

BIOCHEMICAL RESPONSES OF *Digitaria commutata* AND *Cenchrus ciliaris* TO WATER STRESS: ANTIOXIDATIVE REACTIONS, PROLINE AND SOLUBLE SUGARS ACCUMULATION

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ABSTRACT

The impact of water stress on antioxidant enzyme activities, proline, soluble sugars, and carotenoids contents found in *Digitaria commutata* and *Cenchrus ciliaris* plants was investigated. Two different watering regimes were used on plants over a period of three months. Water stress decreased total chlorophyll content in plants, but increased carotenoids content. Interestingly, no change was observed in the quantum yield of PSII photochemistry (F_v/F_m). Malondialdehyde (MDA) content increased to a higher extent in both species. Enhanced activities of all the enzymes (peroxidase, catalase, and superoxide dismutase) studied, except for catalase in the roots were observed. Proline and soluble sugars contents increased significantly following water stress exposure. No clear differences were found between both species. The results link drought tolerance of *Digitaria commutata* and *Cenchrus ciliaris* plants with better capabilities of anti-oxidative system. Additionally, it is linked to the accretion of osmoprotectants proline and soluble sugars when exposed to drought.

Additional keywords: Antioxidative enzymes, carotenoid, proline, soluble sugars, water stress

RESUMEN

Respuestas de *Digitaria commutata* y *Cenchrus ciliaris* al estrés hídrico: reacciones antioxidativas, prolina y azúcares solubles

Se investigó el impacto del estrés hídrico en la actividad de las enzimas antioxidantes, así como de prolina, azúcares solubles y carotenoides en plantas *Digitaria commutata* y *Cenchrus ciliaris*. Se utilizaron dos regímenes diferentes de riego durante un período de tres meses. El estrés hídrico disminuyó el contenido total de clorofila en las plantas, pero aumentó el contenido de carotenoides. Curiosamente, no se observó ningún cambio en el rendimiento cuántico del fotosistema PSII (F_v/F_m). El contenido de malondialdehído (MDA) aumentó en mayor medida en ambas especies. Se observe una actividad mejorada en todas las enzimas (peroxidasa, catalasa y superóxido dismutasa), excepto la catalasa en las raíces. Los contenidos de prolina y azúcares solubles aumentaron significativamente después de la exposición al estrés hídrico. No se encontraron diferencias claras entre ambas especies. Los resultados relacionan la tolerancia a la sequía de *D. commutata* y *C. ciliaris* con mejores capacidades del sistema antioxidante, así como también con la acumulación de los osmoprotectores prolina y azúcares solubles.

Palabras clave adicionales: Carotenoides, enzimas antioxidantes, estrés hídrico, prolina, sólidos solubles

INTRODUCTION

Among environmental constraints, water stress is a significant threat to the plants. Today, water scarcity is listed as a serious global problem jeopardizing the aim to achieve sustainable agriculture. In the plant, water stress induces severe disturbances in the physiological processes, affecting vital functions and ultimately causing steep decline in agricultural output. Most evidently, water deficit is accompanied by growth retardation and inhibition of diverse metabolic processes including photosynthesis, respiration,

uptake/translocation of ions, and assimilation of nutrients. Water stress triggers a domino of molecular mechanisms, as it has been largely documented (Foyer and Noctor, 2004; Pirasteh-Anosheh et al., 2016; Fang et al., 2017). More research has pointed towards a link between oxidative stress and exposure to drought conditions (Manivannan et al., 2008; Filippou et al., 2014; Laxa et al., 2019), as evident from the buildup in the concentration of Reactive Oxygen Species (ROS) (OH^\cdot , O_2^\cdot , $^1\text{O}_2$, H_2O_2). These species can attack sensitive membranes and biomacromolecules causing damage at cellular level,

