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Foliar applications of alpha-tocopherol improves the composition of fresh pods of *Vigna radiata* subjected to water deficiency

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Abstract: Alpha-tocopherol (α-Toc), so-called vitamin E, is a low molecular weight lipophilic antioxidant that generally protects plants from stress-induced cellular oxidation. It is well known that exogenously applied α-Toc is effective in improving plant growth and developmental processes under adverse environmental conditions. The current study was performed to determine the best concentration of α-Toc [0 (no spray), 100, 200, and 300 mg L^{-1}] that could improve the biological yield and chemical constituents of fresh green pods of mung bean (*Vigna radiata*) and hence their quality under varying water regimes. Foliar spray of α-Toc significantly improved total soluble proteins, chlorophyll *a* and *b*, total soluble sugars, proline, phenolics, total free amino acids, nonreducing sugars, and activities of SOD, POD, and CAT under water stress conditions. However, no prominent change was observed in reducing sugars and biological yield due to externally applied α-Toc in either mung bean cultivar subjected to either water regime. Both mung bean cultivars (Cyclone 7008 and Cyclone 8009) showed similar behavior in chlorophyll *a* and *b*, while cv. Cyclone 7008 was superior with respect to the concentrations of total soluble sugars and total free amino acids, whereas Cyclone 8009 was better in activities of CAT, SOD, and POD. Overall, externally applied 200 and 300 mg L^{-1} α-Toc considerably enhanced the activities of antioxidant enzymes (SOD, POD and CAT), chlorophyll *a* and *b*, proline, and total phenolic contents in both mung bean cultivars.

Key words: Alpha-tocopherol, antioxidants, water stress, green pods, mung bean

1. Introduction

Drought is one of the most prominent adversities for plant growth and development. Crop plants often come across periods of water shortage in their life span particularly in regions where annual rainfall is below the average limit (Naderi et al., 2014). Water stress causes dysfunction of several metabolic processes including alterations in hormone actions (Knight and Knight, 2001), thereby leading to a suppression in plant activities (Ghassemi-Golezani et al., 2008). However, the extent of yield reduction is determined by the timing, severity, and duration of the water deficiency. Water deficiency at the vegetative stages of crop plants has a severe effect on plant biomass production but drought stress during flower formation imposes considerable adversities on yield productivity (Ghassemi-Golezani et al., 2008). Grain filling is a key factor of yield and is characterized by period and rate of filling (Yang et al., 2008). The composition of amino acids, proteins, and carbohydrates has a direct impact on the nutritious value of seed crops (Khamssi, 2011). Water deficiency imposition during grain production terminates the kernel sink potential by reducing the endosperm cells and amyloplast formation, thus decreasing grain yield due to starch deprivation (Saini and Westgate, 2000). Prevailing drought suppresses growth and development, leading to reduction in flowers, as well as smaller and fewer grains (Farooq et al., 2009).

Application of antioxidants including vitamins has gained considerable attention for alleviating the negative impact of water and salinity stress on plants in terms of plant growth and yield quantity and quality (Sadak and Dawood, 2014). Vitamins could be regarded as natural and safety bio-regulators; low concentrations can exert considerable effects on different chemical activities (Sadak and Dawood, 2014). In extension to xanthophylls cycle, alpha-tocopherol (α-Toc) completes at least two different functions in chloroplasts: it preserves membrane lipids and protects PSII from photo-inactivation (Havaux et al., 2005). Vitamins E level varies in response to climatic cues, depending on the species or stress. Changes in α-Toc concentrations depend on related genes' expression and it is well confirmed that high α-Toc concentration results in

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plant stress resistance/tolerance (Munne-Bosch, 2005). For example, plants such as wheat raised from seeds treated with α-tocopherol showed tolerance to various stresses by minimizing oxidative damage (Kumar et al., 2012).

Mung bean is one of the most nutritious grain legumes and it is utilized mainly as a pulse crop in different parts of the world (Ghassemi-Golezani et al., 2014). However, fresh pods and sprouts of mung bean can be eaten as vegetables and are a source of vitamins and minerals (Minh, 2014). Of different leguminous crops, it is relatively drought tolerant; however, low soil moisture can result in considerable yield loss of this crop (Ghassemi-Golezani et al., 2014).

