic slides.
Xylem and phloem diameter and mesophyll, bundle sheath and epidermal cells area were measured in the transverse sections prepared from middle parts of the leaves. Significant differences were observed among genotypes under drought stress in terms of leaf characteristics. Results also showed that drought stress changes the diameter and the surface area of the cells. However, the changes were not the same in the winter and spring leaves. The changes in some cases such as diameter of xylem vessels were considerable. For example, in the cultivars Sahra and Jonob, the winter leaves had bigger xylem vessels under drought stress as compared to the normal condition while in the spring leaves the xylem diameter was smaller under the same condition.

Keywords: Anatomical characteristics; Barley; Drought; Leaf

Introduction
Barley (Hordeum vulgär L.) is a major crop ranked fourth in the world among cereals (Baik and Ullrich 2008). Barley is typically cultivated in the arid and semi-arid regions of Iran generally in areas with low precipitation that is not suitable for wheat (Talame et al. 2007). Drought is a significant limiting factor for agricultural productivity and generally inhibits plant growth through the reduction in water absorption and nutrient uptake. Different plant features such as leaf anatomy have been considered as characters useful for increasing drought stress tolerance (Lee et al. 2011). 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 (Bohnert and Jensen 1996) and stomatal frequency (Rebetzke et al. 2010) are anatomical characteristics which are believed to be useful for breeding drought stress tolerant genotypes. Leaf morphological characters including leaf area (Zagdanska and Kozdoj 1994), shape (Reddy et al. 2004) and developing behavior (Hu et al. 2000) are also considered as effective characters for environmental stress tolerance.

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 (Zagdanska and Wisniewski 1996). 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 (Hallik et al. 2009). However, the xylem vessel diameter is considered as the main factor limiting flow rate though the changes of the xylem conductivity along the leaf axis also depends on the different stages of xylem maturation (Payvandi et al. 2014) 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 drought 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 (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 lignification level (Moore et al. 2008), increased succulence, increased water storage capacity and accumulation of mucilage (Harb et al. 2010).

Growth responses of plants to drought 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 (Verma et al. 2004). Drought stress has shown to prevent cell division and growth (Zagdanska and Kozdój 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 the water stress tolerant sugarcane genotypes cell wall thickness increased under stress condition (Vasantha et al. 2005). Smaller epidermal cells were found in Lolium perenne under drought stress condition (Japp and Newman 1987). Epidermal and mesophyll cell sizes were shown to reduce under water stress environment (Arteimos et al. 2002).

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 (Hallik et al. 2009; Payvandi et al. 2014). 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; Talame et al. 2007).

Barley 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. 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 30 mm from ligule in which cells are dividing. In the second region, 30-60 mm 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 (Kazemi Arbat 2005). 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 drought stress on the size of epidermal, mesophyll and bundle sheath cells and vascular tissues of barley leaves.

Materials and Methods
In this research, two separate experiments were conducted in pot and field conditions at the experimental field of Miandoab Azad University, Iran. The pot experiment was factorial based on completely randomized design with four replications in which half of the pots were drought stressed under a rain shelter. Plants were sampled at fourth leaf stage in order to obtain winter leaves. Four barley facultative cultivars including Sahra and Jonob as drought tolerant, Zarjo as semi-tolerant and Valfajr as a sensitive genotype were used. Seeds were germinated on wet tissue papers and were then sown at Nov 22, 2014 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 drought stressed pots, respectively. In each replication there were two pots for each genotype, one was drought 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 60-65 mm from the blade base were again sampled.

The genotypes were sown at the same time in the 2 × 3 meter square experimental plots in the field condition in order to obtain typical spring leaves. The experiment was factorial and the treatments were completely randomized using four replications. In the drought stressed plots, irrigation withheld before booting stage so that when flag leaves emerged, the soil water content was at about 60 percent of soil water holding capacity. In the normal condition, plots were 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. After that, sections were 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 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. MSTATC and SPSS software were used to analyze the data obtained. Duncan’s multiple range test was used to compare means, and Excel software was used to construct diagrams.

Results

Winter leaves characteristics

Xylem vessel diameter: The effect of genotype, drought stress and interaction of these two factors on xylem vessel diameter was significant (Table 1). Under drought stress condition xylem vessels diameter was increased in Sahra, Jonob and Zarjo while decreased in Valfajr as compared to the normal condition (Figure 1A).

Phloem sieve tubes diameter: Phloem sieve tubes’ diameter was significantly affected by genotype, drought stress and their interaction (Table 1). Drought stress decreased phloem sieve tubes diameter in Valfajr and Jonob while increased in Sahra and Zarjo as compared to the normal condition (Figure 1B).

