Influence of ascorbic acid foliar application on chlorophyll, flavonoids, anthocyanin and soluble sugar contents of sunflower under conditions of water deficit stress

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Abstract
Water stress is one of the most important environmental factors that reduce growth, development and production of plants, thus, the effects of water deficit stress and foliar application of ascorbic acid were studied on chlorophyll a and b, flavonoids and anthocyanin contents and soluble sugar composition (glucose, xylose and mannose) in leaves of sunflower. A factorial pot experiment based on completely randomized design was used with three replications. Stress levels were consist, -0.03 (control), -0.6 and -1.2 MPa as the first factor and ascorbic acid concentrations namely 0, 50, 100 and 150 mM were allocated to the second factor. The results showed that water deficit stress decreased chlorophyll content while increased flavonoids, anthocyanin and soluble sugars contents. Ascorbic acid foliar application had significant effect on decrease of flavonoids, anthocyanin and soluble sugars content in stressed plants. All these results suggest that ascorbic acid foliar application increases water stress resistance and we can use it on stressed plants in order to decrease adverse effect of water deficit stress.

Key words: Anthocyanin, ascorbic acid, flavonoid, sugars, sunflower, water stress.

Introduction
Plants are exposed to a variety of biotic or abiotic stresses, such as drought, salinity and freezing that influence on their development, growth and productivity. Water stress is one of the most important abiotic stress factors 1, which affects almost every aspect of plant growth in agricultural systems. Water stress causes a wide range of morphological, physiological and metabolic responses in plants: including reduced growth, reduced leaf area and an increase in some pigments such as flavonoids and anthocyanin 2 and a decrease in the efficiency of photosynthesis due to chlorophyll degradation. Also, accumulation of sugars in different parts of plants in response to the variety of environmental stresses has been reported 3. A secondary aspect of water stress in plants is the stress-induced production of reactive oxygen species (ROS) including superoxide radicals, hydrogen peroxide and hydroxyl radicals. These oxygen species cause oxidative damage to different cellular components including membrane lipids, proteins and nucleic acids 4, 5. Plants possess efficient systems for scavenging ROS that protect them from destructive oxidative reactions. These systems consist of non-enzymatic and enzymatic antioxidant systems such as carotenoids, ascorbic acid, tocopherols, peroxidase and catalase 6, 7. One approach for inducing oxidative stress tolerance would be to increase the cellular level of enzyme substrates such as ascorbic acid (vitamin C). Ascorbic acid is a small, water-soluble anti-oxidant molecule which acts as a primary substrate in the cyclic pathway of enzymatic detoxification of hydrogen peroxide. As presented by Dolatabadian et al. 8 application of ascorbic acid alleviates reactive oxygen species and this will be beneficial for plants’ tolerance to oxidative stresses 9. In this study, we present details on chlorophyll, flavonoids, anthocyanin and status of soluble sugars in the vegetative stage of sunflower under conditions of water deficit stress and ascorbic acid foliar application. Thus, we have selected three different levels of water potential and four concentrations of ascorbic acid. The main objective of this study was reducing detrimental effects of water stress by ascorbic acid as an antioxidant molecule.

Material and Methods
Plant material and growth condition: The experiment was conducted in a glasshouse at Tabriz during the spring of 2008. The seeds of sunflower (Helianthus annuus L. cv. Blizlar) were grown in plastic pots (20 litres volume) that contained clay–loam soil (36% clay, 34% silt and 30% sand) in a glasshouse under conditions of 25/20°C day/night temperature and supplementary photon flux density of 300 µmol m⁻² s⁻¹. Five surface-sterilized seeds were planted in each pot, and after full germination the number of plants was reduced to two seedlings per pot. The pots were irrigated at field capacity level until seed germination.

Water deficit stress and ascorbic acid foliar application: When plants had two or three expanded leaves water deficit stress was created by stopping irrigation. Time domain reflectometry was used to monitoring of soil moisture in all pots. In order to water deficit stress induction irrigation was stopped until soil water potential reached to -0.6 and -1.2 MPa in stressed plants and -0.03 in control plants. Soil moisture was monitored daily. It took 2 days for control plants and about 4 and 5 days for stressed plants to reach these water potentials. Ascorbic acid was sprayed on plants by backpack sprayer at two times. First was at five-leaves stage and second was at flowering initiation. Ascorbic acid in four
concentrations, 0, 50, 100 and 150 mM, was applied. Plant leaves were sampled at the stage of flowering, frozen in liquid N₂, and stored at −80°C until biochemical analysis.