The endogenous levels of amino acids, carbohydrates, and protein in seeds determine the nutritive value of a crop (Khamssi, 2011). There are reports that deficiency of water has a prominent effect on seed chemical composition, but very little research has been reported on the impact of water deficiency on the composition of mung bean fresh green pods in relation to chlorophyll; contents of phenolics, sugars, proteins, and amino acids; and antioxidant activity. Therefore, we examined the impact of α-Toc and water stress on the biological yield and seed chemical composition in relation to the above-mentioned attributes in two cultivars of mung bean.

2. Materials and methods

To study the effect of foliage spray of α-Toc on the chemical composition of fresh green pods of mung bean under water-deficit conditions, seeds of cvs. Cyclone 7008 and Cyclone 8009 were collected from the Pulses Section, AARI, Faisalabad, Pakistan. The two lines used were chosen on the basis of their high yield potential and wide cultivation. However, their drought tolerance had not been determined earlier. An experiment with a completely randomized design with four replicates was carried out in the research area of G.C. University Faisalabad, Pakistan, during February to June 2015. The seed was planted in plastic pots (depth 24.5 cm and radius 10.5 cm). Each pot (plastic) was filled with 7 kg of soil. The seeds were kept in deionized water for 60 min before sowing. The results of analysis of the soil used for the trial were as follows: N, 170.0 mg kg^{-1}; P, 7.2 mg kg^{-1}; pH, 8.1; saturation percentage, 36%; EC, 1.89 dS m^{-1}; K, 80 mg kg^{-1}; Ca^{2+}, 122.4 mg kg^{-1}; organic matter, 0.35%; Zn, 0.68 mg kg^{-1}; clay, 7.5%; sand, 65%; silt, 27.5% and Fe, 1.92 mg kg^{-1}. For each pot six healthy seeds of each cultivar were selected. After 7 days of sowing, germination started and thereafter three seedlings were maintained in each pot. Water-stress conditions, i.e. control (100% FC), and water-deficit conditions (60% FC) were imposed on 3-week-old mung bean seedlings. The 60% field capacity was regulated by measuring the weights of related treatment. After 4 weeks of water stress treatment, the plants were treated with 0, 100, 200, and 300 mg L^{-1} α-Toc (Company MP Bio USA and 100% pure) as a foliage spray. All plants were allowed to grow until complete formation of pods (about 65 days after seed germination). Green pods were collected uniformly from each of three plants, weighed, and used for the chemical analyses described below.

2.1. Chlorophyll content

The protocol described by Arnon (1949) was adopted to determine chlorophyll contents. Fresh green pods (0.5 g) were extracted with 10 mL of 80% acetone (v/w) and optical densities read at 645 and 663 nm using a single-beam spectrophotometer.

2.2. Determination of free proline

Following the procedure proposed by Bates et al. (1973), fresh pods (0.5 g) were homogenized in 3% sulfosalicylic acid and OD was recorded at 520 nm using a spectrophotometer.

2.3. Total phenolics

Fresh green pods (0.5 g) were ground in 80% acetone (10 mL) following Julkunen-Titto (1985). Then homogenate mixture was collected. After that, Folin-Ciocalteu reagent (1 mL) + H_2O (2 mL) and the supernatant (0.1 mL) were homogenized, followed by vigorous shaking. To it was added 5 mL of Na_2CO_3 (20%) and the volume was raised to 10 mL using distilled H_2O. The absorbance was noted at 750 nm and the results expressed as mg g^{-1} fresh weight of sample.

2.4. Total soluble proteins

Fresh green pods (0.25 g) were ground in 10 mL of 50 mM potassium phosphate buffer (pH 7.8) in a cool environment. The samples were centrifuged at 10,000 × *g* at 4 °C for 15 min as described by Bradford (1976). The aliquot (0.1 mL) was mixed with 2 mL of Bradford reagent and the absorbance read by spectrophotometer at 590 nm.

2.5. Total free amino acids

Fresh green pods (0.25 g) were triturated in 10 mL of K phosphate buffer (50 mM; pH 7.8). After centrifugation, 0.5 mL of the aliquot was added to the cultural tubes and pyridine (0.5 mL; 10%) and ninhydrin (0.5 mL; 2%) were added. The mixture was heated in a boiling water bath for 30 min as instructed by Hamilton and Van Slyke (1943). The volume of the contents was raised to 25 mL in each case and the OD was recorded at 570 nm.

