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The Effects of Exogenous Application of Ascorbate and Glutathione on Antioxidant System in Cultivated *Cicer arietinum* and Wild Type *C. reticulatum* under Drought Stress

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Abstract: The roles of ascorbate and glutathione as key antioxidant molecules involves in environmental stress responses have already been well indicated. We conducted experiments in order to study the effects of exogenous ascorbate (ASC) and glutathione (GSH) treatments on antioxidant enzyme activities and ASC and GSH levels of cultivated and wild type chickpea plants under drought stress. We determined that ASC and GSH accumulation, antioxidant enzyme activities increased due to drought stress, except for CAT activity, in both species. ASC treatment increased ASC level and APX activity in *C. arietinum* under drought stress, except high concentration of ASC treatment. Antioxidant treatments increased antioxidant accumulation of *C. reticulatum* under drought stress. Antioxidant treatments did not lead to significantly changes in GR activity, in both species. However, exogenous ASC and GSH via eliminating of reactive oxygen species decreased SOD, CAT and APX activities in *C. reticulatum*. The results of present study indicate that ASC and GSH may contribute to the improvement of tolerance against drought stress in chickpea. Also, drought tolerant chickpea *C. reticulatum* showed a better protection mechanism against oxidative damage than the sensitive chickpea *C. arietinum*.

Kuraklığa Maruz Bırakılan Kültür Nohut *Cicer arietinum* ve Yabani Nohut *C. reticulatum*'un Antioksidan Sistemi Üzerine Dışsal Askorbat ve Glutatyon Uygulamalarının Etkisi

Anahtar Kelimeler
Kuraklık stresi
askorbat
glutatyon
antioksidan
nohut.

Özet: Askorbat ve glutatyonun çevresel streste anahtar moleküller oldukları iyi bilinmektedir. Kültür nohut *Cicer arietinum* ve yabani *Cicer reticulatum*'un antioksidan enzimler, askorbat (ASC) ve glutatyon (GSH) birikimi üzerine kuraklık, ASC ve GSH'ın etkisini çalışmak üzere bir deney hazırlandı. Her iki türde kuraklığa bağlı olarak ASC ve GSH birikiminin ve antioksidan enzim aktivitelerinin katalaz (CAT) aktivitesi hariç arttığını belirledik. ASC uygulaması kuraklık stresi altındaki *C. arietinum*'da ASC seviyesini ve askorbat peroksidaz (APX) aktivitesini (askorbatın yüksek konsantrasyonu hariç) arttırdı. *C. reticulatum*'da kuraklık stresi altında antioksidan uygulamaları antioksidan içeriklerini arttırdı. Antioksidan uygulamaları her iki türde de, GR aktivitesinde önemli bir değişikliğe yol açmamıştır. Bununla birlikte, her iki türde ASC ve GSH uygulamaları reaktif oksijen türlerini azaltarak superoxide dismutase (SOD), katalaz (CAT) ve askorbat peroksidaz (APX) aktivitelerini azalttı. Çalışmamız, yapraklara ASC ve GSH uygulamalarının kuraklık stresinin zararlı etkisini azalttığı ve nohutun kuraklığa direnci arttığı hipotezini desteklemektedir. Ayrıca sonuçlarımızdan yola çıkarak *C. reticulatum* türünün *C. arietinum* türüne göre oksidatif stres altında daha iyi bir koruma mekanizmasına sahip olduğunu söyleyebiliriz.

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1. Introduction

Chickpea (*Cicer arietinum* L.), an ancient legume crop which is believed to be originated from the Southeastern Turkey and the adjoining part of Syria (Günes et al. 2008). Chickpea is valued for its nutritive seeds with high protein content, 25.3–28.9%, after dehulling. It is the second most important pulse crop in the world, grown in at least 33 countries in South Asia, West Asia, North Africa, East Africa, southern Europe, North and South America, and Australia. It covers 15% of the cultivated area and contributes to 14% (7.9 million tons) of the world's pulse harvest of about 58 million tons (Singh, 1997).