**Chlorophyll assay:** Chlorophyll was extracted in 80% acetone from the leaf samples. Extracts were filtrated and absorbance of chlorophyll a and b were determined by spectrophotometer (UV-S, Sinco 2100) at 645 and 663 nm. The content of chlorophyll was expressed as mg g⁻¹ FW.

**Flavonoid assay:** Flavonoids were measured by method of Krizek et al. Leaf samples were homogenized in a mortar and pestle with 3 ml 1% acetic acid-ethanol solvent (1: 99 v:v). The homogenate was centrifuged at 18,000 g for 30 min, and the supernatant was incubated in a water bath for 10 min at 80°C and allowed to cool to room temperature. The amount of flavonoids was determined from the absorbance at 270, 300 and 330 nm. The content of flavonoids were determined using the extinction coefficient of flavonoids (ε = 33,000 mol⁻¹ cm⁻¹). Flavonoid content was expressed as µmol cm⁻².

**Anthocyanin assay:** Anthocyanin content was estimated according to the method of Dubois et al. Leaf samples were homogenized in a mortar and pestle with 3 ml 1% HCl-methanol solvent (1: 99 v:v). The homogenate was centrifuged at 18,000 g for 30 min and the supernatant was filtered through Whatman #1 to remove particulate matter and stored in darkness at 5°C for 24 h. The amount of anthocyanin was determined from the absorbance at 520, 485 and 320 nm. The content of anthocyanin was determined using the extinction coefficient of anthocyanin (ε = 33,000 mol⁻¹ cm⁻³). Anthocyanin content was expressed as µmol cm⁻².

**Soluble sugars assay:** Soluble sugars including glucose, xylose and mannose were estimated according to the method of Dubois et al. Leaf samples were homogenized in a mortar and pestle with 3 ml distilled water, homogenate was filtered by filter paper and 0.5 ml of 5% phenol and 2.5 ml of 98% sulfuric acid were added to homogenate. After reaction, the test tubes were allowed to cool to room temperature. The amounts of glucose, xylose and mannose were determined from the absorbance at 480, 485 and 490 nm, respectively. The sugar concentration was calculated from a glucose, xylose and mannose standard curve.

**Statistical analysis:** All data were analyzed by analysis of variance (ANOVA) by SAS. Duncan’s Multiple Range Tests was used to measure statistical differences between treatment methods and controls.

**Results and Discussion**

The analysis of variance demonstrated that water deficit stress and ascorbic acid had significant effect on all assayed traits except flavonoid 270 and chlorophyll a, respectively. Also, interaction between water deficit stress and ascorbic acid foliar application was significant on chlorophyll b, flavonoids and soluble sugars (Table 1). Water deficit stress significantly decreased total chlorophyll when soil water potential was -1.2 MPa while there was no significant difference between -0.03 and -0.6 MPa irrigation treatments (Fig. 1). Foliar application of ascorbic acid had significant effect on total chlorophyll content and increased it but the highest total chlorophyll content was in those plants which were treated by 50 mM ascorbic acid (Fig. 2). It’s possible that high concentrations of ascorbic acid have some phytotoxic effect on chlorophyll function. In other word increase of ascorbic acid concentration up to 100 and 150 mM decreased chlorophyll content. It’s reported that the chlorophyll content decreased to a significant level at higher water deficit in sunflower plants. Also, a reduction in chlorophyll content was reported in drought-stressed cotton. Similar reports exist for decreasing chlorophyll in wheat, pea and white mulberry under drought condition. The accumulation of active oxygen species produced during drought stress damages many cell compounds like fat, protein and photosynthetic pigments. The present results show that chlorophyll a was decreased by high level of water deficit stress (Table 1, Fig. 3). Sanchez et al. reported that drought stress changes chlorophyll content. Interaction between water deficit stress and ascorbic acid foliar application was significant on chlorophyll b (Table 1). Comparison of means showed that the highest chlorophyll b content was obtained from highly stressed plants which received 50 mM ascorbic acid (Table 2). It seems that increase of chlorophyll content due to ascorbic acid application (50 mM) depends on scavenging of reactive oxygen species by this antioxidant molecule. Also it’s implied that ascorbic

![Figure 1](image1.png)

**Figure 1.** Changes in total chlorophyll content due to water deficit stress. Columns followed by the same letter are not significantly different (p<0.05)