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protein oxidation, nucleic acid mutations, and lipid degeneration (Bian and Jiang, 2009; Choudhury et al., 2013; La et al., 2019). Water scarcity stress instigates oxidative pressure in view of restraint on photosynthetic action because of discrepancy between light captured and the amount used (Foyer and Noctor, 2004). Alterations are observed in functioning of chloroplasts in leaves of plants exposed to water stress. The consequence is the degeneracy of excess light captured leading to reactive oxygen species. Malondialdehyde (MDA), a by-product of degeneration of polyunsaturated fatty acids found in membranes, is considered a dependable indicator of oxidative stress (Demiral and Türkan, 2005). MDA is an important sign of membrane system injury. It is used as a biomarker of lipid peroxidation (Gawel et al., 2004; Moussa and Abdel-Aziz, 2008).

In order to cope with water stress plants have evolved numerous systems. These are mainly oxidative systems which are either enzymes based or are non-enzymatic. These systems can reduce oxidative damage caused to cells by scavenging ROS species. In all plants, mechanisms exist for detoxification of active oxygen species. These include stimulation of antioxidant enzymes e.g. superoxide dismutase (SOD) that catalyzes the O_2^- to H_2O_2 and O_2 conversion and catalase (CAT) which breaks down H_2O_2 into water, a process that can also be carried out by a number of other peroxidases (Miller et al., 2010). Antioxidants which are Non-enzymatic include numerous compounds such as ascorbate (AsA), nitrogenous metabolites, phenols and carotenoids (Milvia, 2013; Laxa et al., 2019). Additionally, they comprise amino acids, among which proline is a powerful antioxidant activity which is needed to reduce the damaging effects of ROS (An et al., 2013; Ejaz et al., 2020). Adaptation of osmotic pressures using buildup of sugars (Delatorre-Herrera et al., 2010; Zhang et al., 2014) and proline (An et al., 2013; Ejaz et al., 2020) is a common metabolic reaction of higher plants designed to maintain turgor pressure and avoid water stress.

Digitaria commutate and *Cenchrus ciliaris* are a perennial species, found mostly in warm regions of both hemispheres, growing in a wide range of habitats (Boukris and Chaieb, 1998). The goal of this study was to assess the antioxidant responses

of *D. commutate* and *C. ciliaris*, when faced with water stress, through study of physiological responses related to lipids peroxidation and different antioxidant system. Morphophysiological changes of those perennial species when subjected to water stress were previously reported (Amari et al., 2017).

MATERIALS AND METHODS

Characteristics of soil. For the purpose of this research, soil was collected from a depth of 0 – 20 cm depth in Gafsa, which is a city located in the south western region of Tunisia. Following attributes were found in soil sample: pH = 7.4; calcium ion ($244.69 \mu\text{equiv}\cdot\text{g}^{-1}\text{soil}$); potassium ion ($0.41 \mu\text{equiv}\cdot\text{g}^{-1}\text{soil}$); sodium ion ($1.21 \mu\text{equiv}\cdot\text{g}^{-1}\text{soil}$); conductivity of soil ($96.68 \mu\text{s}\cdot\text{cm}^{-1}$) and organic particles (0.87 %). For the study a sample of 5 kg of dried soil was placed in imperforated pot and watered intensively with tap water over a period of two days. Evaporation was mitigated through use of aluminum cover placed on pot. Soil field capacity was measured after desiccation at 105°C for 48 hours.

Plant material and culture conditions. Seed collection took place in vicinity of Bouhedma National Park (East of Tunis). Germination was conducted in pots filled with 2 kg of soil. 3 plants were placed in each pot. To study only water loss through leaf transpiration, aluminum foil was used for covering the pots four weeks into the experiment for preventing water loss via evaporation. As a control, 24 plants were subject to irrigation at 70 per cent of field capacity for 3 months. It was performed under natural sunlight and temperature to imitate conditions found in field.