Environmental stress causes significant crop losses. The stresses are numerous and often crop or location specific. They include drought, high salinity, temperature extremes, mineral nutrient deficiency, metal toxicity, pollutants, and increased UV-B radiation. Drought stress, which is a natural stress factor, has the highest percentage, 26%, when the usable areas on the earth are classified in view of stress factors (Kalefetoglu and Ekmekci 2005). A common effect of drought stress, similar to other environmental stresses, is to cause oxidative damage (Unyayar et al. 2005, Fazeli et al. 2007). Reactive oxygen species (ROS) are partially reduced forms of atmospheric oxygen. They typically result from the excitation of O_2 to form singlet oxygen ($^1O_2$) or from the transfer of one, two, or three electrons to O_2, respectively, a superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), or a hydroxyl radical (OH^-) (Unyayar et al. 2005). The levels of ROS are regulated by their rates of generation, their rate of reaction with target substances, such as proteins, lipids, and/or nucleic acids, their potential rate of degradation, and their rate of scavenging/buffering by enzymatic and/or nonenzymatic antioxidants. The removal of ROS is essential for the prevention of oxidative damage to a wide range of biomolecules (Nayyar et al. 2006). Oxidative damage in the plant tissue is alleviated by a concerted action of both enzymatic and nonenzymatic antioxidant mechanisms. Cooperation between these enzymes is essential for effective scavenging of ROS (Fazeli et al. 2007). There are many reports in the literature that underline the intimate relationship between enhanced or constitutive antioxidant enzyme activities and increased resistance to environmental stress (Aqıl et al. 2006).

The degrees to which the activity of enzymatic antioxidants and the levels of nonenzymatic antioxidants are elevated under drought stress vary considerably among several plant species and can even vary between two cultivars of the same species (Nayyar et al. 2006). Moreover, recent studies reported varying responses of plant antioxidant enzymes specific for species and tissues. One approach for inducing oxidative stress tolerance would be to increase the cellular level of enzyme substrates such as ascorbic acid (ASC) (vitamin C). ASC is a small, water-soluble antioxidant molecule that acts as a primary substrate in the cyclic pathway of enzymatic detoxification of hydrogen peroxide. ASC reacts directly with a number of ROS including superoxide, singlet oxygen, ozone, and hydrogen peroxide (Dolatabadian et al. 2008).

Functioning of GSH as an antioxidant under oxidative stress has received much attention during the last decade. It scavenges cytotoxic H_2O_2, and reacts nonenzymatically with other ROS. The central role of GSH in the antioxidative defense is due to its ability to regenerate another powerful water - soluble antioxidant, ASC, via the ascorbate-glutathione cycle (Blokhina et al. 2003).

Ascorbic acid and glutathione play important role in the regulation of a number of metabolic processes in plants exposed to drought stress. However, information on how exogenous ascorbic acid and glutathione regulate antioxidant defense in cultivated and wild chickpea plants under drought stress is not much available in the literature. Thus, the main objective of the present study was to examine whether the adverse effects of drought stress on chickpea plants could be alleviated by exogenous application of ascorbic acid and glutathione as foliar spray. We also want to provide information about how far the exogenous application of these molecules regulates the antioxidant defense system in chickpea plants.

2. Materials and Methods

Plant materials: *Cicer arietinum* L. ILC8617 (drought susceptible) and *Cicer reticulatum* Ladiz. AWC611 (drought tolerant) were used for physiological and biochemical analysis. *C. reticulatum* L. AWC611 was obtained from Akdeniz University, Faculty of Agriculture. *C. arietinum* ILC8617 L. was provided from the Ministry of Agriculture, Field Crops Central Research Institute, Ankara. Seeds were sterilized with 3% sodium hypochlorite for 10 min, rinsed in distilled water, and imbibed for 24 h with aerated water. After imbibition, the seeds were planted in plastic pots containing soil, volcanic tuff, manure (2/1/1). The plants were grown at 26/22 °C (day/night) temperature and 65 ± 5% relative humidity in a growth chamber with 480 μmol/ m^2/s light (day/night 16/8 h). When plants were 35 days old, drought was initiated by stopping irrigation. Pots of each species were randomly divided into two sets,

one of which served as the control group and the other was subjected to drought for 10 days. For the first three days of the drought stress, ascorbate ((+) - sodium L-ascorbate) (12.5 mM and 25 mM) and glutathione (L - glutathione reduced) (10 mM and 100 mM) were applied to each both groups by spraying. At the end of 10 days, leaves of the plants (5 grams) were harvested and frozen in liquid nitrogen and stored at - 80°C for further analysis.