![Figure 2](image2.png)

**Figure 2.** Changes in total chlorophyll content due to ascorbic acid foliar application. Columns followed by the same letter are not significantly different (p<0.05)

<table>
<thead>
<tr>
<th>S.O.V</th>
<th>df</th>
<th>Chlt</th>
<th>Chla</th>
<th>Chlb</th>
<th>270 nm</th>
<th>300 nm</th>
<th>330 nm</th>
<th>Anth</th>
<th>Glu</th>
<th>Xyl</th>
<th>Man</th>
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<td>2</td>
<td>**</td>
<td>**</td>
<td>**</td>
<td>ns</td>
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<td>ns</td>
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<td>**</td>
<td></td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>3</td>
<td>**</td>
<td>ns</td>
<td>**</td>
<td>**</td>
<td>**</td>
<td>**</td>
<td>**</td>
<td>**</td>
<td>**</td>
<td>**</td>
</tr>
<tr>
<td>Water stress x Ascorbic acid</td>
<td>6</td>
<td>ns</td>
<td>**</td>
<td>**</td>
<td>**</td>
<td>**</td>
<td>**</td>
<td>ns</td>
<td>**</td>
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</tr>
<tr>
<td>C.V</td>
<td>0.75</td>
<td>21.50</td>
<td>16.55</td>
<td>11.66</td>
<td>11.83</td>
<td>8.21</td>
<td>15.01</td>
<td>8.89</td>
<td>10.45</td>
<td>13.74</td>
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</table>

Chlt total chlorophyll; Chla chlorophyll a; Chlb chlorophyll b; Flavonoid 270 nm; Flavonoid 300 nm; Flavonoid 330 nm; Anth anthocyanin; Glu glucose; Xyl xylose; Man mannose

**Table 1.** Analysis of variance of chlorophyll, flavonoids, anthocyanin, glucose, xylose and mannose affected by water stress and ascorbic acid foliar application.

<table>
<thead>
<tr>
<th>Journal of Food, Agriculture &amp; Environment, Vol.10 (1), January 2012</th>
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Our results showed that water stress decreased the total chlorophyll concentration while the flavonoid concentration increased. Thus, this implies a negative relation between chlorophyll and flavonoid concentration by water relations.

Main effects of water deficit stress and ascorbic acid foliar application were significant on anthocyanin content in leaves of sunflower while interaction between them was not significant (Table 1). Fig. 4 shows that water deficit stress increased anthocyanin content, however, there was no significant difference between two levels of water stress (-0.6 and -1.2 MPa). On the other hand ascorbic acid decreased anthocyanin content, especially at high concentration namely 150 mM (Fig. 5). Anthocyanins are water-soluble pigments derived from flavonoids via the shikimic acid pathway. Anthocyanins and phenolic compounds such as flavonoids are largely responsible for the antioxidant capacity in plant tissues. In addition, anthocyanins have been reported to help reduce damage caused by free radical activity such as low-density lipoprotein oxidation, platelet aggregation, and endothelium-dependent vasodilation of arteries. Since drought induces the generation of reactive oxygen species, the antioxidant properties of anthocyanins can prevent membrane injury, protein degradation, enzyme inactivation and the disruption of DNA. These characteristics of anthocyanin have been described in leaves by Gould et al. Also, anthocyanins may protect photosynthetic tissues against photo-inhibition.

### Table 2. Interaction between water deficit stress and ascorbic acid foliar application on chlorophyll b, flavonoids, glucose, xylose and mannose.

