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Thiamin-induced variations in oxidative defense processes in white clover (*Trifolium repens* L.) under water deficit stress

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Abstract: Vitamins are regarded as vital growth substances. They act as cofactors of various key enzymes involved in metabolism and also have vital influence on metabolic activities of plants. They are believed to play a significant role in plants against abiotic stresses, particularly during water stress. The current experiment was conducted to evaluate the role of exogenously applied thiamin on two white clover cultivars, Super Late Fsd and Layalpur Late, at varying [100% (control), 80%, and 60% field capacities] water regimes. A significant suppression was noted in plant biomass, plant height, chlorophyll *a* and *b* contents, total soluble proteins, and shoot and root P, Ca^{2+}, and K^{+} contents particularly at 60% field capacity in both cultivars. Drought stress had no significant effect on phenolic contents, but MDA concentration was increased in white clover cultivar Layalpur Late at both 80% and 60% drought levels. However, in Super Late Fsd, it was increased at 80% and decreased at 60% field capacity. Drought stress significantly enhanced levels/activities of ascorbic acid and antioxidant enzymes (CAT, SOD, and POD). Foliar spray of 50 mM thiamin improved the shoot and root fresh weights and shoot dry weight. Varying levels of thiamin were found significantly effective for shoot length, root length, and photosynthetic pigments (chlorophyll *a* and *b*). However, no considerable change was observed in ascorbic acid, total soluble proteins, and activities of antioxidants (SOD, CAT, and POD) due to exogenous spray of thiamin on two white clover cultivars. Of the two white clover cultivars, Super Late Fsd was better in shoot and root P and Layalpur Late in root dry weight and root length. Overall, 100 mM of thiamin was found effective in improving growth of both white clover cultivars, which can be attributed to thiamin-induced improvement in photosynthetic pigments and phenolic contents.

Key words: Water deficit stress, *Trifolium repens*, thiamin, exogenous application, oxidative defense system

1. Introduction

World food security is being significantly hampered due to water scarcity (Mitter et al., 2015). Water stress is different from other stresses because unlike other natural disasters its effects remain even after its period terminates (Ashraf et al., 2010; Strauss et al., 2013). Soil water deficiency affects three major mechanisms in plants: reduced canopy absorption of photosynthetically available radiation (PAR), radiation use efficiency, and harvest index, and all of these lead to reduced plant yield (Fiala et al., 2014). Seedling growth and productivity are also severely affected, mainly due to low supply of water (Atkinson and Urwin, 2012). Due to drought stress, germination potential (Wang et al., 2011), hypocotyl length, and plant biomass are commonly hampered (Wang et al., 2015). During the initial stage of growth and germination, relative water content was high in wheat, which decreased as dry matter accumulated and leaf matured (Hajheidari et al., 2007; Kaur et al., 2007). Nutrient uptake and tissue level concentrations along with water relations are perturbed under water-limited conditions in crop plants (Losak et al., 2010).

Drought stress causes the production of reactive oxygen species (ROS), which affect the ultrastructure of cell membranes and DNA as well as the functioning of cellular processes (Turkan, 2011; Sun et al., 2013; Kaya et al., 2015). ROS including hydroxyl radical, anion radical, hydrogen peroxide, alkyl radicals, and singlet oxygen are injurious to plant metabolic activities. These species may react to lipids, proteins, and DNA, which causes oxidative damage and abnormal functions of cells (Sarvajeet and Tuteja, 2010; Shafiq et al., 2015). It is known that absorption of thiamin by plant root, leaves, and

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seeds has beneficial effects on plant growth under adverse conditions (Mateikene et al., 1988; Goyer, 2010; Kaya et al., 2015). An increase in endogenous thiamin level by its exogenous application or by activation of its biosynthesis could enhance oxidative defense system by activating enzymes including transketolase, a major enzyme of glycolysis (Kruger and von Schaewen, 2003). It is observed that this enzyme is involved in the generation of NADPH, which enhances the generation of ascorbate and glutathione (Mozafar and Oertli, 1992). It has been found in Arabidopsis seedlings that, under oxidative stress, high thiamin levels are interrelated with mRNA transcripts of different biosynthetic genes (Tunc-Ozdemir, 2009).