2.6. Total soluble sugars

For measuring total soluble sugars, fresh green pods (0.1 g) were ground in ethanol solution (10 mL; 80%) and then shaken for 6 h at 60 °C. The supernatant (0.1 mL) was taken and mixed in anthrone reagent (3 mL). Then the mixture was heated for 10 min, ice cooled, and incubated for 20 min. The OD of the aliquot was recorded at 625 nm using a spectrophotometer. Total soluble sugars were determined from a standard curve of glucose following Yemm and Willis (1954).

2.7. Reducing sugars

The fresh pods (0.5 g) were homogenized in alcohol (80%). A 1-mL aliquot was added to 5 mL of O-toluidine, followed by heating for 15 min at 97 °C. The OD was read at 630 nm using a spectrophotometer after cooling the mixture following Nelson (1944).

2.8. Nonreducing sugars

The nonreducing sugars were determined according to the method of Loomis and Shull (1937). Ethanol extract of fresh green pods (0.1 mL) was added to 0.1 mL of 5.4 N KOH, heated, and then cooled. Then 3 mL of anthrone was added to the mixture, which was again heated, cooled, and then finally incubated at room temperature and read at 620 nm.

2.9. Determination of antioxidant enzymes

Fresh green pods (0.5 g) were ground in 10 mL of potassium phosphate buffer (50 mM; pH 7.8) and the supernatant was used to determine the activities of the following enzymes.

2.9.1. Activity of CAT enzyme

The activity of catalase enzyme was measured according to the method described by Luck (1974). Green pods extract (0.1 mL) was mixed with H_2O_2 (1 mL; 5.9 mM) and phosphate buffer (1.9 mL; 50 mM; pH 7.0) and then the absorbance was recorded at 240 nm for 3 min.

2.9.2. Activity of POD enzyme

According to the method described by Chance and Maehley (1955), the phosphate buffer (1.8 mL; pH 7.8) was taken in a cuvette and H_2O_2 (0.1 mL; 0.5%), guaiacol (0.1 mL; 0.5%), and enzyme extract (0.1 mL) were added to it. The absorbance was read at 470 nm for 3 min.

2.9.3. Activity of SOD enzyme

The reaction mixture (250 µL phosphate buffer + 400 µL H_2O + 100 µL Triton-X + 100 µL L-methionine + 50 µL NBT + 50 µL enzyme extract + 50 µL riboflavin) was taken in a cuvette and kept under light for 15 min. The absorbance was recorded at 560 nm following Van Rossum et al. (1997).

2.9.4. Biological yield

The plucked fresh pods were air-dried and placed in an oven at 60 °C for 3 days. Then their dried weights were recorded.

2.10. Statistical analysis

A completely randomized design with four replicates was used to arrange the experiment. The three factors were cultivars, water stress, and tocopherol treatments. A three-way analysis of variance method was applied for the determination of significance level. Standard errors were applied to appraise the difference between replicates. The least significance difference (LSD) values at 5% probability were worked out and are presented in each figure.

3. Results

Chlorophyll *a* and *b* contents decreased significantly ($P \le 0.001$, 0.05) in the fresh green pods of both mung bean cultivars subjected to water-deficit conditions (Figure 1). Foliar-applied varying levels (100, 200 and 300 mg L^{-1}) of α-Toc were effective in improving photosynthetic pigments in both mung bean cultivars particularly under water-stress conditions. Of all Toc levels used, 200 and 300 mg L^{-1} were superior in raising chlorophyll *a* and *b* contents in both mung bean cultivars. Both cultivars (Cyclone 7008 and Cyclone 8009) showed a similar response in terms of chlorophyll *a* and *b* contents under water stress and tocopherol application (Figure 1).

Water stress remained noneffective for free proline accumulation in the fresh green pods of both mung bean cultivars. The exogenous application of α-Toc had a significant ($P \le 0.01$) increasing effect on proline accumulation in both mung bean cultivars, particularly under water stress conditions. Of all Toc levels, 200 and 300 mg L^{-1} were considerably effective in improving proline contents only in cv. Cyclone 8009 under stress conditions (Figure 1).

An increase in total phenolics was observed in the fresh green pods of mung bean subjected to water deficit conditions ($P \le 0.01$) and foliar-applied varying levels of α-Toc ($P \le 0.001$). Of all Toc levels, 300 mg L^{-1} Toc was found to be most effective in improving total phenolics in both cultivars in all water regimes (Figure 1).