Antioxidant enzyme assays: Extraction processes for SOD, CAT and GR were conducted in the same method. Fresh leaves (1 g) of plants were homogenized in 5 mL of 0.1 mol / L potassium phosphate buffer (pH 6.8) containing 0.1 mM EDTA and 100 mg of PVP. The homogenate was centrifuged at 15.000 g for 20 min at + 4 °C and the supernatant was immediately used for the following enzyme assays.

Total SOD activity was determined by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) as described by Beyer and Fridovich (1987). The reaction mixture consisted of 150 µL of enzyme extract, 2.4 mL, 0.1 M of potassium phosphate buffer (pH 7.8), 200 µL of 0.25 M methionine, 200 µL of 5 mM NBT, 1 mL of sodium carbonate, and 150 µL of 0.1 mM riboflavin. One unit of SOD activity was defined as the amount of enzyme that was required to cause 50% inhibition of the reduction of NBT as monitored at 560 nm.

CAT activity was assayed by measuring the rate of decomposition of H_2O_2 at 240 nm, as described by Aebi (1983). The reaction mixture consisted of 120 µL of enzyme extract, 2.8 mL of 50 mM potassium phosphate buffer (pH 7 without EDTA), and 80 µL of 0.5 M H_2O_2.

GR activity was measured by following the change in 340 nm as oxidized glutathione (GSSG) dependent oxidation of NADPH, as reported by Carlberg and Mannervik (1985). The reaction mixture consisted of 200 µL of enzyme extract, 1.5 mL of 0.1 M phosphate buffer (pH:7), 150 µM of 2 mM $NADPH_2$, 150 µL of 200 mM oxide glutathione (GSSG), and 1 mL of H_2O.

To determine APX activity, fresh leaf tissue (1 g) was homogenized in 15 mL of extraction medium containing 200 mM HEPES, 2 mM EDTA, 5 mM $MgCl_2$, and 4 mM sodium ascorbate. The crude extract was centrifuged at 16.000 g for 5 min at + 4 °C, and the supernatant was used for the measurements. The reaction mixture contained 50 mM potassium phosphate buffer (pH 7), 500 µM ascorbate, 1 mM H_2O_2, and extract. A fall in absorbance at 290 nm was measured as ascorbate was oxidized. APX activity was calculated using an extinction coefficient of 2.8 mM/cm for ascorbate at 290 nm (Bonnet et al. 2000; Vanaker et al. 1998).

Nonenzymatic antioxidant assays

Ascorbate: Leaves (1 g) were homogenized with 0.1 M sodium acetate buffer (pH:3). Homogenate was centrifuged at 16.000 g for 5 min at + 4 °C and the supernatant was collected for analysis of ASC. Chromatography separation was performed by using an Agilent 1100 HPLC Analytical system. A C18 column was used with 0.1 M sodium acetate buffer (pH 5) as the mobile phase. ASC analysis was carried out according to Schmieden and Wild (1994).

Glutathione: The total glutathione (reduced form GSH and oxidized form GSSG) was extracted from frozen leaves according to Hawrylak and Szymanska (2004). Leaves (1 g) were homogenized with 2 vol/g 5-sulfosalicilic acid. Homogenate was centrifuged at 10 000 g for 10 min at + 4 °C, and the supernatant was used for measurements. The reaction mixture consisted of 700 µL of daily buffer (545 µL of 143 mM sodium phosphate buffer, 5 µL of 6.3 mM Na_4 - EDTA, and 150 µL 0.248 mg/mL NADPH), 100 µL of 6 mM 5 - 5'Dithiobis (2-nitrobenzoic acid), and 175 µL of ultrapure water. The reaction tube was left at 30 °C for 15 min. 25 µL of the supernatant and 5 µL of glutathione reductase from baker's yeast 168 U/mg protein (Sigma) were added to initiate the assay. One minute later spectrophotometric measurements were made at 412 nm. Total GSH content was found from the standard curve made for the reduced form of glutathione (L - GSH, Sigma).