Thiamin is a vitamin B complex that is found in green plants and some microbes (Al-Hakimi and Hamada, 2011). Thiamin diphosphate (vitamin B1) basically acts as a cofactor in many metabolic activities such as glycolysis, the tricarboxylic acid cycle, and the pentose phosphate pathway (Goyer, 2010). Recently, it was detected that thiamin plays a protective role in plants during exposure to environmental stresses. Foliar spraying of thiamin neutralizes harmful effects of abiotic stresses on most plants (Sayed and Gadallah, 2002). Thiamin pyrophosphate (TPP) plays a central role in intermediary metabolism and is required for all biological systems. It can also alleviate drought stress in plants (Ajjawi et al., 2007).

White clover (*Trifolium repens* L.) is common fodder crop for cattle and race animals. White clover also plays a potential role in atmospheric nitrogen fixation as it contains nitrogen-fixing bacteria in its roots forming nodules (Pryor and Lowther, 2002), and it contains high levels of proteins, sugar contents, low fiber contents, and high mineral contents. However, production of white clover is affected by water shortage conditions (Li et al., 2013). Thus, this study was conducted to assess the role of foliar applied thiamin in minimizing the adverse effects of drought stress on white clover in terms of its growth and various physiobiochemical characteristics.

2. Materials and methods

This study was carried out to investigate the effect of varying levels of exogenously applied thiamin (vitamin B_1) on different morphological and physiobiochemical parameters of white clover (*Trifolium repens* L.) under water stress regimes. An experiment in pots was established in a research area located at the Government College University Faisalabad, Pakistan. Seeds of two cultivars (Super Late FSD and Layalpur Late) of white clover were collected from the Ayub Agricultural Research Institute, Faisalabad. The experiment was completely randomized with three replicates in plastic pots, each containing 8 kg of sandy-loam soil. The electrical conductivity of the soil was 0.24 dS/m and pH was 6.75. After 1 week, four plants of almost equal size were allowed to grow in each pot and all remaining seedlings were removed. In addition to a control (100% field capacity), drought stress levels were maintained at 60% and 80% of field capacity, which were initiated after 15 days of seedling growth. All plants were kept under varying water regimes for 4 weeks. After that time period, three levels of thiamin (0, 50, and 100 mM) were sprayed exogenously at various field capacities. After 2 weeks of thiamin application, data for the following attributes were recorded.

2.1. Plant biomass

Two plants per pot were uprooted for measuring different growth parameters. Fresh weights (shoots and roots) of the clover plants were recorded individually at the time of harvest. Then the uprooted plants were placed in an oven at 65 °C for 3 days.

2.2. Chlorophyll contents

Fresh leaf (0.5 g) was ground in 10 mL of acetone (80%, v/v). After centrifuging, the aliquot was read at 663 and 645 nm using a spectrophotometer for the determination of chlorophyll *a* and *b* contents as described by Arnon (1949).

2.3. Ascorbic acid determination

Ascorbic acid was measured following Mukherjee and Choudhuri (1983). Plant material (0.25 g) was extracted in 10 mL of trichloroacetic acid [TCA (6%); MP Biomedicals, de Kaysersberg Illkirch, France]. Each 4 mL of extract was mixed with 2 mL of dinitrophenyl hydrazine. Afterwards, one drop of thiourea (10%) was added. The reaction mixture was placed in a water bath at 100 °C for 15 min and cooled at room temperature, and then 5 mL of H_2SO_4 (80%, v/v; Merck, Germany) was added to it. After shaking the mixture, using a spectrophotometer, its absorbance was noted at 530 nm.

2.4. Malondialdehyde (MDA)

Fresh sample material (0.25 g) was ground in 3 mL (5%; w/v) of TCA and centrifuged at 12,000 × *g* for 15 min. An aliquot of 1 mL of the supernatant was mixed with 4 mL of thiobarbituric acid (0.5%, w/v; mol. wt. 144.5; Sigma-Aldrich Chemie GmbH, Steinheim, Germany). The mixture was incubated at 95 °C for 50 min and then cooled in chilled water. The optical density was read at 532 and 600 nm following Cakmak and Horst (1991).