<table>
<thead>
<tr>
<th>Water potential</th>
<th>Ascorbic acid (mg g⁻¹ FW)</th>
<th>Chlb (µmol cm⁻¹)</th>
<th>270nm (µmol cm⁻¹)</th>
<th>300nm (µmol cm⁻¹)</th>
<th>330nm (µmol cm⁻¹)</th>
<th>Glu (µg g⁻¹ FW)</th>
<th>Xyl (µg g⁻¹ FW)</th>
<th>Man (µg g⁻¹ FW)</th>
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<tbody>
<tr>
<td>0 mM</td>
<td>0.50def</td>
<td>0.07def</td>
<td>0.11a</td>
<td>0.06dc</td>
<td>20407de</td>
<td>10807fg</td>
<td>12042e</td>
<td></td>
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<tr>
<td>50 mM</td>
<td>0.69bcd</td>
<td>0.09bcd</td>
<td>0.07cd</td>
<td>0.07cd</td>
<td>22308de</td>
<td>13899de</td>
<td>15905bde</td>
<td></td>
</tr>
<tr>
<td>100 mM</td>
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<td>0.09bc</td>
<td>0.06d</td>
<td>0.08c</td>
<td>27503b</td>
<td>16392bde</td>
<td>19019b</td>
<td></td>
</tr>
<tr>
<td>150 mM</td>
<td>0.60cde</td>
<td>0.09bcde</td>
<td>0.06d</td>
<td>0.06de</td>
<td>26885b</td>
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<td></td>
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<tr>
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<td>0.12a</td>
<td>36307a</td>
<td>21620a</td>
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<tr>
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<td>0.11b</td>
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<tr>
<td>100 mM</td>
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<td>0.07ef</td>
<td>0.07bcd</td>
<td>0.10b</td>
<td>27399b</td>
<td>17094b</td>
<td>19119b</td>
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<tr>
<td>150 mM</td>
<td>0.65bced</td>
<td>0.07f</td>
<td>0.08bc</td>
<td>0.05e</td>
<td>17844ef</td>
<td>10948fg</td>
<td>11967c</td>
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<tr>
<td>0 mM</td>
<td>0.52def</td>
<td>0.13a</td>
<td>0.12a</td>
<td>0.13a</td>
<td>37441a</td>
<td>22536a</td>
<td>25524a</td>
<td></td>
</tr>
<tr>
<td>50 mM</td>
<td>1.03a</td>
<td>0.07ef</td>
<td>0.08bcd</td>
<td>0.06dc</td>
<td>16230f</td>
<td>9231g</td>
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<td>0.08def</td>
<td>0.11a</td>
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<td>16556b</td>
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<tr>
<td>150 mM</td>
<td>0.76bc</td>
<td>0.07ef</td>
<td>0.09b</td>
<td>0.04f</td>
<td>22520bc</td>
<td>13546def</td>
<td>13812de</td>
<td></td>
</tr>
</tbody>
</table>

-0.03  0  1  2  3  4  5  6  7  8  9  10  11  12

### Figure 3. Changes in chlorophyll b content due to water deficit stress. Columns followed by the same letter are not significantly different (p<0.05).

### Figure 4. Changes in anthocyanin content due to water deficit stress. Columns followed by the same letter are not significantly different (p<0.05).

### Figure 5. Changes in anthocyanin content due to ascorbic acid foliar application. Columns followed by the same letter are not significantly different (p<0.05).
Decrease of anthocyanin content in plants treated with ascorbic acid can be due to removing of reactive oxygen species by ascorbic acid.

The highest contents of soluble sugars were in untreated plants under conditions of water deficit stress (Table 2). In general, application of ascorbic acid strongly decreased soluble sugar content when plants were under stress but under normal condition we observed an increase in soluble sugars due to ascorbic acid foliar application. It seems that ascorbic acid improves photosynthesis due to increase of chlorophyll content and consequently assimilates such as sugars will increase. Also the alleviation of sugar under drought stress can be as a result of photosynthesis reduction, because the decreasing of water causes alleviation of turgor and losing of turgor pressure causes closing of stomata and finally decreasing photosynthesis. In contrast, decrease of soluble sugars by ascorbic acid under water deficit stress conditions can be related to neutralizing adverse effect of water stress by this antioxidant. Because carbohydrate accumulation during various abiotic stresses has been reported, decrease of sugars due to removing of stress is acceptable. Adaptation to water stress is associated with metabolic adjustment that leads to the accumulation of several organic solutes, for example sugars. Plants with increasing sugar content in roots (in case of water deficit condition) help decreasing osmotic potential on root and so absorb more water.

In case of water stress adaptation to this stress has been attributed to the stress-induced increase in carbohydrate level. Soluble carbohydrates have a potential role in adaptation to water stress. They can act in water replacement to maintain membrane phospholipids in the liquid crystalline phase and to prevent structural changes in soluble proteins.

Conclusions

Our results showed that ascorbic acid was involved in the plant response to water deficit stress, by changes in pigment contents and soluble sugars. We conclude that ascorbic acid decreases adverse effects of water stress and it can improve plant growth via increase of water deficit resistance. Plants, living in stressful environments, have evolved complex enzymatic and non-enzymatic antioxidant defense systems to regulate endogenous ROS concentrations throughout their development; however, oxidative damage may occur when ROS formation and antioxidant defenses become unbalanced.

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References