The data indicated that water stress (60% field capacity) significantly suppressed the total soluble proteins in the fresh green pods of both mung bean cultivars. Exogenous application of α-Toc was significantly ($P \le 0.001$) effective in improving total soluble proteins in the green pods of both mung bean cultivars. Of both mung bean cultivars, cv. Cyclone 8009 was better than the other cultivar in this characteristic under water-stress conditions.

Imposition of water stress significantly raised total free amino acids in fresh green pods of both mung bean cultivars (Figure 1). A prominent increase ($P \le 0.001$) was observed in total amino acids in the fresh green pods of both mung bean cultivars due to foliar-applied α-Toc. Of the two mung bean cultivars, cv. Cyclone 7008 was better in the contents of total amino acids under water-stress conditions.

Water stress significantly increased total soluble sugars (TSSs) as well as reducing sugars ($P \le 0.001$) in the fresh green pods of both mung bean cultivars, while no change was observed in nonreducing sugars in response to water stress. The exogenously applied α-Toc had a significant increasing effect ($P \le 0.001$) on the total soluble sugars and

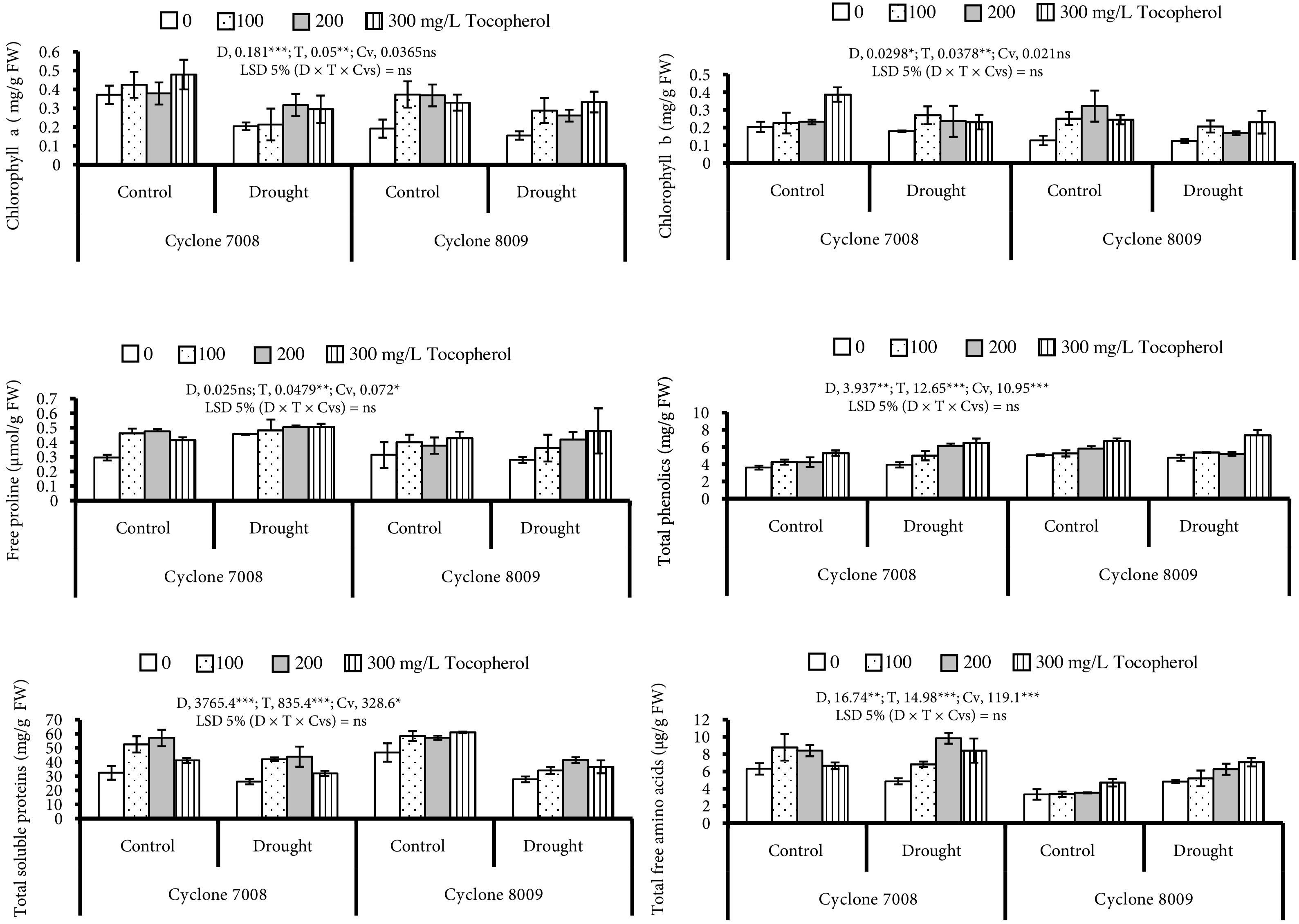

Figure 1. Chlorophyll *a* and *b* pigments, free proline, total phenolics, total soluble proteins, and total amino acids of two mung bean [*Vigna radiata* (L.) Wilczek] cultivars treated with varying levels of tocopherol (0, 100, 200, and 300 mg/L) as a foliar spray under nonstress and water-stressed conditions (Mean ± S.E.); FW, Fresh weight; *, **, and *** significant at 0.05, 0.01, and 0.001 levels, respectively; ns, not significant; D, Drought; T, Tocopherol; Cv, Cultivars.

nonreducing sugars, while no effect of Toc was observed on the reducing sugars in the green pods of either mung bean cultivar grown under either water regime. The differential response of both mung bean cultivars was observed only in the case of total soluble sugars, and cv. Cyclone 7008 was better than Cyclone 8009 under moisture-deficit conditions (Figure 2).

A significant increase ($P \leq 0.001$) was observed in the activities of CAT, SOD, and POD enzymes in the fresh green pods of both mung bean cultivars under water-deficit conditions (Figure 2). Exogenously applied α-Toc significantly ($P \leq 0.001$) improved the activities of CAT, SOD, and POD in the fresh green pods of both mung bean cultivars (Figure 2). However, for the activities of SOD and CAT enzymes, 300 mg L^{-1}, while for that of POD enzyme, 200 mg L^{-1} was the most prominent in both mung bean cultivars. Cyclone 8009 was superior to Cyclone 7008 in the activities of SOD, POD, and CAT enzymes under water-stress conditions.

