Sains Malaysiana 48(3)(2019): 533–541
http://dx.doi.org/10.17576/jsm-2019-4803-05

Assessment of Biochemical Changes in Spinach (*Spinacea oleracea* L.) Subjected to Varying Water Regimes

(Penilaian Perubahan Biokimia dalam Bayam (*Spinacea oleracea* L.) Tertakluk kepada Rejim Air Berbeza)

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ABSTRACT

*It is known that leafy vegetables including spinach (*Spinacea oleracea *L.) contain relatively high amount of water, therefore, their water requirement during the life cycle is comparatively more than the other vegetables. In addition, there is an association between osmoprotection and antioxidants with reference to drought stress tolerance. Keeping in mind these facts, the present study was conducted to assess the changes in plant growth, osmoprotectants, chlorophyll pigments and activities/levels of antioxidative system in spinach (*Spinacea oleracea *L.) grown under varying water deficit regimes with 40%, 60%, 80% and 100% field capacity (FC). Imposition of varying water regimes significantly decreased shoot and root fresh and dry weights, shoot plus root lengths, and chlorophyll* b *contents of spinach plants. Increase in proline, glycinebetaine (GB), total phenolics, ascorbic acid and malondialdehyde (MDA) contents as well as the activities of antioxidant enzymes including superoxide dismutase, peroxidase and catalase were observed in the spinach plants particularly at 40% FC. The most effective level of water stress for elevating the proline, GB and antioxidant levels/ activities was observed at 40% FC followed by 60% FC. Hence, the results of this study suggested that upregulation of antioxidants and osmoprotectants is positively associated with the drought tolerance of spinach which depends on the severity of water stress level. These results can be used to narrow the gap between selection of plant species and requirement of irrigated water for the crops grown on dry land areas.*

Keywords: Antioxidants; osmoprotection; reactive oxygen species; spinach; water stress

ABSTRAK

*Adalah diketahui bahawa sayur-sayuran berdaun termasuk bayam (*Spinacea oleracea *L.) mengandungi jumlah air yang agak tinggi, oleh itu, mereka memerlukan lebih air sepanjang kitaran hidup berbanding sayur-sayuran lain. Di samping itu, terdapat hubungan antara osmoperlindungan dan antioksidan berkaitan toleransi tekanan kemarau. Dengan mengambil kira fakta tersebut, kajian ini dijalankan untuk menilai perubahan dalam pertumbuhan tanaman, osmoperlindungan, pigmen klorofil serta aktiviti/tahap sistem antioksidatif pada bayam (*Spinacea oleracea *L.) yang ditanam di bawah rejim defisit air berbeza dengan 40%, 60%, 80% dan 100% kapasiti lapangan (FC). Pengenaan rejim air yang berbeza dengan ketara mengurangkan berat segar dan kering pucuk dan akar, panjang pucuk dan akar serta kandungan klorofil* b *pada pokok bayam. Pertambahan kandungan prolin, glisinbetain (GB), jumlah fenolik, asid askorbik dan malondialdehid (MDA) serta aktiviti enzim antioksida termasuk superoksida dismutase, peroksidase dan katalase telah diperhatikan pada tanaman bayam terutamanya pada 40% FC. Tahap tekanan air paling berkesan untuk meningkatkan tahap/aktiviti proline, GB dan antioksidan diperhatikan pada 40% FC diikuti 60% FC. Oleh itu, keputusan kajian ini mencadangkan pengawalaturan atas antioksidan dan osmoperlindungan dikaitkan secara positif dengan toleransi kemarau oleh bayam yang bergantung pada keterukan aras tekanan air. Keputusan ini boleh digunakan untuk mengurangkan jurang antara pemilihan spesies tumbuhan dan keperluan pengairan untuk tanaman yang ditanam di kawasan tanah kering.*

Kata kunci: Antioksidan; bayam; osmoperlindungan; spesies oksigen reaktif; tekanan air

INTRODUCTION

Drought stress is the most crucial environmental constraint to plant growth and development (Srivastava & Kumar 2014). It is well established that plants subjected to drought stress show alterations in a multitude of physio-biochemical processes (Ashraf 2010). For example, drought causes osmotic stress, over-production of reactive oxygen species (ROS), stomatal closure, impairment in carbohydrate assimilation and disturbance in CO_2 uptake in most plants (Kosar et al. 2015). Due to overproduction of ROS in mitochondria, chloroplasts and peroxisomes, membrane damage, ion leakage and inactivation of enzymes occur in drought-stressed plants (Noreen et al. 2009). However, to tolerate stress conditions, only stress resistant plants are able to regulate growth and development (Hammad & Ali 2014) by improving photosynthesis, osmoprotection, ion flux, respiration, carbohydrate metabolism, oxidative defence system and plant growth promoters that are

significantly perturbed under stress conditions (Kusaka et al. 2005).