2.5. Total phenolics

Plant material (0.1 g) was triturated in 5 mL of acetone (80%). The contents were centrifuged at 10,000 × *g* for 10 min. The reaction mixture contained 100 µL of the supernatant, 2 mL of distilled H_2O, and 1 mL of Folin–Ciocalteu solution (MP Biomedicals). It was shaken vigorously and then 5 mL of sodium carbonate (20%) was added and the final volume was made 10 mL using distilled water. Following Julkenen-Titto (1985), the absorbance was recorded at 750 nm.

2.6. Activities of antioxidant enzymes

In a prechilled pestle and mortar, fresh leaf material (0.5 g) was ground in 5 mL of 50 mM sodium phosphate buffer (pH 7.8). The homogenate was centrifuged at 15,000 × *g* for 15 min at 4 °C. The supernatant was separated and used for the determination of activities of the following enzymes.

2.6.1. Superoxide dismutase (SOD) activity

The reaction mixture contained distilled H_2O, phosphate buffer, L-methionine, Triton-X, NBT, enzyme extract, and riboflavin (MP Biomedicals). as described in the method of Giannopolitis and Ries (1977). The SOD activity was measured at 560 nm.

2.6.2. Catalase (CAT) activity

Following the protocol of Chance and Maehly (1955), the enzyme extract was treated step-wise and the activity of catalase enzyme was measured at 240 nm.

2.6.3. Peroxidase (POD) activity

Adopting the protocol of Chance and Maehly (1955), the reaction mixture was prepared and absorbance was recorded at 470 nm.

2.7. Statistical analysis

Data for all variables were subjected to analysis of variance (ANOVA) and significant differences between the treatment means were determined with the least significant difference (LSD) test at $P < 0.05$ level using SAS (9.1) software.

3. Results

Analysis showed that shoot and root fresh and dry biomasses of both white clover (*Trifolium repens* L.) cultivars were reduced significantly ($P \leq 0.001$) under varying (80% and 60% field capacities) drought stress conditions (Figure 1). However, exogenously applied thiamin (50 and 100 mM) significantly ($P \leq 0.05$) improved the shoot and root fresh weights and shoot dry weight of both white clover cultivars under drought stress conditions. The responses of both white clover cultivars to water deficit conditions were similar with respect to root fresh and dry weights. Of the thiamin levels, 100 mM thiamin was most effective in improving root fresh weight, particularly at 80% field capacity.

Shoot and root lengths decreased significantly ($P \leq 0.001$) in both cultivars under water deficit conditions. Exogenous spraying of 100 mM thiamin was found to be effective in promoting shoot length in cultivar Layalpur Late, particularly at 80% field capacity. Overall, the response of both white clover cultivars was almost unchanged for shoot length, although a highly significant ($P \leq 0.001$) difference between the cultivars was observed in root length (Figure 1). Of the white clover cultivars, Layalpur Late had better performance in terms of root length than the other cultivar under all water regimes. Of the thiamin levels, 50 mM thiamin as a foliar spray showed higher root length in Super Late Fsd at 60% field capacity and in Layalpur Late at 80% field capacity.

Chlorophyll *a* and *b* contents declined significantly ($P \leq 0.01$; 0.05) under water deficit conditions in both white clover cultivars. Both cultivars depicted the same trend in this parameter. Exogenously applied thiamin had a marked effect on chlorophyll *a* and chlorophyll *b* contents. However, 100 mM foliar application was found to be the very useful for chlorophyll pigments in both white clover cultivars, particularly under moisture-scarce conditions (Figure 1).

Data analysis of ascorbic acid concentration showed a significant effect ($P \leq 0.05$) due to low water supply in both white clover cultivars. Both cultivars were nonresponsive in this characteristic. Thiamin treatment did not show any remarkable change in the concentration of this nonenzymatic antioxidant (Figure 2).

Malondialdehyde (MDA) contents increased under 80% and 60% drought conditions in Layalpur Late, while they decreased at 60% and increased at 80% field capacities in Super Late FSD. Mean data showed that 50 mM of thiamin elevated the MDA contents under water stress and nonstressed conditions (Figure 2).

Water deficit conditions had a nonsignificant effect on total phenolic contents in both cultivars of white clover (Figure 2). However, exogenously applied thiamin had a positive effect ($P \leq 0.05$) in improving total phenolics in both cultivars during all field conditions. A similar trend in both white clover cultivars to drought stress or thiamin was observed for this attribute.