Maximum efficiency of photosystem II. Measurements of mature leaves were recorded according to procedure outlined by Genty et al. (1989). Chlorophyll fluorescence measurements (6 replicates per treatment) were also recorded at the end of the experiment, before the last harvest. Measurements were taken at midday using a portable-modulated fluorimeter PAM-2000 (Walz, Germany). Leaves were dark-adapted for at least 20 min using leaf clips. Florescence level (F_0) was measured with 100 % PSII reaction centers open by employing modulated light low enough not to induce variance in fluorescence. Maximal

fluorescence level (F_m) was measured by 0.8 saturating pulses at $8000 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ in dark-adapted leaves. The values of F_0 and F_m were then employed for determining variable fluorescence ($F_v = F_m - F_0$) and maximum efficiency of photosystem II (F_v/F_m).

Chlorophyll content. Chlorophyll concentration of leaves of 6 replicates was measured through UV spectrophotometer employing the absorption spectra. Small pieces of fresh leaves weighing 300 mg were extracted and placed in 3 mL 80 % acetone. As recorded by Lichtenthaler and Welburn (1983), measurements were made for extract absorption at 470, 646.8 and 663.2 nm.

Lipid peroxidation estimation. A quick and easy method was deployed for the measurement of cellular oxidative stress. MDA production during the oxidation of polyunsaturated fatty acids was used as the basis of this method (Hodges et al., 1999). The technique accounts for the possibility of amalgamating the compounds in the thiobarbituric acid (TBA)-reactive substances assay. Fully expanded leaves were used for MDA contents. Ultrasonic cleaner was used to freeze dry samples extracted with 80-20 ethanol and water solution (Bransonic, Danbury, CT). 10-minute centrifugal treatment of homogenate at 15,000 g was employed. Following centrifuging, same solvent was used to re-extract pellet twice.

Analytical procedure of proline. For making measurements of proline, samples of frozen leaves and roots were used, homogenized in solution of 3 vol of 1 mM tridecafluoroheptanoic acid (TDFHA), 50 % (v/v) methanol. Dual treatment was rendered to the samples, while they were first shaken for 10 min at 4 °C, they were later centrifuged twice at 14,000 g for 20 min at 4 °C. 0.5 mM TDFHA, 25 % (v/v) methanol solution was used for dilution of underivatized supernatant. Agilent Technologies 1200 Series capillary pump along with aQ-TOF mass spectrometer was employed for LC-ESI-MS analysis, a technique already employed by Armstrong et al. (2007).

Soluble sugars content. Dissolvable sugars content was measured using Sturm et al. (2003) technique with some small adjustment utilizing liquid chromatography (HPLC) (Agilent Technologies 1200 arrangement) coupled with refractive index detector RID and Zorbax Carbohydrate 5 μm segment (4.6×250 mm). Test conditions: blend of mobile phase of acetonitrile

with deionized water in proportion 65: 35 (v/v); consistent rate for stream of $0.8 \text{ mL}\cdot\text{min}^{-1}$; temperature of 30 °C was maintained. Samples of dried gel were set up by adding 2 mL of deionized water and blending for 1 min.

Enzyme assays. Complete superoxide dismutase (EC 1.11.1.5) activity and associated activities were accessed according to Scebbba et al. (1999). Gradually increased volumes (5, 10, 20, and 40 μL) of extracts of root and leaf were added to the mixture to achieve final volume of 3 mL. The composition of reaction mixture was 50 mM potassium phosphate buffer (pH 7.8), 0.1 mM EDTA, 13 $\text{mM}\cdot\text{L}^{-1}$ methionine, 2 μM riboflavin and 75 μM NBT (nitroblue tetrazolium). Technique employed by Luck (1965) was used for measurement of the total catalase (EC 1.11.1.6) activity. As H_2O_2 was being consumed, a decline in absorbance was measured against a plant extract-free blank. The 3 mL mixture for the experiment contained 66 mM sodium phosphate buffer (pH 7.0), to which 30 % (w/v) H_2O_2 was added (optical density of 0.5 at 240 nm was achieved with a 1 cm light path). A diluted leaf extract was added to the solution to start the reaction.