Under water deficit conditions, many physiological parameters such as chlorophyll contents, tissue water content and membrane stability are generally measured as signs of improved plant growth and development (Razzaq et al. 2013). However, physiological and morphological adaptations of plants to water stress conditions may vary among species or even among the cultivars/lines of the same crop (Cranston et al. 2016). However, purposeful use of irrigation is essential because all ontogenic stages are not equally susceptible to drought stress conditions. It was reported earlier that drought stress significantly altered growth and other physio-biochemical processes in many plant species (Darvishan et al. 2013; Galahitigama & Wathugala 2016; Shafiq et al. 2015, 2014). For example, some metabolites including total phenolics, glycinebetaine and proline help to sustain plant growth by neutralising ROS (Patel & Aranjan 2013). The scavenging network of ROS which involves enzymatic (catalase (CAT), peroxidase (POD), superoxide dismutase (SOD), ascorbate peroxidase (APX)) and non-enzymatic antioxidants (tocopherol, ascorbate, glutathione) help to mediate the harmful effects of stress conditions (Lehtimaki et al. 2010).

Since most vegetables including spinach (*Spinacea oleracea* L.) are prone to water deficit conditions either naturally or man-made, therefore, it is natural to expect under such adversaries a myriad of physio-biochemical changes in such crops. Of the most common vegetable crops, spinach is a favorite vegetable throughout the world, because of the reason that it is rich in important minerals and vitamins particularly vitamin C essentially required for human health (Ekinci et al. 2015). Furthermore, it has a smaller number of calories but large quantities of bioactive molecules such as glucuronic acid derivatives of flavonoids and *p*-coumaric acid derivatives which exhibit strong antioxidant activity (Lamhamdi et al. 2013; Xu & Leskovar 2015). Generally, spinach plants require sufficient amount of water for optimum growth, but if they experience water deficit conditions, a considerable restraint to its production takes place (Xu & Leskovar 2015). A remarkable reduction in the yield of spinach plants was also reported in some earlier studies under water deficit conditions; particularly 50% irrigation showed a marked suppression in crop yield (Leskovar et al. 2012). Xu and Leskovar (2015) reported that water stress suppressed the growth, leaf area, relative water contents and gas exchange attributes of spinach plants, while application of a seaweed extract was found to be beneficial for improving the growth, leaf water relations and gas exchange attributes. In another study, Du et al. (2015) observed that salinity-induced oxidative damage in spinach plants was alleviated by the application of nitric oxide.

Although the processes of osmoprotection and antioxidative defense system are generally upregulated in most plants due to drought stress, there are reports which show otherwise association of these two mechanisms with drought tolerance (Maevskaya & Nikolaeva 2013). For example, in wheat, coordination between osmoprotectants and antioxidants was retained at moderate water stress level, but was disrupted at severe water stress conditions (Maevskaya & Nikolaeva 2013). Thus, the present study was conducted to assess the extent that drought stress could alter growth, and the mechanisms of osmoprotection and antioxidative defense in spinach plants and how far these mechanisms were involved in the drought tolerance of this plant.

Materials and Methods

To assess the osmoprotection and antioxidative defense system in spinach (*Spinacea oleracea* L.) plants, a pot experiment with four replicates laid out in a completely randomised design, was carried out at the Research Area of GC University, Faisalabad, Pakistan from October to December 2015. For this study, seed of the spinach cultivar, White Desi Spinach, was obtained from the Vegetables Section, Ayub Agricultural Research Institute (AARI), Faisalabad, Pakistan. The seed samples were surface sterilised in 0.05% (w/v) sodium hypochlorite solution and soaked in water before germination. Eight seeds were sown in each plastic pot. After 1 week of germination, thinning was done to maintain uniform size seedlings of five plants. Plants were subjected to different water stress levels with 40%, 60%, 80% and 100% field capacity (FC). Two plants from each replicate were harvested after 28 days of stress treatment and washed with deionised water. Then, growth parameters such as shoot and root fresh and dry weights as well as shoot and root lengths were determined. The following attributes were determined before harvesting the plants.