A significant reduction ($P \leq 0.001$) was observed in total soluble proteins in both white clover cultivars under both water deficit conditions. Foliar applications of thiamin had a negligible effect on the accumulation of total soluble proteins in both white clover cultivars under drought stress conditions. No considerable change was observed in this attribute in both white clover cultivars under stress or nonstressed conditions (Figure 2).

Water deficit conditions (80% and 60% field capacity) had an enhancing effect on the activities of CAT, POD, and SOD enzymes in both white clover cultivars. Foliar application of thiamin had a nonsignificant effect on the activities of all antioxidant enzymes determined in both cultivars. The response of both cultivars to water deficit conditions or thiamin was also similar for all three antioxidant enzymes (Figure 2).

Water shortage in the growth medium significantly decreased the leaf and root P, Ca^{2+}, and K^+ concentrations in both white clover cultivars (Figure 3). Exogenous application of thiamin showed a nonsignificant effect on the accumulation of all mineral nutrients observed in the present study in different parts of both white clover

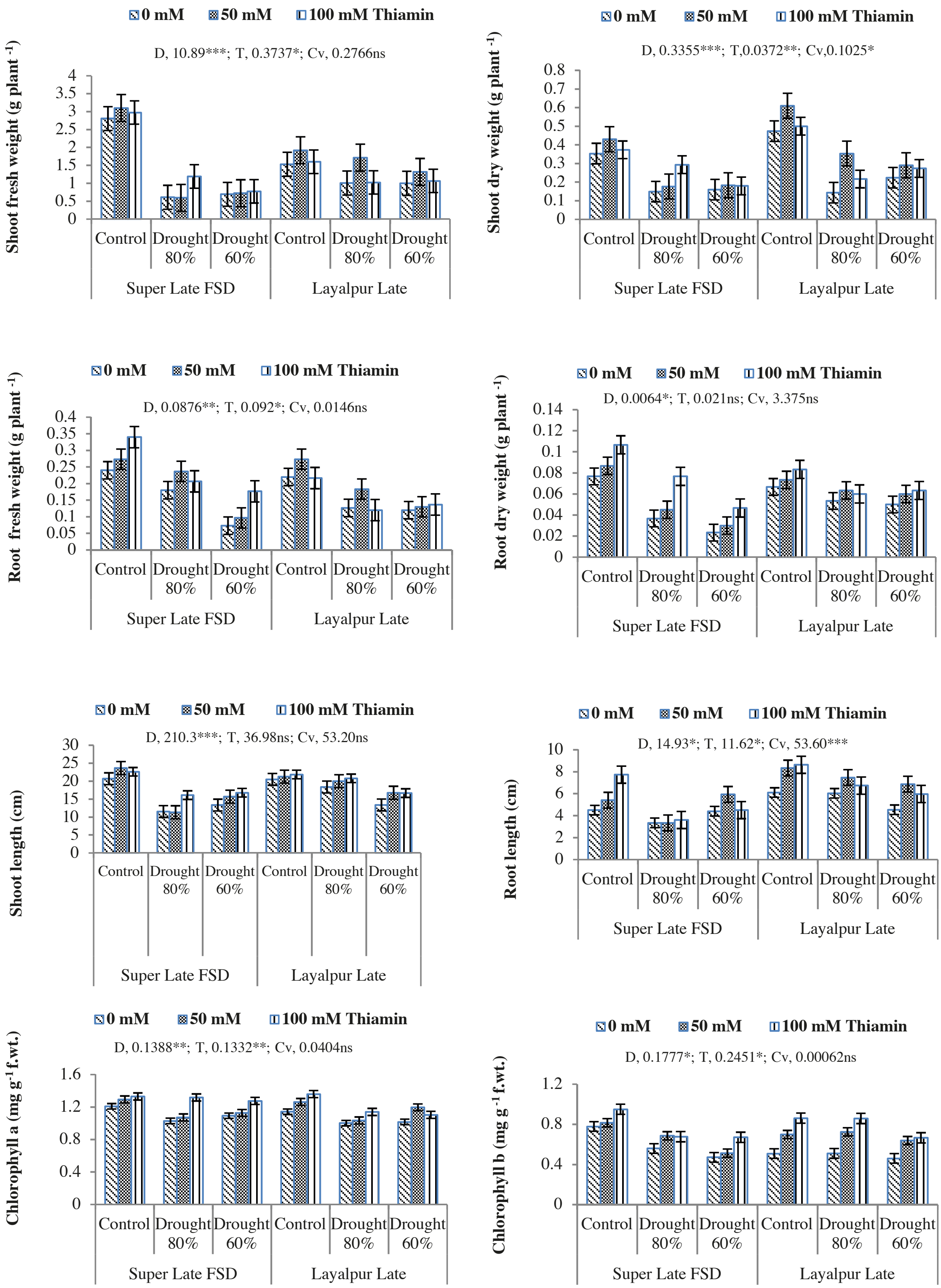

Figure 1. Shoot and root fresh, dry weights and lengths, and chlorophyll *a* and *b* contents of water-stressed and nonstressed plants of two cultivars of white clover (*Trifolium repens*) subjected to varying levels of foliar applied thiamin (mean ± S.E.). Cv, Cultivar; D, drought; T, thiamin; ns, nonsignificant; *, **, and ***, significant at 0.05, 0.01, and 0.001 levels, respectively.