Protein assay: The total protein content was determined by Lowry's method as modified by Hartree (1972), using purified bovine serum albumin as a standard.

Statistical analysis: Antioxidant data were analyzed using Statistica 6.0. Tukey's HSD (Honestly Significant Difference) was used in the determination of different groups ($P<0.05$). Three biological samples were used. Each treatment was analysed in three replications.

3. Results

ASC levels were increased in drought stress, ASC and GSH treated groups as compared to control groups in both chickpea species (Figure 1, Figure 2). In the foliar GSH treated groups, GSH level was increased up to two folds in *C. reticulatum* while non significant changes was observed in *C. arietinum*.

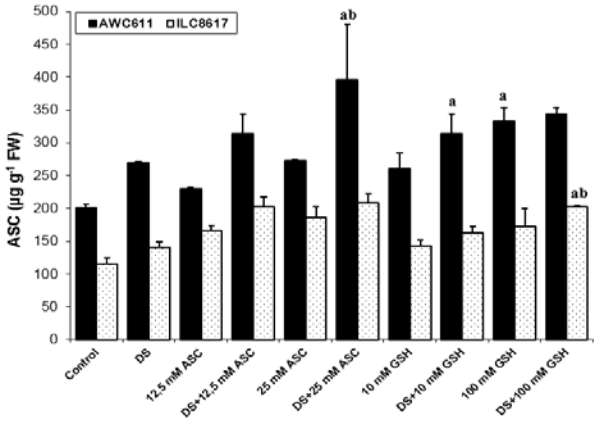

Figure 1. Effect of exogenous ASC and GSH on ASC in *C. arietinum* (ILC8617) and *C. reticulatum* (AWC611) under drought stress. Control: well watered, DS: drought stress **a:** Statistically different from control group **b:** Statistically different from drought stress group (P<0,05) **absence letter:** Non significant

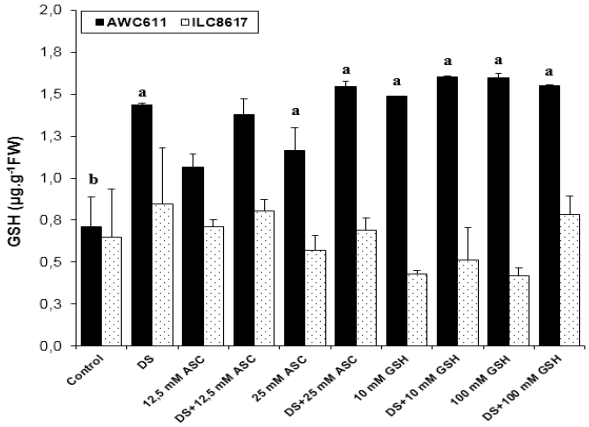

Figure 2. Effect of exogenous ASC and GSH on GSH in *C. arietinum* (ILC8617) and *C. reticulatum* (AWC611) under drought stress. Control: well watered, DS: drought stress **a:** Statistically different from control group **b:** Statistically different from drought stress group (P<0,05) **absence letter:** Non significant

SOD activity in leaves of *C. reticulatum* was significantly greater than that in *C. arietinum* leaves in all treatments. Exogenous 100 mM GSH decreased SOD activity in *C. arietinum* and *C. reticulatum* under drought stress. Exogenous 12.5 mM ASC decreased SOD activity of *C. reticulatum* under drought stress, compared with the drought stress group (Figure 3).