Chlorophyll contents

Fresh leaf (each 500 mg) sample was triturated with 10 mL 80% (v/v) acetone following Arnon (1949). The absorbance was read at 480, 645 and 663 nm using a spectrophotometer.

Determination of proline contents

The protocol outlined by Bates et al. (1973) was used to determine the proline contents in spinach leaves. Fresh leaf (500 mg) was ground in 10 mL 30% (w/v) sulfosalicylic acid, and the mixture was then filtered. Acid ninhydrin (2 mL) and 2 mL glacial acetic acid were added in 2 mL filtrate. Then, the mixture was incubated at 100°C in a water bath for 30 min. After cooling, toluene was added to the mixture and the absorbance was recorded at 520 nm using a spectrophotometer.

Determination of glycinebetaine (GB) content

Following Grieve and Grattan (1983), GB content was determined by extracting 500 mg leaf sample in 10 mL 0.5% (v/v) toluene. The mixture was kept at 4°C overnight, thereafter to 1 mL of the supernatant, 1 mL 2N sulfuric

acid was added after centrifugation. In a test tube, 0.5 mL of the mixture was taken and 0.2 mL KI_3 added to it. Then the contents were shaken and cooled in an ice bath for 90 min. The absorbance of the colored solution was read at 365 nm using a spectrophotometer.

DETERMINATION OF HYDROGEN PEROXIDE (H_2O_2) CONTENT

The tri-chloroacetic acid (TCA) assay was used to determine H_2O_2 content in the spinach leaves. Fresh leaf (each 250 mg) sample was extracted in 5 mL 0.1% (v/v) TCA in pre-chilled pestle and mortar (Velikova et al. 2000). The mixture was centrifuged at 12,000 × g for 15 min. Then 1 mL of potassium iodide was added to the mixture following the addition of 0.5 mL of potassium phosphate buffer in 0.5 mL of aliquot. The mixture was vortexed and absorbance read at 390 nm.

DETERMINATION OF MALONDIALDEHYDE (MDA) CONTENT

The procedure of Cakmak and Horst (1991) was used to assess the lipid peroxidation induced in the spinach leaves under drought stress. Leaf sample (250 mg) was homogenised in 3 mL 1% (v/v) TCA (3 mL) solution. The extract was centrifuged for 15 min at 20,000 × g and then 4 mL TBA (0.5% prepared in 20% TCA) was added in 1 mL aliquots. The samples were incubated for 50 min at 95°C and after cooling, the absorbance was recorded at 532 and 600 nm.

DETERMINATION OF TOTAL PHENOLICS

According to the protocol outlined by Julkunen-Tiitto (1985), 100 mg fresh leaf was homogenised in 5 mL 80% (v/v) acetone. After centrifugation, 2 mL deionised water was added with 1 mL Folin-Ciocalteau's phenol reagent to 100 µL of aliquot. Each mixture was shaken and the volume was raised to 10 mL by adding 5 mL 20% (w/v) sodium carbonate and deionised water. Then the absorbance was read at 750 nm using a spectrophotometer.

DETERMINATION OF ASCORBIC ACID (ASA) CONTENT

For the determination of ASA content, 10 mL 6% (w/v) TCA solution was used to grind 250 mg fresh leaf sample following Mukherjee and Choudhuri (1983). Two milliliters of dinitrophenyl hydrazine (2% in 9 N H_2SO_4) was mixed with 4 mL of the extract following the addition of one drop of thiourea (10% in 70% ethanol). The mixture was incubated for 15 min in a water bath and then cooled at room temperature. Five milliliters of H_2SO_4 (80%) was added and absorbance was read at 530 nm.

DETERMINATION OF ENZYMATIC ANTIOXIDANTS

Fresh leaf (each 500 mg) sample was extracted in 10 mL of phosphate buffer (pH7.8) using a pre-chilled pestle and mortar. The extract was centrifuged at low temperature. The aliquot was separated to assess the activities of antioxidants enzymes as mentioned below in the following methods.