Bundle sheath cells area: Genotype did not have significant effect on bundle sheath cells area. However, the effect of drought stress and interaction of genotype by drought stress was significant on this trait (Table 1). Highest and lowest bundle sheath cell areas were found in Valfajr and Zarjo under normal condition, respectively. Compared to the control condition bundle sheath cells area decreased in Valfajr and Jonob under drought stress condition while it was increased in Zarjo and Sahra under the same condition (Figure 1C).

Mesophyll cells area: Mesophyll cells area was significantly affected by genotype, drought stress and genotype by drought stress interaction (Table 1). Under drought stress condition mesophyll cells area decreased in Valfajr, Jonob and Zarjo while increased in Sahra. Highest and lowest mesophyll cells area were found in Sahra and Zarjo under drought stress condition, respectively (Figure 1D).

Upper epidermal cells area: Upper epidermal cells area was also significantly affected by genotype, drought stress and genotype by drought stress interaction (Table 1). Epidermal cells area decreased in Valfajr, Zarjo and Sahra under drought stress condition while it was increased in Jonob. Highest and lowest epidermal cell areas were found in Valfajr and Zarjo under stress condition (Figure 1E).
Lower epidermal cells area: Drought stress and genotype by drought stress interaction effects on the lower epidermal cells area were significant (Table 1). In the Jonob cultivar lower epidermal cells area increased significantly under drought stress condition while in others the changes were not significant (Figure 1F).

Spring leaves characteristics
Xylem vessel diameter: Xylem vessel diameter was significantly affected by genotype, drought stress and their interaction (Table 2). Xylem vessel diameter in Valfajr significantly decreased under drought stress condition. Meanwhile, there were no changes in the xylem diameter in Zarjo, Sahra and Jonob under stress as compared to the normal condition (Figure 2A).
Phloem sieve tubes diameter: Genotype had significant effect on phloem sieve tubes diameter (Table 2). Generally, phloem sieve tubes diameter was significantly higher in the Jonob cultivar as compared to Valfajr and Sahra while the difference between Jonob and Zarjo was not significant (Figure 2B).
Bundle sheath cells area: Data analysis showed that genotype, drought stress and their interaction were not significant on the bundle sheath cells area (Table 2).
Mesophyll cells area: Genotype, drought stress and their interaction on the mesophyll area were significant (Table 2). In Valfajr, mesophyll cells area significantly increased under drought stress condition while it decreased in the Jonob cultivar. Changes in the mesophyll cells area of genotypes Zarjo and Sahra under drought stress condition were not significant (Figure 2C).

Upper epidermal cells area: Drought stress, genotype and their interaction on the upper epidermal cells area were significant (Table 2). Results showed that upper epidermal cells area increased in Sahra, Jonob and Valfajr under drought stress condition while it was decreased in the cultivar Zarjo as compared to the normal condition (Figure 2D).
Lower epidermal cells area: This trait was significantly increased in Valfajr under drought stress condition and decreased in Zarjo. There was no change in the epidermal cells area of Sahra (Figure 2E).

Discussion
In this experiment xylem vessel diameter was increased under drought stress condition in the winter leaves except in the Valfajr cultivar; xylem vessel diameter in Valfajr reduced significantly under drought stress condition. Furthermore, 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. Decreasing xylem diameter may play a role in adaptation of plants to drought stress condition since smaller diameter decreases the hydraulic conductivity of the xylem (Fitter and Hay 2002). It was shown that in the water stressed susceptible winter wheat cultivars xylem diameter is greater compared to the tolerant ones (Verma et al. 2004).

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
(Verma et al. 2004; Talame et al. 2007) 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 day times of high water demand. A
bottleneck is shown to exist at the basal region
of the leaf which could limit water transport (Fitter
and Hay 2002).

The existence of genetic variation in this case
shows that changing the capacity of the phloem
sieve tubes can be targeted in the breeding of
cultivars for higher assimilate translocation to the
grains. Values of phloem cross-sectional area of
different vein types along the barley leaf blade
supported the idea that lateral veins are responsible
for translocation of assimilates while intermediate
veins are working as collecting reservoirs
(Minchin et al. 2002). It has been shown that
phloem sieve tubes area depends on the sink with
which they are related (Fitter and Hay 2002). This
is in contrast with what is expected from the
phloem sieve tubes diameters implying that there
may be other limiting factors affecting grain size
in these cultivars.

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. 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. On the other hand, they can work
as a light penetration system helping the light to
penetrate into the deep cell layers and increase
photosynthetic capacity (Niinemets and Sack
2006). There is, however, no report about 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.