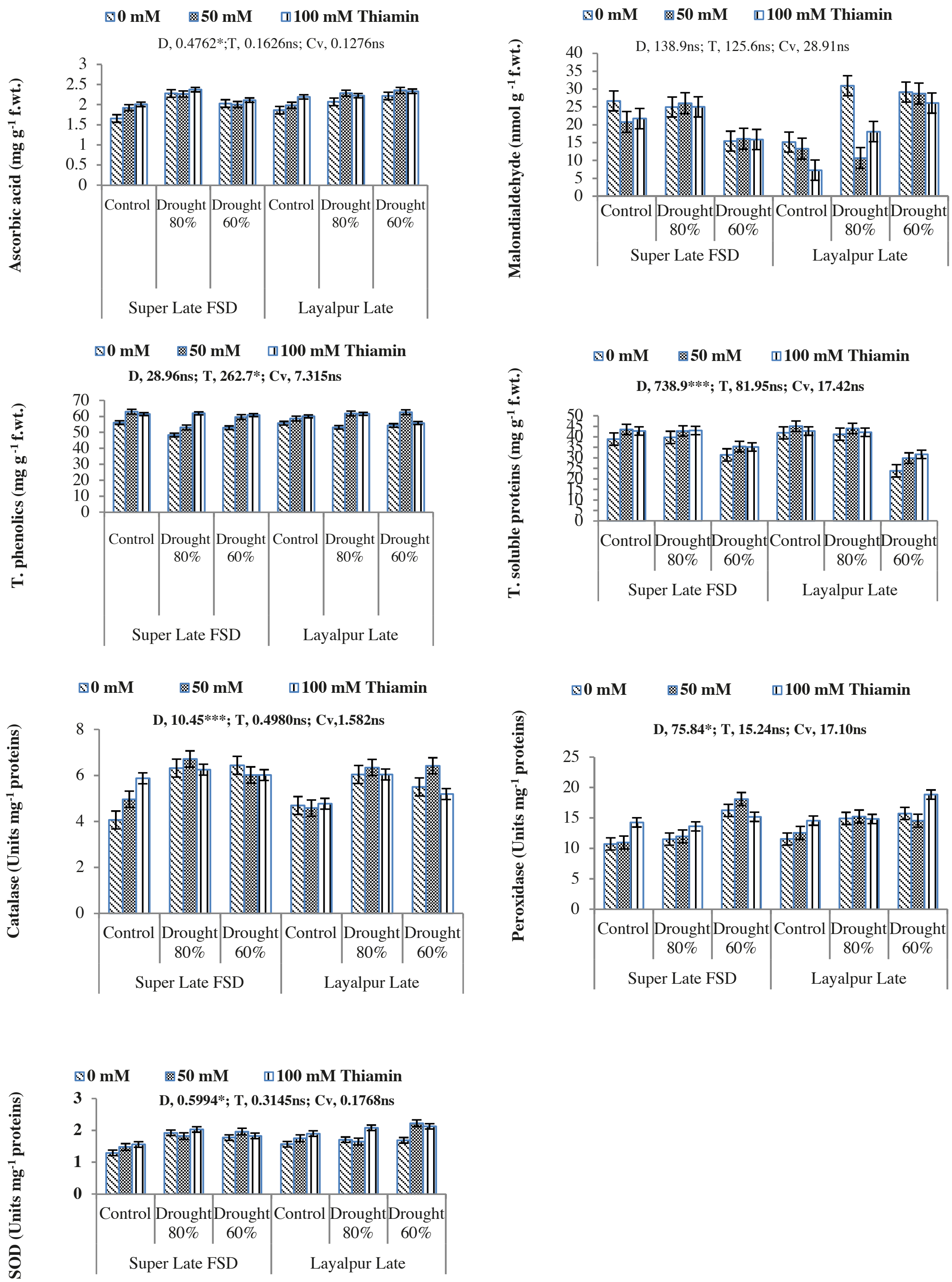

Figure 2. Ascorbic acid, malondialdehyde, total phenolics, total soluble proteins, and activities of catalase, peroxidase, and superoxide dismutase enzymes of water-stressed and nonstressed plants of two cultivars of white clover (*Trifolium repens*) subjected to varying levels of foliar applied thiamin (mean ± S.E.). Cv, Cultivar; D, drought; T, thiamin; ns, nonsignificant; * and ***, significant at 0.05 and 0.001 levels, respectively.