ACTIVITY OF SUPEROXIDE DISMUTASE (SOD)

The method proposed by Giannopolitis and Ries (1977) was used to determine the activity of SOD enzyme. The reaction mixture contained distilled H_2O (400 µL), phosphate buffer (250 µL), 0.1% triton-X (100 µL), 13 mM L-methionine (100 µL), 50 µL NBT, 1.3 µM (50 µL) riboflavin and 50 µL enzyme extract in cuvettes and kept the mixture under light for 15 min. The absorbance was measured at 560 nm and the activity of enzyme per unit was defined as the amount necessary for the photo-reduction of 50% NBT.

ACTIVITY OF PEROXIDASE (POD)

According to the protocol of Chance and Maehly (1955), 1 mL 20 mM guaiacol, 900 µL 40 mM H_2O_2 and 1 mL 50 mM phosphate buffer were added into a cuvette already containing 100 µL enzyme extract. The change in absorbance was recorded every 30 s at 470 nm and 1 unit of enzyme activity was considered to be equal to a change of 1.0 A_{470} unit per min.

ACTIVITY OF CATALASE (CAT)

For the determination of CAT activity, 5.9 mM H_2O_2 and 50 mM buffer were added into 100 µL of enzyme extract (Chance & Maehly 1955). The change in absorbance was read at 240 nm for 3 min and 0.01 A_{240} unit per min was considered to be equivalent to 1 unit of CAT activity.

STATISTICAL ANALYSIS

One-way analysis of variance was worked out for each parameter using a Cohort Statistical software (Cohort software, Costat V6.303). The LSD (least significance difference) test was used to compare the means of all treatments.

RESULTS

Mean data as well as analysis of variance (ANOVA) showed that varying water regimes (100%, 80%, 60% and 40%) significantly ($P \leq 0.001$, 0.05, 0.01 and 0.01, respectively) decreased the shoot and root fresh and dry weights of spinach (*Spinacea oleracea* L.) cv. Desi White (Figure 1). A consistent decrease in all mentioned growth attributes of spinach was observed with a progressive decrease in water supply. Shoot and root lengths also decreased significantly ($P \leq 0.05$) due to imposition of varying water deficit levels. Of all water regimes, the most effective level in decreasing the shoot and root lengths of spinach plants was 40% field capacity (Figure 1).

Drought had no significant effect in altering the chlorophyll *a* content, while a significant reduction in chlorophyll *b* content was observed (Figure 1). The