The complete ascorbate peroxidase (EC 1.11.1.11) movement was estimated spectrophotometrically as indicated by Nakano and Asada (1981) by following the decrease in absorption at 290 nm as solution was oxidized ($R = 2.8 \text{ mM}^{-1}\cdot\text{cm}^{-1}$). The pace of oxidation was assessed somewhere in the range of 1 and 60 s in the wake of beginning the response with the expansion of H_2O_2 . The 1 mL reaction fluid contained 50 mM Hepes-NaOH (pH 7.6), 0.22 mM ascorbate and 1 mM H_2O_2 . The control reaction blend was set up without the enzyme. Amendments were made for the low, non-enzymatic oxidation of ascorbate by H_2O_2 and for the oxidation of ascorbate without H_2O_2 . The activity was expressed in terms of units (μmol of oxidized ascorbate per min) per mg of protein. The total guaiacol peroxidase activity was determined following the increase in absorption at 470 nm by adding the enzyme solution to 2 mL of guaiacol (0.5 %) and hydrogen peroxide (9 mM) in K-phosphate buffer (50 mM, pH 7.0) (Fielding and Hall, 1978).

Statistical analysis. The techniques of ANOVA, coupled with orthogonal contrasts and mean comparison was employed to study differences

between different treatments. Duncan's multiple range tests were employed for mean separation procedures.

RESULTS

Maximum efficiency of PSII. No significant changes were seen in the maximal quantum yield of PSII photochemistry (F_v/F_m) measured in leaves adapted for dark in either *Cenchrus ciliaris* or *Digitaria commutata* water-stressed plants (Figure 1). Almost constant F_v/F_m ratio was observed in both species, the figure was close to 0.80.

Pigment content. Exposure to water stress was observed to decrease total chlorophyll content in both plants (Figure 2A). The percentage reduction in chlorophyll content observed in both species reached *ca.* 28 and 23.4 % in comparison to control, respectively. However, on the other end, water scarcity led to an increase of carotenoid content in plants (Figure 2B) in comparison to the controls, the increase recorded was around 50 % in both *Cenchrus ciliaris* and *Digitaria commutata*.

Lipid peroxidation (MDA content) A nominal increase in lipid peroxidation was noticed in leaves of both species, and a significant difference was observed in MDA content between stressed and control plants in *C. ciliaris* (Figure 3).

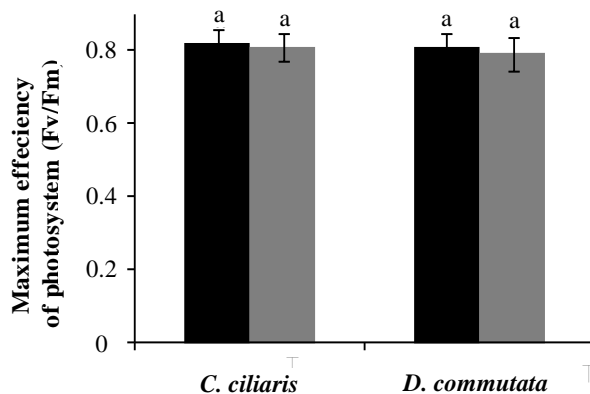


Figure 1. Means of maximal quantum yield of photosystem II (F_v/F_m) in *Cenchrus ciliaris* and *Digitaria commutata* leaves of control (black bars) and water stressed plants (gray bars). $n = 6$; bars indicate SE. Equal letters indicate no significant differences (Duncan test, $P > 0.05$)