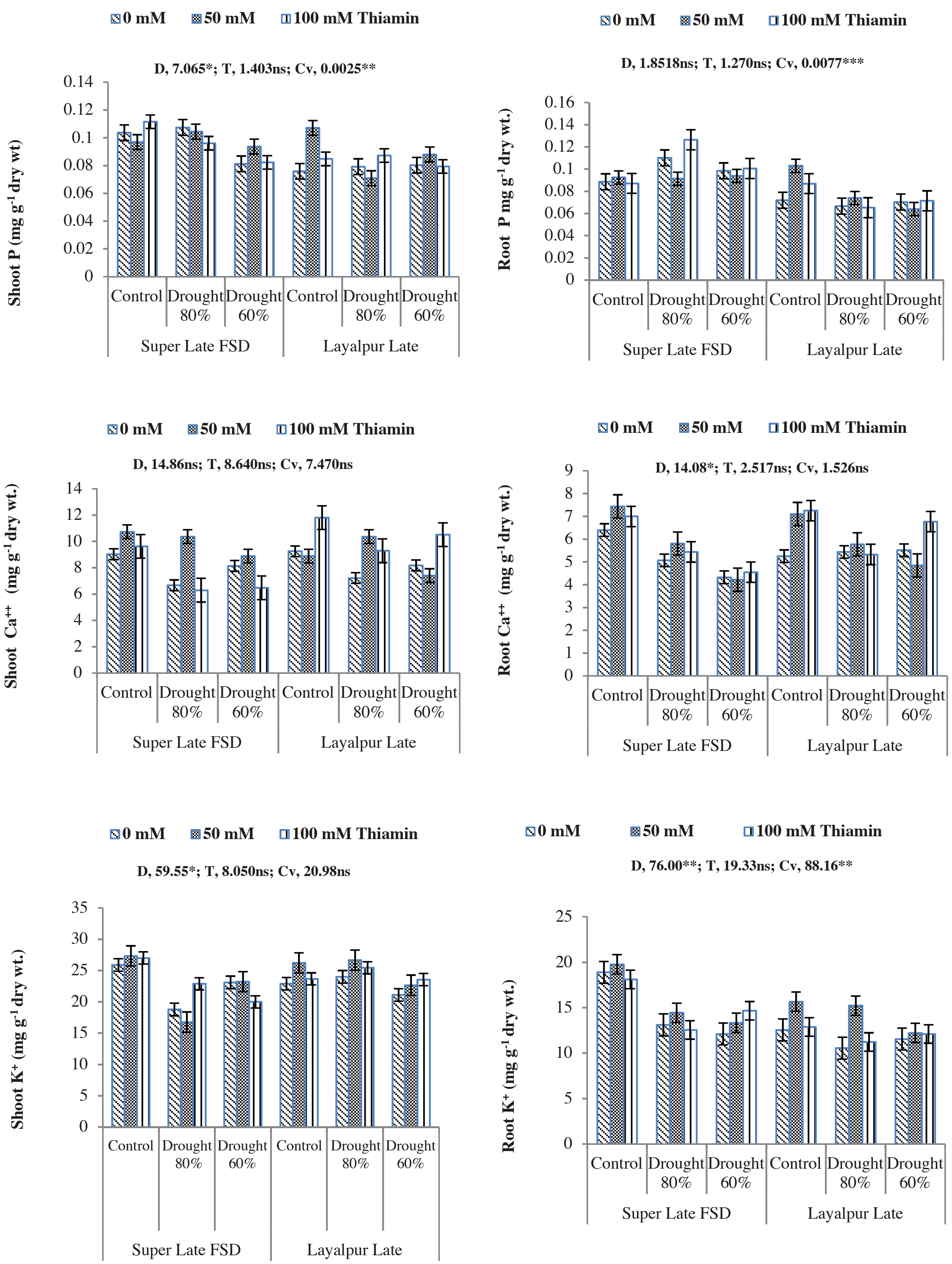

Figure 3. Shoot and root phosphorus, Ca^{++}, and K^{+} concentrations of water-stressed and nonstressed plants of two cultivars of white clover (*Trifolium repens*) subjected to varying levels of foliar applied thiamin (mean ± S.E.). Cv, Cultivar; D, drought; T, thiamin; ns, nonsignificant; *, **, and ***, significant at 0.05, 0.01, and 0.001 levels, respectively.

cultivars. Of the two white clover cultivars, Super Late Fsd was significantly better in leaf and root P and root K^+ concentrations, while accumulation of all the other nutrients was similar in both white clover cultivars under varying water regimes.

4. Discussion

Exogenous application of plant growth regulators is believed to be very effective in ameliorating the adverse effects of abiotic stresses on different crops (Ashraf et al., 2008, 2010; Shafiq et al., 2015; Waseem et al., 2016). Of various plant growth promoters, thiamin (vitamin B1) plays a very important role in mitigating the adverse effects of abiotic stress including drought stress on different crops (Sayed and Gadallah, 2002; Tunc-Ozdemir, 2009; Rapala-Kozik et al., 2012). Application of thiamin under stress conditions has been suggested to reduce membrane injury and increase the uptake of K^+, relative water contents, soluble sugars, total free amino acids, and dry mass production in plants (Sayed and Gadallah, 2002; Rapala-Kozik et al., 2012). In the present study, under both water stress regimes (80% and 60% field capacity), shoot and root fresh and dry weights of both white clover cultivars decreased significantly (Figure 1). Cultivar Layalpur Late was relatively better in biomass accumulation as compared to Super Late Fsd under both water deficit conditions. Recently, Ranjbar et al. (2014) reported that application of 100 mg/L thiamin enhanced the biomass production