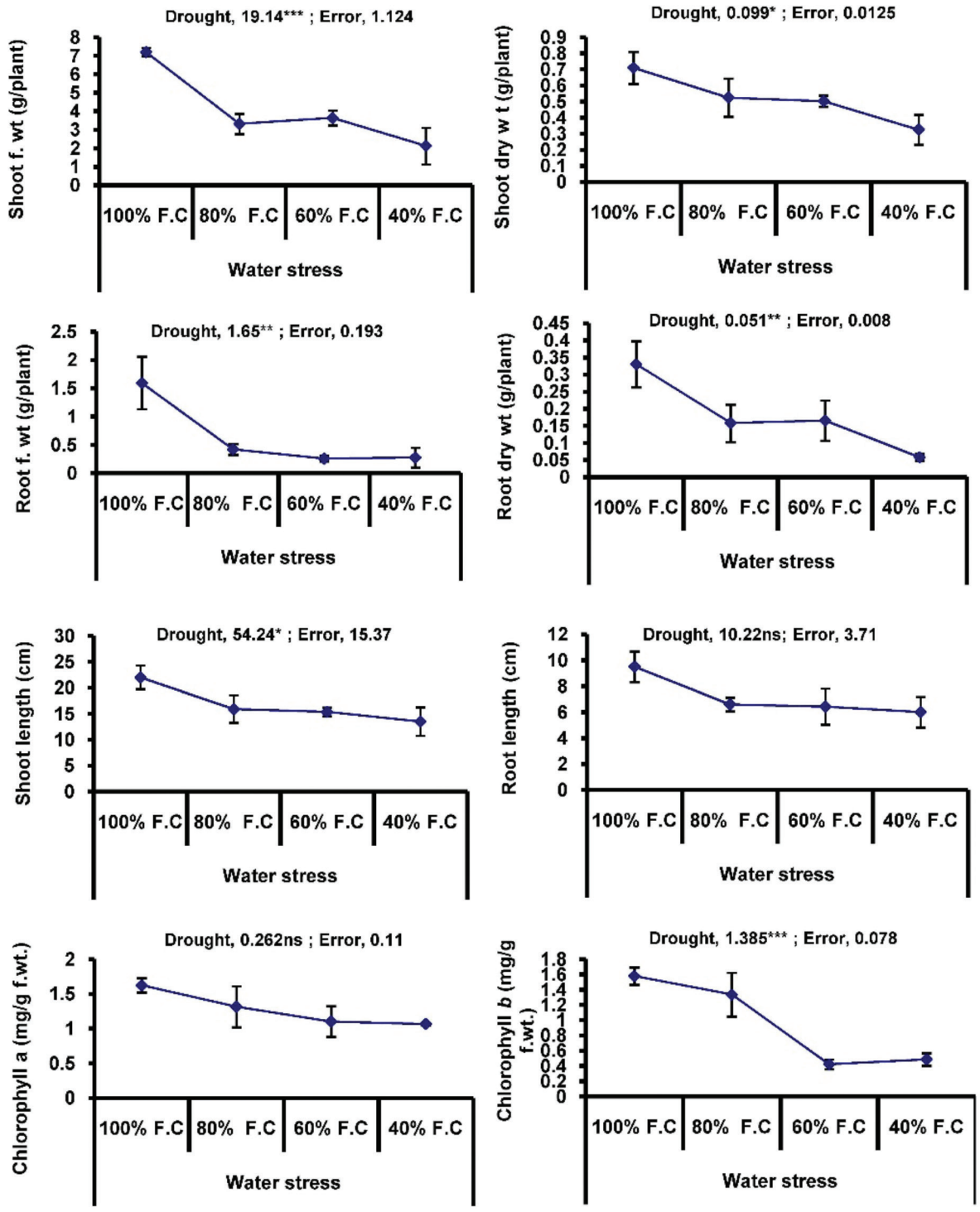

FIGURE 1. Shoot and root fresh and dry weights, shoot and root lengths, chlorophyll *a* and *b* pigments of spinach (*Spinacia oleracea* L.) cultivar, Desi White grown under varying (100%, 80%, 60% & 40% of field capacity) water regimes (Mean ± S.E.; mean square error analyzed by ANOVA; *, **, ***; significant at 0.05, 0.01 and 0.001 levels; ns, non-significant)

decreasing trend in chlorophyll *b* content was observed as the water supply progressed from 80% to 40% field capacity.

Significant increases in proline and glycinebetaine (GB) contents were observed in the spinach leaves at lower levels of water supply. The most effective level of water stress for elevating the proline and GB contents was observed 40% FC followed by 60% FC (Figure 2).

Both non-enzymatic antioxidants, total phenolics and ascorbic acid contents increased significantly in spinach plants as water stress progressed from 80% to 40% field capacity (Figure 2).

Water stress considerably ($P \leq 0.05$) increased the malondialdehyde (MDA) contents of spinach plants at varying water stress levels. A maximum increase in MDA

contents was observed at 40% FC. However, no significant change was observed in hydrogen peroxide (H_2O_2) contents except a marked increase in its level at 40% FC (Figure 2). The activities of antioxidant enzymes including SOD, POD and CAT remained un-affected due to varying water regimes except at 40% FC where a slight increase was observed in the activities of all the above-mentioned enzymes in spinach plants (Figure 3).

Total soluble proteins also remained unaffected due to imposition of varying water regimes to spinach plants (Figure 3).

Discussion

This study was conducted to evaluate the contribution of antioxidant defense system and osmoprotection in reducing the drastic effects of water deficit stress in spinach (*Spinacea oleracea* L.) plants. Drought stress is a mutual and most vital abiotic stress that adversely affects almost all plants occurring in the biosphere (Shafiq et al. 2014). Drought affects the plant water relations at all stages from molecular, cellular and tissue to the whole plant level (Muscolo et al. 2015). Turgor pressure is reduced by the water deficit conditions and wilting cells experience decrease in expansion thereby resulting in reduced growth. It is now widely known that antioxidant defense system comprising non-enzymatic and enzymatic antioxidants are helpful in reducing the oxidative stress caused by water deficit conditions in plants (Ashraf 2010, 2009). In the present study, plant biomass (shoot and root fresh and dry weights) and root and shoot lengths of spinach plants were decreased by the imposition of drought stress. Similar results were observed by Khaki-Moghadam and Rokhzadi (2015) and Simon-Grao et al. (2016) in tomato in safflower plants. Under water shortage, plants tend to close their stomata leading to lowering of the rate of transpiration as