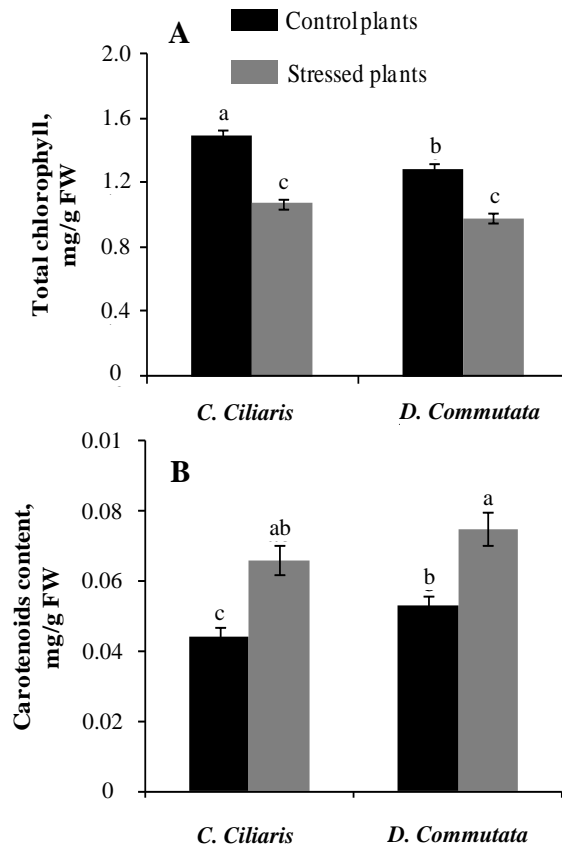


Figure 2. Means of total chlorophyll (A) and carotenoids (B) contents in leaves of *Cenchrus ciliaris* and *Digitaria commutata* subjected to water stress. $n = 6$; bars indicate SE. Different letters indicate significant differences (Duncan test, $P \leq 0.05$)

Leaf antioxidant enzymes activities. Responses of antioxidant enzyme activities in leaves of *C. ciliaris* and *D. commutata* seedlings are summarized in Table 1. Data shows the effects of irrigation treatments on CAT, APX and GPX activities. The activities of these enzymes in water stress were higher than control. Activity of CAT in both species increased significantly compared with control plants. CAT activity values recorded under water deficit were 37 and 34 % higher than those of the control, for *C. ciliaris* and *D. commutata*, respectively. In treated plant, the ascorbate and guaiacol peroxidase activities also increased significantly in leaves and roots of both species. In contrast, for both species, the superoxide dismutase activity remained almost unchanged in water-stressed plants.

Proline and soluble sugars content accumulation. Exposure to water stress augmented proline and sugars accumulation in leaves of *Cenchrus ciliaris* and *Digitaria commutata*. Proline level increased significantly in response to water deficit (Figure 4A), and there was also a significant accumulation of soluble sugars in leaf tissue of both species (Figure 4B).

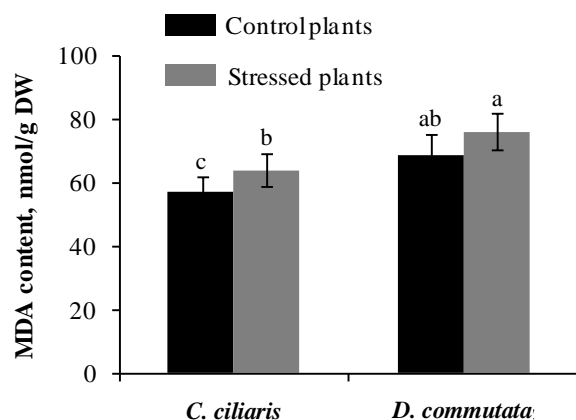


Figure 3. Means of Malondialdehyde (MDA) content on leaves of *Cenchrus ciliaris* and *Digitaria commutata* subjected to water stress. n = 6; bars indicate SE. Different letters indicate significant differences (Duncan test, $P \leq 0.05$)