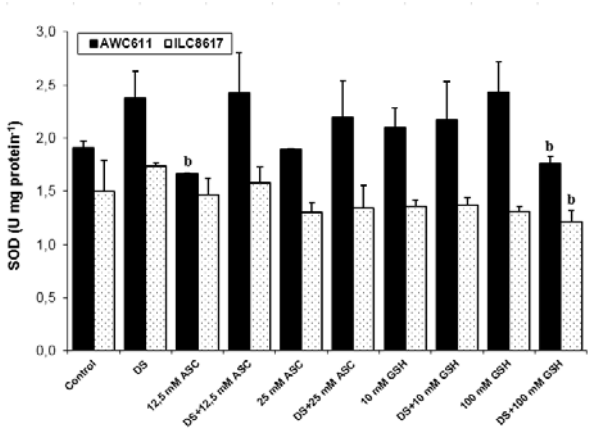

Figure 3. Effect of exogenous ASC and GSH on SOD activity, in *C. arietinum* (ILC8617) and *C. reticulatum* (AWC611) under drought stress. Control: well watered, DS: drought stress **a:** Statistically different from control group **b:** Statistically different from drought stress group (P<0,05) **absence letter:** Non significant

Drought stress increased APX activity in both species (Figure 4). Exogenous ASC and GSH treatments decreased APX activity in *C. arietinum* and *C. reticulatum* under drought stress.

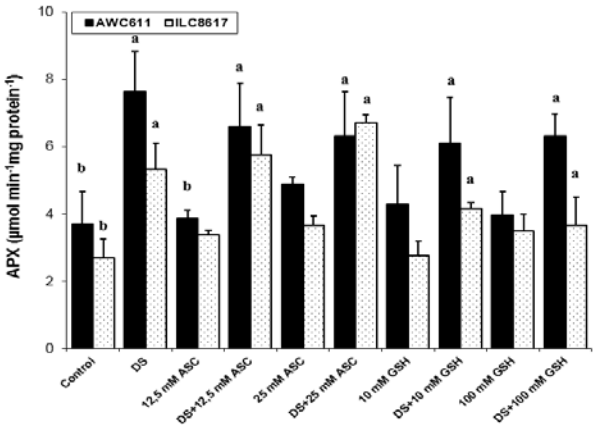

Figure 4. Effect of exogenous ASC and GSH on APX activity, in *C. arietinum* (ILC8617) and *C. reticulatum* (AWC611) under drought stress. Control: well watered, DS: drought stress **a:** Statistically different from control group **b:** Statistically different from drought stress group (P<0,05) **absence letter:** Non significant

There was no statistically significant differences in GR activity between groups in both species (Figure 5).

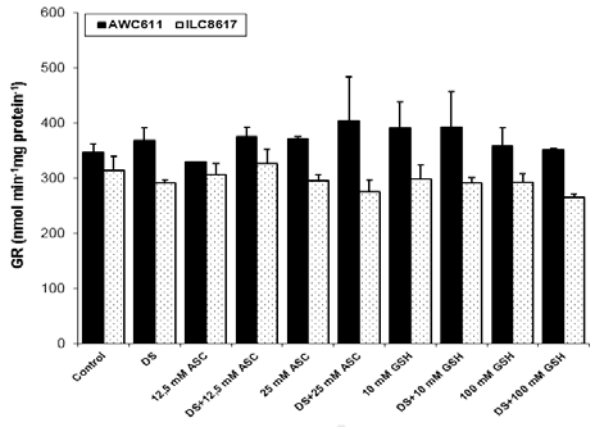

Figure 5. Effect of exogenous ASC and GSH on GR activity, in *C. arietinum* (ILC8617) and *C. reticulatum* (AWC611) under drought stress. Control: well watered, DS: drought stress **a:** Statistically different from control group **b:** Statistically different from drought stress group (P<0,05) **absence letter:** Non significant

As shown in Figure 6, drought stress, ASC and GSH treatments significantly decreased CAT activity in both species, compared with control plants. CAT activity decreased with ASC and GSH in both species under drought stress.