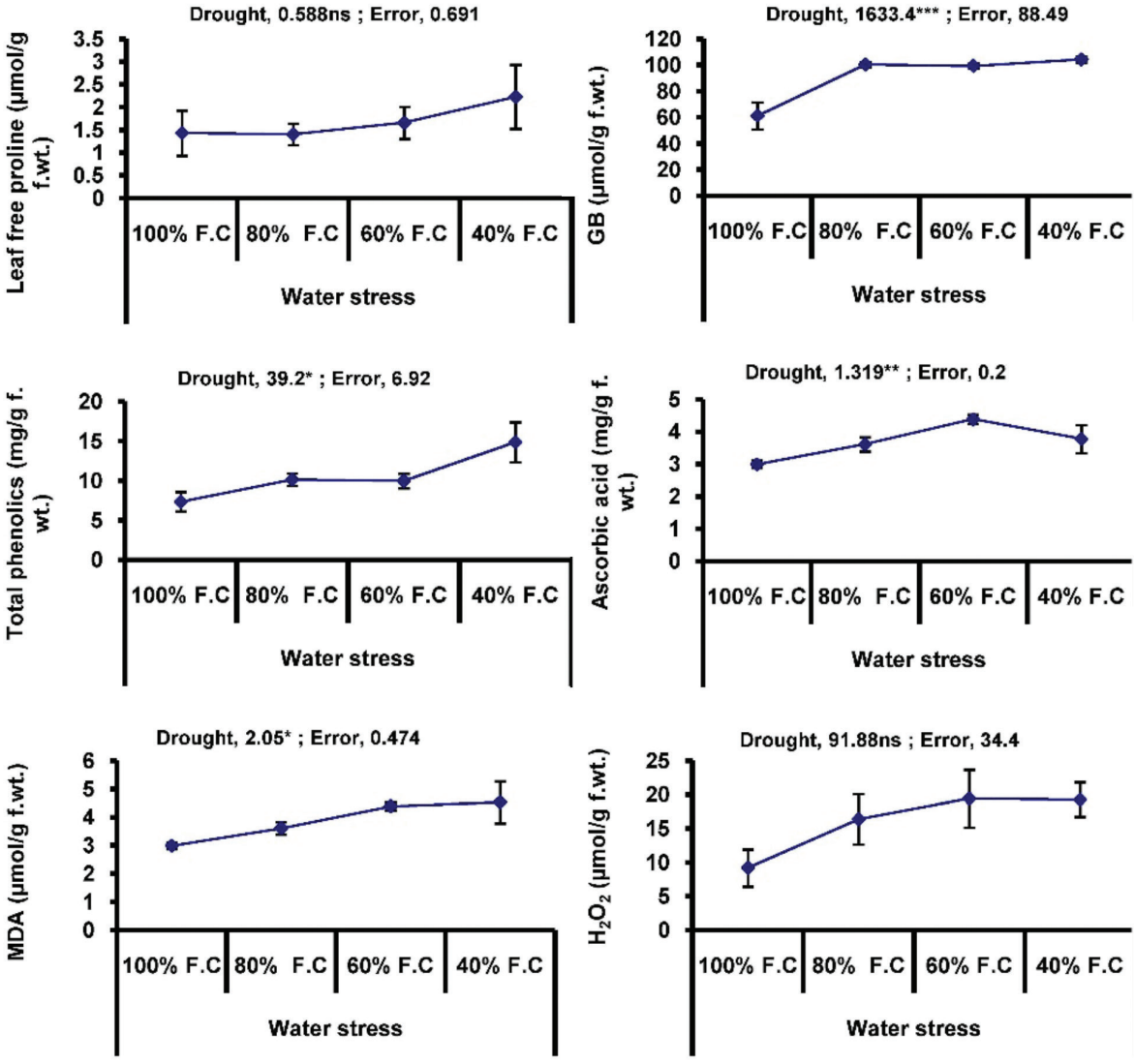

FIGURE 2. Accumulation of proline, glycinebetaine, total phenolics, ascorbic acid, malondialdehyde and hydrogen peroxide in spinach (*Spinacia oleracea* L.) cultivar, Desi White grown under varying water (100%, 80%, 60% & 40% of field capacity) regimes (Mean ± S.E.; mean square error analyzed by ANOVA; *, **, ***; significant at 0.05, 0.01 and 0.001 levels; ns, non-significant)