Water stress significantly ($P \leq 0.001$) decreased the biological yield of both mung bean cultivars. The foliar application of α-Toc remained nonresponsive. Both mung bean cultivars exhibited a similar trend in this attribute (Figure 3).

4. Discussion

Crop plants during their life cycle are expected to be exposed to a variety of environmental stresses, which influence adversely plant growth and development, thereby leading to reduced productivity (Siddiqi et al., 2007; Wei et al., 2014). α-Toc is a well reputed chloroplastic antioxidant in plants (Orabi and Abdelhamid, 2014). It preserves adequate redox reactions in chloroplasts and stabilizes thylakoid functions and membrane structure during plant development under stress (Munne-Bosch, 2005). Chlorophyll content is often linked to visual symptoms of plant and photosynthetic activity (Akram and Ashraf, 2013). In the current study, under water-deficit conditions, chlorophyll pigments decreased significantly under water stress. Of all foliar-applied Toc levels, 200 and 300 mg L^{-1} were superb in maintaining chlorophyll contents in both mung bean cultivars (Figure 1). Earlier, Farouk (2011) showed that contents of total chlorophyll in the flag leaf of wheat decreased significantly under NaCl salt stress, but Toc application increased the total chlorophyll content. Our results for chlorophyll content are in agreement with those reported by Kumar et al. (2012) on stressed wheat and Al-Qubaie (2012) on sunflower, wherein α-tocopherol was reported to protect the organization of the chloroplast to minimize chlorophyll loss.

Accumulation of organic solutes like proline is a common response of plants subjected to a stress as a defense mechanism to overcome stress-induced harmful effects (Munns and Tester, 2008). In the present case, water stress remained noneffective in terms of proline accumulation in the green pods of both mung bean cultivars. The exogenous application of α-Toc had a significant increasing effect on proline accumulation in both mung bean cultivars, particularly under water-stress conditions. Of all Toc concentrations used as a foliar spray, 200 and 300 mg L^{-1} were found to be considerably effective in improving proline contents under stress conditions (Figure 1). Similarly, Orabi and Abdelhamid (2014) reported the effect of α-Toc on proline content in the leaves of faba bean plants grown under seawater salinity. These results indicated that high proline is an indicator of intense stress (Ain-Lhout et al., 2001; Rai et al., 2003), while others are of the view that proline at high concentration acts as a osmoprotectant (Taie et al., 2013).