of German chamomile plants. In another case, Al-Hakimi and Hamada (2011) pretreated seeds of wheat with vitamin B_1 and they found prominent gain in shoot and root weights as compared with the untreated plants under Cu stress. These results also correlate with those of Alipoor and Mohsenzadeh (2012), in which application of vitamin B complex to *Aloe vera* seedlings under nickel and cadmium stress improved plant biomass. Nahed et al. (2009) used 100 mg/L thiamin for *Gladiolus* plants and reported similar results. El-Aziz et al. (2007) found that foliar applied thiamin at 50 mg/L improved plant height of *Syngonium podophyllum*. In another study with sunflower, it was reported that the application of thiamin (5 and 10 mg L^{-1}) improved dry biomass production of sunflower plants, which was ascribed to thiamin-induced reduced membrane injury, enhanced uptake of K^+, and increased leaf RWC, chlorophyll pigments, soluble sugars, and total amino acids under dehydration conditions (Sayed and Gadallah, 2002).

Chlorophyll pigments decreased significantly in both cultivars under water deficit conditions. Thiamin application remained significant for chlorophyll *a* and *b* pigments. However, 100 mM foliar applied thiamin was proved to be effective in increasing chlorophyll contents, particularly in Super Late Fsd under both water-scarce conditions. No literature was available about exogenously applied thiamin in plants exposed to drought stress. However, an earlier study by El-Aziz et al. (2007) showed that 50 mg/L thiamin caused improvement in chlorophyll pigments of *Syngonium podophyllum*. Alipoor and Mohsenzadeh (2012) reported that vitamin B improved chlorophyll pigments in *Aloe vera* seedlings under Ni stress. Earlier Sayed and Gadallah (2002) reported that thiamin enhanced chlorophyll contents in sunflower plants under salinity stress.