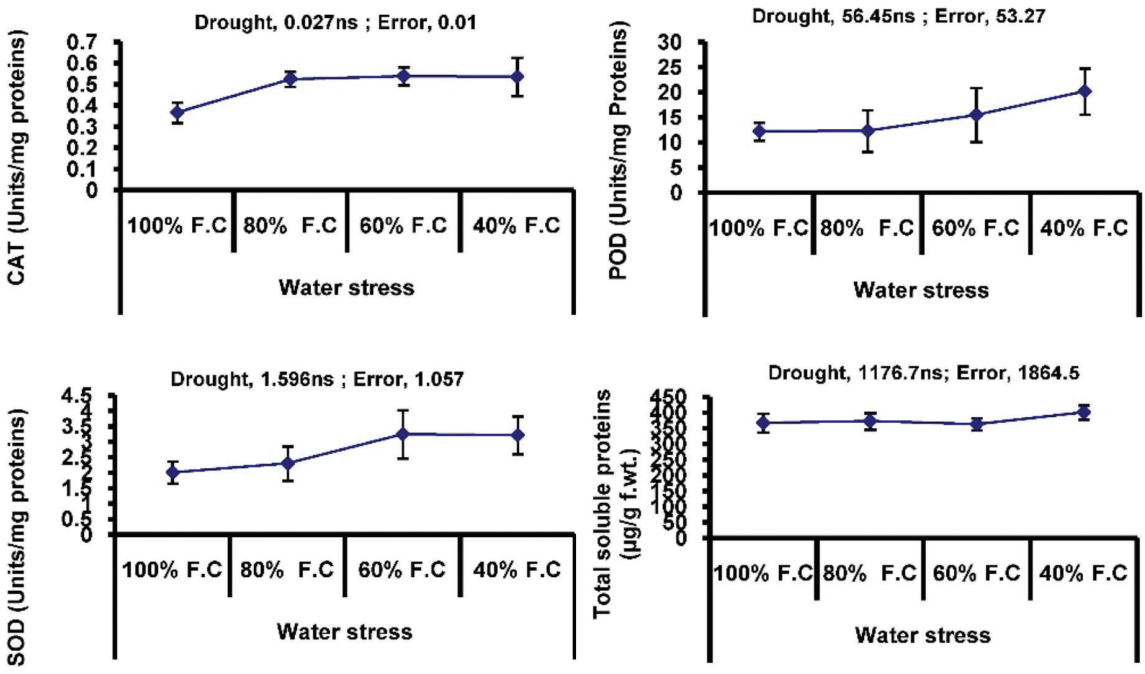

FIGURE 3. Activities of enzymatic antioxidants (CAT, POD & SOD) and total soluble proteins contents in spinach (*Spinacia oleracea* L.) cultivar, Desi White grown under varying water (100%, 80%, 60% & 40% of field capacity) regimes (Mean ± S.E.; mean square error analysed by ANOVA; ns, non-significant)

well as respiration significantly (Diaz-Lopez et al. 2012). Drought stress decreases the growth of root and shoot, partitioning of dry matter, upsets the plant water relations and photosynthesis, thus, ultimately affecting crop yield (Ashraf 2010).

The process of photosynthesis is involved in the regulation of plant development and yield. Imbalance in rate of photosynthesis and production of high amount of oxygen due to drought stress lead plants to experience oxidative stress caused by the generation of a large amount of ROS in plants (Ashraf 2009). The ROS cause lipid peroxidation and damage the lipids, proteins, DNA and biological membranes (Mittova et al. 2000). In this study, chlorophyll *b* content decreased in spinach plants when they were subjected to varying levels of drought stress. Previously, Akram et al. (2016) in radish and Yasmeen et al. (2013) in wheat observed that drought stress decreased the contents of chlorophyll *b* where they attributed this decrease in chlorophyll pigments to water-stress induced damages to oxidative defense system. Chlorophyll *b* is a premier light harvesting pigment, and relatively more drought-induced reduction in chlorophyll *b* content compared to chlorophyll *a* could have been due to the reason that water deficiency adversely affected the PS-I, light harvesting/capturing system, energy transfer and antenna complex accessories (Ashraf & Harris 2013).