In fresh green pods of mung bean, levels of phenolics increased under water-deficit conditions as well as by foliar spray of α-Toc. Previously, in citrus leaves Kostopoulou et al. (2014) showed that phenol contents were accumulated in the leaves exposed to NaCl, but were decreased by resveratrol or by resveratrol + α-Toc treatment in relation to NaCl alone application. However, phenol concentration in roots was increased by α-Toc treatments. It is thought that stress conditions lead to an increase in phenolic compounds in most plants (Sakr and El-Metwally, 2009) and thus phenolic compounds could be a cellular adaptive mechanism for scavenging oxygen free radicals during drought stress conditions.

Water stress (60% field capacity) significantly reduced total soluble proteins (TSPs) in fresh green pods of mung bean plants. Foliar-applied α-Toc proved effective in improving TSPs in the green pods of both mung bean cultivars (Figure 1). Analogous to this study, Al-Qubaie (2012) reported that treating plants three times with vitamin E either singly or in all combinations resulted in significantly enhanced protein percentage in the fresh sunflower leaves with respect to the control treatment. Orabi and Abdelhamid (2014) showed that although salinity (sea water) induced considerable suppression in total proteins in the seeds of faba bean, foliar application of α-Toc at the rate of 50 or 100 mg L^{-1} caused a significant increase in protein content but in both stressed and nonstressed plants. An α-Toc–induced rise in TSP contents has already been reported in sunflower (Sadak et al., 2010; Rady et al., 2011), *Vicia faba* (Hala et al., 2005), and geranium (Ayad et al., 2009) plants under stress conditions. The increases in protein content as observed in the present study could be partially related to the increase in the dry weight of mung bean plants at all levels of α-Toc. It could be concluded that the inhibitory effect of abiotic stresses on plants can be mitigated by vitamin (α-Toc) treatment.