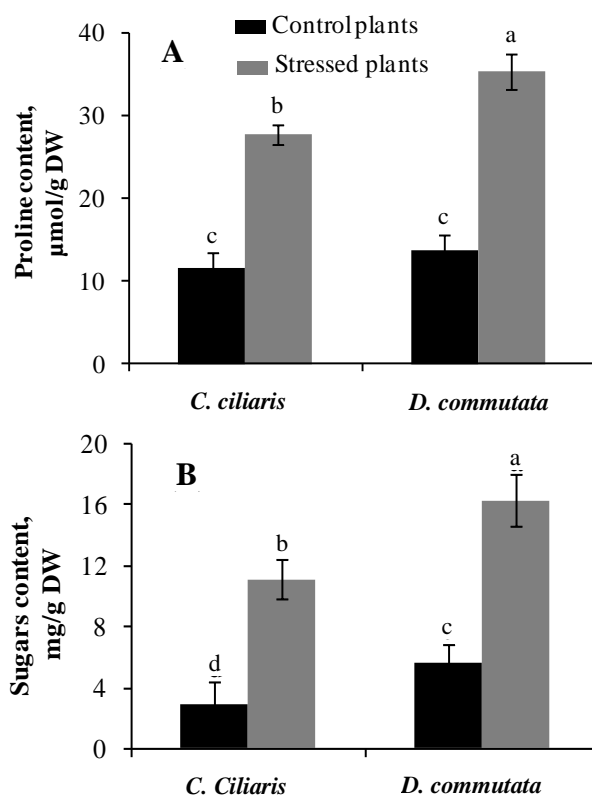


Figure 4. Means of proline (A) and total soluble sugars content (B) in leaves of *Cenchrus ciliaris* and *Digitaria commutata* subjected to water stress. n = 6; bars indicate SE. Different letters indicate significant differences (Duncan test, $P \leq 0.05$)

Table 1. Catalase (CAT), Superoxide dismutase (SOD), Guaiacol peroxidase (GPX) and Ascorbate peroxidase (APX) activity of *Cenchrus ciliaris* and *Digitaria commutata* plants in response to water stress

Treatment	Leaves			
	CAT (U·mg ⁻¹ protein)	APX (U protein·min ⁻¹)	SOD (U·mg ⁻¹ protein ⁻¹)	GPX (U·mg ⁻¹ protein)
<i>C. ciliaris</i>				
Well-watered	9.82 b	39.64 d	13.45 c	1.17 c
Stressed	13.45 a	64.72 b	27.39 a	2.19 b
<i>D. commutata</i>				
Well-watered	8.81 b	45.21c	14.67 c	2.38 b
Stressed	11.79 ab	69.73 a	24.75 b	3.76 a
Roots				
<i>C. ciliaris</i>				
Well-watered	4.12 a	17.81 b	41.3 b	0.97 d
Stressed	3.98 a	25.16 a	45.1 ab	3.23 a
<i>D. commutata</i>				
Well-watered	1.88 a	11.75 c	53.41 a	1.34 c
Stressed	1.96 a	19.85 ab	52.90 a	2.71 b

Different letters in each column for each plant organ indicate significant difference according to Duncan's multiple range test ($P \leq 0.05$)

DISCUSSION

Total chlorophyll content of *C. ciliaris* and *D. commutata* was observed to decrease as a result of water scarcity (Figure 1). According to literature, the chlorophyll reduction could be due to altered chlorophyll metabolism or induced disintegration of the chloroplast molecules and the complex molecules of pigment-protein (Singh and Dubey, 1995). Yet, this does not seem to be the case in *C. ciliaris* and *D. commutata*. In spite of the fact that the reduction seen for chlorophyll content was generally significant, both plant species demonstrated a better ability to preserve the PSII functional integrity when tested against water deficiency. The efficiency of light utilization of PSII was observed to be unhinged by water stress-exposure since F_v/F_m ratio remained consistent over the course of treatment (Figure 2). The maximum efficiency of photosystem II was 0.80, in line with levels observed in healthy plants (Maxwell and Johnson, 2000). Additionally, higher MDA content of water-stressed plants of both species, compared to control (Figure 3), demonstrated good ability of these species to preserve their membranes, especially chloroplast lipids, when exposed to water stress. Occurrence of complex membrane systems in chloroplasts rich in polyunsaturated fatty acids is a well-known fact. Hence these membranes are potential targets for ROS for peroxidation (Halliwell and Gutteridge, 1999).