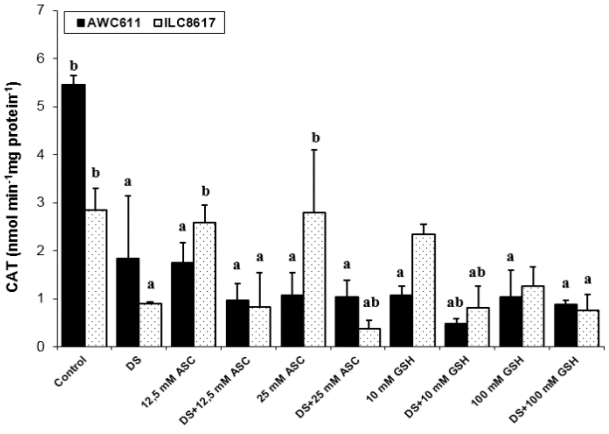

Figure 6. Effect of exogenous ASC and GSH on CAT activity, in *C. arietinum* (ILC8617) and *C. reticulatum* (AWC611) under drought stress. Control: well watered, DS: drought stress **a:** Statistically different from control group **b:** Statistically different from drought stress group (P<0,05) **absence letter:** Non significant

4. Discussion

When plants are subjected to drought stress, a number of physiological responses have been observed (Çevik et al. 2014). Activities of antioxidant enzymes and levels of non - enzymatic antioxidant and metabolites are increased under drought stress (Basu et al. 2010). In this study, drought stress increased the levels of ASC and GSH in both species. The tolerant species *C. reticulatum* showed higher ASC and GSH contents than the cultivated *C. arietinum* under drought stress (Figure 1 and 2). Drought tolerant species generally respond with significant increases in antioxidants during water deficit, whereas the susceptible strain maintains a lower protection from oxidants (Basu et al. 2010). Moreover, drought - sensitive species have lower antioxidative capacity than that of the tolerant cultivars (Sairam et al. 1998). Marked increases in both ASC and GSH were observed in *C. reticulatum* with exogenous ASC and GSH, except for low concentration of ASC under drought stress. These results are in good agreement with other authors (Dolatabadian et al. 2008). Drought stress remarkably increased SOD (Figure 3) and APX (Figure 4) activities in both species. SOD and APX activities were increased under the effect of drought stress as reported by various authors (Dolatabadian and Jouneghani 2009; Çevik et al. 2014). SOD scavenges the toxic O_2^- in different cell organelles (Noctor and Foyer 1998). It converts O_2^- to H_2O_2 which is then scavenged by APX activity. Leaf antioxidant enzyme activities, except for CAT activity, of the drought stressed plant were higher than control plants. Drought significantly decreased CAT (Figure 6) activity in both species. The CAT deactivation by drought stress might be resulted from the prevention of new enzyme synthesis or catalase photo-inactivation (Basu et al. 2010). A marked decline in CAT activity was also reported stressed pea (Morán et al. 1994) and tomato (Unyayar et al. 2005). Exogenous ASC and GSH significantly decreased CAT activities in both species. Our results show that SOD and APX activities increased with drought stress, similar results have been determined in drought stressed *Lycopersicon* sp. (Unyayar et al. 2005) and *Trifolium repens* (Wang et al. 2011) were studied. Ascorbic acid and glutathione are ASC - GSH cycle components, which can scavenge O_2 - and H_2O_2 non - enzymatically, take part in APX mediated scavenging of H_2O_2 (Asada 1999, Dolatabadian and Jouneghani 2009). The decline in the activities of SOD, CAT and APX could be due to the elimination of free radicals by non - enzymatic antioxidant molecules which are increased by exogenous ASC and GSH treatments. Ascorbate is oxidized by oxygen free radicals and dehydroascorbate is generated (Noctor and Foyer 1998). This leads to a decline in antioxidant activities (Dolatabadian and Jouneghani 2009). It has been reported that limitation of one the components of antioxidant defense can be compensated through up regulation of other components (Selote and Khanna - Chopra 2004).

In conclusion, result of this study demonstrated that foliar application of ASC and GSH decreased enzyme activities which was increased by drought stress. The decline in the enzyme activities could be due to elimination of free radicals by ASC and GSH molecules. According to these results, it can be suggested that usage of ASC and GSH can reduce

the harmful effects of ROS and improve plant resistance under drought stress conditions.

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