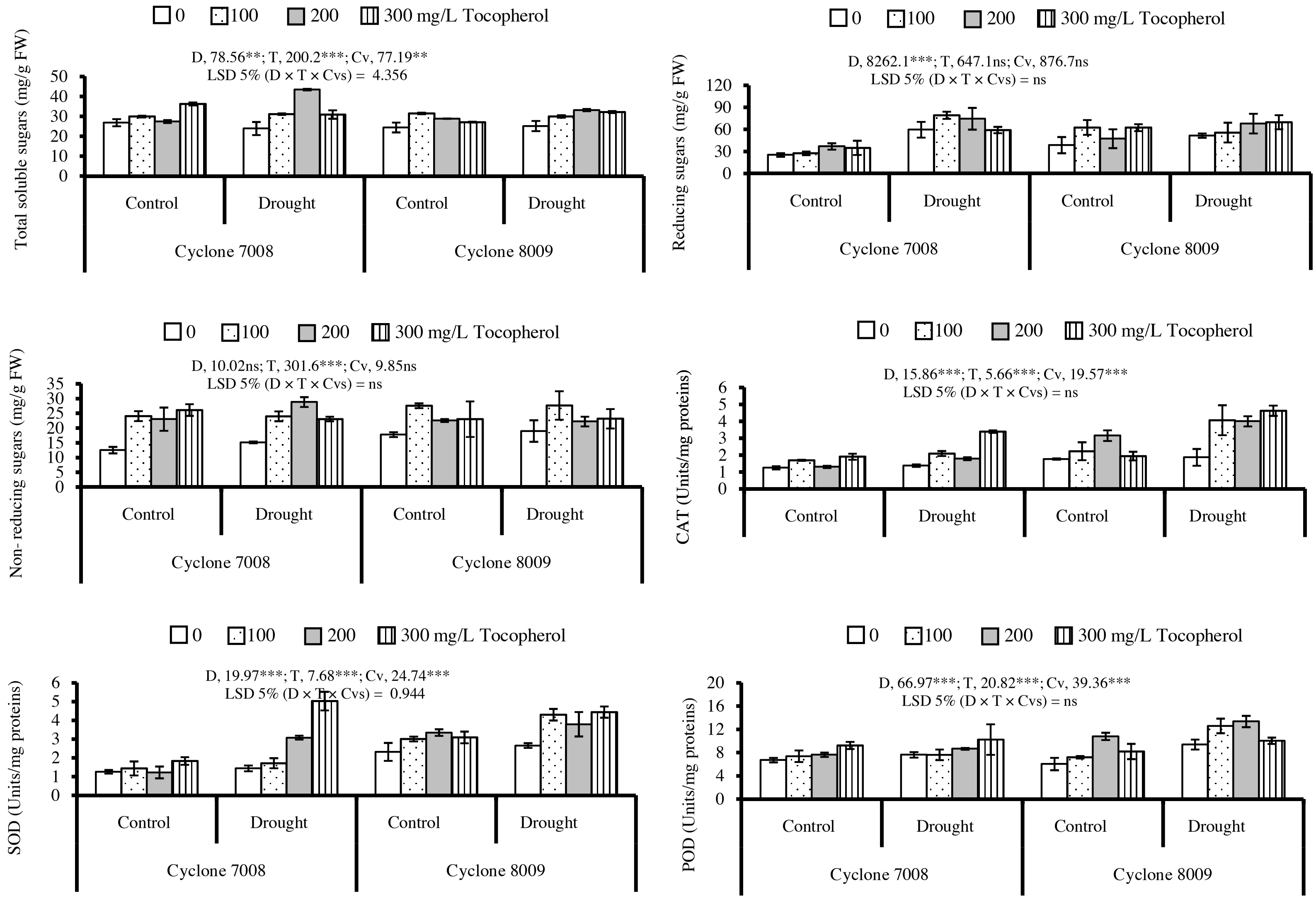

Figure 2. Total soluble sugars, reducing sugars, nonreducing sugars, and activities of catalase (CAT), superoxide dismutase (SOD), and peroxidase (POD) enzymes of two mung bean [*Vigna radiata* (L.) Wilczek] cultivars treated with varying levels of tocopherol (0, 100, 200, and 300 mg/L) as a foliar spray under nonstress and water-stressed conditions (Mean ± S.E.); FW, Fresh weight; ** and *** significant at 0.01 and 0.001 levels; ns, not significant; D, Drought; T, Tocopherol; Cv, Cultivars.

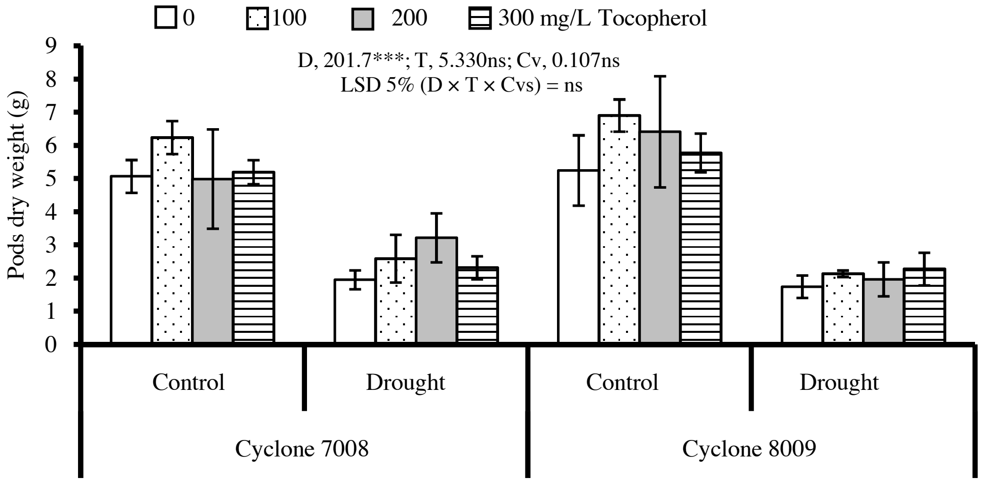

Figure 3. Pods dry weight of two mung bean [*Vigna radiata* (L.) Wilczek] cultivars treated with varying levels of tocopherol (0, 100, 200, and 300 mg/L) as a foliar spray under nonstress and water-stressed conditions (Mean ± S.E.); FW, Fresh weight; *** significant at 0.001 level; ns, not significant; D, Drought; T, Tocopherol; Cv, Cultivars.