It has been reported that reduction in the
size is the major response of the cells to drought
stress condition. It was shown that mesophyll cells
size decreased in olive plants leaves under drought
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). This
may help plants to have higher photosynthesis
rates. There is no report about the effect of drought
stress on barley 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, CO₂ 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 (Chartzoulakis et al. 2002; Chaves and Oliveira 2004).

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. Reductions of the epidermal cell size in the winter and spring leaves in cultivar Zarjo and also in the winter leaves of Valfajr are in accordance with the findings of Bosabalidis and Kofidis (2002) in olive and Jones et al. (1980) in ryegrass.

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

Table 1. Analysis of variance of winter leaf traits of barley varieties under normal and drought stress conditions

<table>
<thead>
<tr>
<th>SOV</th>
<th>df</th>
<th>Xylem vessel diameter</th>
<th>Phloem sieve tubes diameter</th>
<th>Bundle sheath cells area</th>
<th>Mesophyll cells area</th>
<th>Upper epidermal cells area</th>
<th>Lower epidermal cells area</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genotype</td>
<td>3</td>
<td>171.96 *</td>
<td>12.02 *</td>
<td>12.20 **</td>
<td>1367.25 *</td>
<td>1030.85 *</td>
<td>293.46 *</td>
</tr>
<tr>
<td>Water condition</td>
<td>1</td>
<td>654.85 **</td>
<td>28.80 *</td>
<td>20.96 *</td>
<td>2152.37 *</td>
<td>2532.22 **</td>
<td>1038.54 *</td>
</tr>
<tr>
<td>Genotype × Water</td>
<td>3</td>
<td>236.50 *</td>
<td>19.52 *</td>
<td>80.38 **</td>
<td>2797.98 **</td>
<td>1133.48 *</td>
<td>1319.86 *</td>
</tr>
<tr>
<td>Error</td>
<td>28</td>
<td>55.83</td>
<td>4.37</td>
<td>6.94</td>
<td>452.28</td>
<td>320.74</td>
<td>233.27</td>
</tr>
<tr>
<td>C.V. (%)</td>
<td></td>
<td>10.2</td>
<td>11.7</td>
<td>4.5</td>
<td>5.9</td>
<td>6.0</td>
<td>4.1</td>
</tr>
</tbody>
</table>

ns, * and **: Not significant and significant at the 5% and 1% levels of probability, respectively.

Table 2. Analysis of variance of spring leaf traits of barley varieties under normal and drought stress conditions

<table>
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<th>df</th>
<th>Xylem vessel diameter</th>
<th>Phloem sieve tubes diameter</th>
<th>Bundle sheath cells area</th>
<th>Mesophyll cells area</th>
<th>Upper epidermal cells area</th>
<th>Lower epidermal cells area</th>
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<tbody>
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<td>6.02 **</td>
<td>342.80 *</td>
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<td>87.38 *</td>
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<tr>
<td>Water condition</td>
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<td>18.67 *</td>
<td>0.09 **</td>
<td>15.79 **</td>
<td>181.64 **</td>
<td>318.03 *</td>
<td>231.04 **</td>
</tr>
<tr>
<td>Genotype × Water</td>
<td>3</td>
<td>26.40 **</td>
<td>1.02 **</td>
<td>2.18 **</td>
<td>462.13 **</td>
<td>137.68 *</td>
<td>119.49 **</td>
</tr>
<tr>
<td>Error</td>
<td>28</td>
<td>2.98</td>
<td>1.13</td>
<td>4.91</td>
<td>90.19</td>
<td>45.90</td>
<td>27.17</td>
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<tr>
<td>C.V. (%)</td>
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<td>9.8</td>
<td>6.3</td>
<td>2.1</td>
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<td>13.4</td>
<td>14.6</td>
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</table>

ns, * and **: Not significant and significant at the 5% and 1% levels of probability, respectively.
Figure 1. Means of barley genotypes for winter leaf characteristics grown under normal (white) and drought stress (black) conditions; A) Xylem vessel diameter, B) Phloem sieve tubes diameter, C) Bundel sheath cells area, D) Mesophyll cell area, E) Upper epidermal cells area, F) Lower epidermal cells area. Means with different letters are significantly different at 0.05 probability level based on Duncan’s multiple range test.
Figure 2. Means of barley genotypes for spring leaf characteristics grown under normal (white) and drought stress (black) conditions; A) Xylem vessel diameter, B) Phloem sieve tubes diameter, C) Mesophyll cell area, D) Upper epidermal cells area, E) Lower epidermal cells area. Means with different letters are significantly different at 0.05 probability level based on Duncan’s multiple range test.
References


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