Osmoregulation is one of the important plant strategies to counteract the drought stress. Glycinebetaine (GB) and proline are essential osmolytes which are accumulated in many crop species under stress conditions and play important role in osmotic adjustment (Raza et al. 2016). Proline and GB act as principal osmoprotectants as they effectively shield the components of cells from injuries caused by water deficiency. Raza et al. (2014) have reported that GB enhances the plant tolerance to different abiotic stresses including water deficit stress. High GB accumulation improves the drought tolerance of plants as it improves the antioxidant enzyme activity (Ma et al. 2014) and maintains turgor pressure (Ashraf & Foolad 2007). Under water deficit conditions, when water potential of leaves is lowered, it causes acceleration in GB synthesis, which in turn maintains leaf osmotic potential (Ashraf & Foolad 2007). Proline is known to protect the plants from the ROS and upregulates osmoregulation in plants (Aranjuelo et al. 2010). During this study, the contents of both proline and GB increased significantly particularly at the severe water stress level, i.e., 40% FC. Various studies have reported that high accumulation of osmoprotectants is interlinked with better survival of plants under stress conditions. For example, Akram et al. (2016) and Galahitigama and Wathugala (2016) in rice in radish plants observed improvement in stress tolerance due to an increase in proline and GB contents under water deficit conditions.

Stress-induced oxidative stress causes the generation of a myriad of ROS which cause damage to several key biomolecules including bio-membranes of vital cellular organelles. Most of the ROS can effectively peroxidise membranous lipids. However, the extent of ROS-induced lipid peroxidation can be assessed indirectly by malondialdehyde (MDA), a product of lipid peroxidation. In the present study, the content of MDA was not significant in the spinach plants exposed to drought stress. Terzi and Kadioglu (2006) in *Ctenantha setosa,* Yadegari et al.

(2014) in tomato and Hameed and Iqbal (2014) in wheat observed that under drought stress, MDA contents were increased which was the indication of the imposition of stress-induced oxidative stress.

Plant oxidative defense system comprises a multitude of biomolecules including enzymes and non-enzymes. Of the non-enzymatic compounds, ascorbic acid is believed to protect the plants from different abiotic stresses by scavenging the free radicals of oxygen (Shafiq et al. 2014). Ejaz et al. (2012) have reported that the amount of ascorbic acid at cellular level is linked with activation of plant defense system. It plays an important role in plant growth as it is involved in cell division and expansion as well as other different physiological processes (De Gara et al. 2003). In our study, an increase in ascorbic acid content in spinach plants exposed to drought was similar to what has earlier been observed in maize showing a marked increase in the levels of ascorbic acid particularly under high intensity of drought (Dolatabadian et al. 2010). Phenolics are also non-enzymatic compounds which are known to play an essential role in transporting their hydrogen atoms to reduce the ROS generated under stress (Frary et al. 2010). Total phenolic content in the spinach plants was found to be increased under drought stress conditions.

Antioxidative defense system also consists of enzymatic antioxidants which protect the plant cells from oxidative damage caused by drought stress. Several studies show that upregulation of antioxidative defense system enhances drought tolerance in different plants such as in rice (Nounjan et al. 2012), canola (Shafiq et al. 2014) and radish (Shafiq et al. 2015). During this experiment, the activities of SOD, POD and CAT were found to increase with increasing levels of drought. These results are similar to other reports on different plants such as maize (Moussa & Abdel-Aziz 2008), radish (Akram et al. 2016) and corn (Darvishan et al. 2013).

Overall, varying water deficit conditions significantly suppressed plant biomass, lengths of root and shoot, chlorophyll *b* and total phenolic contents, and significantly increased the contents of proline, GB, MDA, and ascorbic acid. Hence, upregulation of osmoprotection and ascorbic acid contents in spinach plants due to a progressive increase in water deficiency can be suggested as potential selection criteria for improving water deficit tolerance in spinach.

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Received: 4 January 2018
Accepted: 30 December 2018