In the current study, water deficit conditions enhanced ascorbic acid concentration in both white clover cultivars, but thiamin spray did not improve these contents. In contrast, Emam et al. (2011) reported that foliar spray of vitamin B complex enhanced endogenous levels of ascorbic acid in flax plants.

Moisture-deficit conditions had a prominent decreasing effect on total soluble proteins in both white clover cultivars. Exogenous use of thiamin caused nonsignificant changes in total soluble protein contents. This study cannot be compared with the existing literature as no single report is available on thiamin-induced changes in total soluble proteins. However, earlier, Alipoor and Mohsenzadeh (2012) found that use of vitamin B ameliorated the adverse effects of Ni stress by increasing protein contents in seedlings of *Aloe vera*. Likewise, Al-Hakimi and Hamada (2011) pretreated wheat grains with thiamin and produced plants with enhanced levels of protein contents, which were reported to be directly associated with enhanced salinity tolerance of wheat plants.

Either short- or long-term exposure to water stress leads to oxidative stress in plants. In response to it, plants have the capacity to generate antioxidants, enzymatic and nonenzymatic, such as SOD, CAT, POD, ascorbate peroxidase, glutathione reductase, phenolics, ascorbic acid, and tocopherols, to maintain normal or nearly normal metabolic processes under extreme environmental conditions. It is evident that drought stress has a significant role in the production of antioxidants and hence in enhancing drought tolerance in plants. In the present study, an increase in production of antioxidants was observed but foliage application of thiamin could not trigger antioxidant potential in the white clover plants. An earlier experiment on *Aloe vera* plants grown under Ni stress by Alipoor and Mohsenzadeh (2012) showed that thiamin treatment caused high accumulation of ROS in *Aloe vera* seedlings. They suggested that the use of vitamin B protects seedlings against ROS by enhancing the oxidative defense system. We observed that total phenolic contents were improved considerably in both white clover cultivars. In contrast to the present study, Nahed et al. (2009) reported an increase in total phenolics in *Gladiolus* plants due to application of 50 to 200 mg/L thiamin. In another study, exogenous thiamin enhanced tolerance to oxidative stress induced by paraquat in *Arabidopsis thaliana*. Thiamin application reduced the overaccumulation of ROS in Arabidopsis plants by reducing protein carbonylation and H_2O_2 accumulation (Tunc-Ozdemir, 2009).

Optimum concentration of nutrients is essential for proper functioning of different metabolic processes taking place within the plant cells/tissues (Ashraf et al., 2010; Akram and Ashraf, 2012). Water deficiency significantly disturbs the uptake/accumulation of different nutrients (Ahmad et al., 2014). In the present study, water stress at different levels (80% and 60% field capacity) adversely affected the accumulation of shoot P and K^+ as well as root Ca^{2+} and K^+. Exogenous application of thiamin at different levels (50 mM and 100 mM) did not affect the accumulation of these nutrients in both white clover cultivars. These findings can be related to those of Yousef and Talaat (2003) while working with rosemary plants, because they found no alteration in shoot P and K^+ concentrations. However, analogous to these studies, Sayed and Gadallah (2002) reported that thiamin application positively influenced the uptake of K^+ under saline conditions.

Overall, drought stress (80% and 60% field capacities) considerably reduced the plant biomass, shoot and root lengths, chlorophyll pigments, total soluble proteins, and shoot and root P, Ca^{2+}, and K^+, while it significantly increased the levels/activities of ascorbic acid and antioxidant enzymes (CAT, SOD, and POD). Foliage spray of thiamin was only effective in improving plant growth, chlorophyll pigments, and total phenolics, while no considerable change was observed in the remaining metabolites observed in the two white clover cultivars. Overall, foliar applied 100 mM thiamin was found effective in improving the growth of both white clover cultivars, which was attributed to thiamin-induced improvement in photosynthetic pigments and antioxidants.

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