Amino acids in high concentrations can lower osmotic potential in several tissues exposed to stress and some of them may act as osmoprotectants (Rady et al., 2011). In the present investigation, imposition of water stress significantly increased total free amino acids in fresh green pods of both mung bean cultivars. A prominent change was observed in both mung bean cultivars due to foliar-applied α-Toc (Figure 1). Mung bean cultivar Cyclone 7008 showed a better response in this regard. These results are in agreement with those observed in sunflower (Rady et al., 2011) and flax (Sadak et al., 2013) plants, where salinity stress was reported to be an activator of free amino acid accumulation, and α-Toc significantly enhanced the stimulatory role of salt stress on the accumulation of free amino acids. Thus, it can be suggested that salt tolerance may have been manifested via hydrolysis of proteins into free amino acids and activation of proline synthesis (Sadak et al., 2010).

Accumulation of organic solutes, like carbohydrates, is a common response of plants subjected to stress conditions as a defense mechanism. In the present study, water stress significantly increased TSSs as well as reducing sugars while no change was observed in nonreducing sugars in either mung bean cultivar. The exogenously applied α-Toc had a highly significant effect on the total soluble sugars and nonreducing sugars, but no effect was observed on reducing sugars (Figure 2). These results are in accordance with those reported by Hala et al. (2005) in which the percentage of carbohydrates in dry seeds of *V. faba* under reclaimed sandy soils was increased significantly by application of α-tocopherol (200 mg L^{-1}). Similarly, Orabi and Abdelhamid (2014) showed that total carbohydrates under seawater salinity caused a significant decrease in total carbohydrates of seeds of faba beans. The reduction in carbohydrates was suggested to be due to the adverse effects of salinity on chlorophyll contents (Sadak and Dawood, 2014). They further established that α-Toc treatment led to the accumulation of total carbohydrates in sunflower plants (Sadak et al., 2010). A significant reduction in the total soluble sugars due to α-Toc application to *Vicia faba* plants was observed by Semida et al. (2014). The authors suggested that this reduction in soluble sugars may be attributed to the crucial role of α-Toc in mitigating the negative effects of soluble salts. However, an α-Toc–induced increase in total soluble sugars was observed in hibiscus (El-Quesni et al., 2009), citrus (Kostopoulou et al., 2014), and flax (El-Bassiouny and Sadak, 2015) plants under stress and nonstress conditions. Accumulation of carbohydrates is thought to be not due to their role as osmoprotectant but could be an index of membrane damage and oxidative stress (Munns and Tester, 2008; Mittal et al., 2012).

Antioxidant enzymes (CAT, SOD, and POD) are considered the most effective biomolecules in preventing cellular damage (Ashraf, 2009). In the present study, activities of CAT, SOD, and POD were enhanced reasonably in the green pods of both mung bean cultivars under water stress. Likewise, in an earlier study with oilseed rape (*Brassica napus* L.), Abedi and Pakniyat (2010) reported a significant stimulation in the activities of SOD and POD enzymes, and a decrease in the activity of CAT enzyme under water-deficit stress. Similarly, an increase in the activities of CAT, GPX, and SOD enzymes was observed in chickpea plants grown under dryland conditions (Mohammadi et al., 2011). However, exogenously applied α-Toc significantly improved the activities of CAT, SOD, and POD in the green pods of both mung bean cultivars. In a comprehensive review by Szarka et al. (2012), it has been reported that stress-induced ROS accumulation is controlled by antioxidants (enzymatic and nonenzymatic) including α-Toc. They are usually capable of chelating metal ions, eliminating their catalytic activity to form excessive ROS. No reports on the role of exogenous application of α-Toc on the antioxidative defense system of plants grown under stress conditions are available in the literature. However, it could be concluded that α-Toc supplementation could compensate for the harmful effects of stress-induced ROS through its powerful antioxidant properties (Bughdadi, 2013).

In conclusion, water stress had adverse effects on the endogenous levels of chlorophyll pigments, total amino acids, total phenolics, TSPs, and TSSs in the fresh green pods and biological yield of both mung bean cultivars. However, exogenous application of α-Toc significantly improved chlorophyll pigments, proline, phenolics, TSPs, total free amino acids, TSSs, nonreducing sugars, and activities of SOD, POD, and CAT under water-deficit stress. Overall, exogenous application of 200 and 300 mg L^{-1} α-Toc was most effective in enhancing the accumulation of chlorophyll, proline, and total phenolic contents along with an increase in the activities of SOD, POD, and CAT in the fresh green pods of both mung bean cultivars.

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