With respect to chlorophyll concentration, the reduction could be due to altered chlorophyll metabolism. The lower pigment concentrations may be also due to the stress effect related cellular disorganization. Interestingly, chlorophyll loss was also described in some plants as a regulatory mechanism to reduce the amount of photons absorbed by leaves, conferring better photoprotection under stress.

Concerning the PSII integrity, plants develop a wide range of defense mechanisms involving enzymatic and non-enzymatic antioxidant systems, which can alleviate cellular oxidative damages. Several antioxidants protect higher plant cells from oxidative stress damage. They play crucial roles in scavenging ROS in the different cell compartments and in response to stress conditions. This supports their possible

implication in preserve the PSII functional integrity when plants challenged with water stress.

Several mechanisms could contribute to crop drought tolerance. Among them, the enzymatic antioxidant system is an important defense system co-evolved with aerobic metabolism as a retort to water scarcity induced stress to counteract the ROS oxidative consequences. Indeed, enzymes which are antioxidant in nature such as, superoxide dismutase (SOD), guaiacol peroxidase (GPX), ascorbate peroxidase (APX) and catalase (CAT) are notably implicated in the ROS-scavenging and contribute towards the regulation of oxidation-reduction balance within the cells (Yin et al., 2008). For instance, SOD is a key enzyme which plays a crucial role in oxidative stress (Halliwell and Gutteridge, 1999). Through the regulation of superoxide levels, SOD plays a critical role in saving cells against oxidative damage, as they act as precursor for highly reactive oxygen derivatives, such as peroxy nitrite or hydroxyl radical (Halliwell and Gutteridge, 1999).

In this study, exposure to water stress was observed to cause a significant increase in SOD activity in both species under examination (Table 1). Previous studies have observed a similar trend (Miller et al., 2010). CAT is a crucial antioxidant enzyme which plays a part in conversion of H_2O_2 to water and molecular oxygen (Arora et al., 2002). This is achieved through β -oxidation of H_2O_2 produced as a by-product of fatty acids and photorespiration (Nayyar and Gupta, 2006). Our results have shown an increase of the catalase activity in leaves of *C. ciliaris* and *D. commutata*, but it remained almost constant in the roots. Similar to our findings, increased CAT in *Triticum aestivum* plant subjected to water stress (Nayyar and Gupta, 2006) was found. Peroxidases constitute a family of enzymes (hemic) found in higher plants, facilitating various processes, such as lignification, linking of cell wall and auxin metabolism (Moussa and Abdel-Aziz, 2008). Therefore, SOD plays the main role in metabolic activity during growth alterations and abiotic stress. An increase in activity of APX and GPX was detected in leaves and roots of both species. Similar results were reported by Nayyar and Gupta (2006). However, the response seems to be related to the kind of stress. In a recent study, it

was found that toxicity stress in rice caused lower activity of CAT, SOD, APX and GPX, although MDA showed higher content (Boorboori et al., 2020).

Increased level of activity of SOD and CAT might help plants in enduring extreme conditions of water scarcity. For example, increased CAT and APX activities reduced H₂O₂ level in cell and improved membranes stability and facilitated process of CO₂ fixation because numerous enzymes involved in Calvin cycle in chloroplasts are very sensitive to H₂O₂ (Yamazaki et al., 2003). Under the extreme conditions of water stress, plants also synthesize solutes such as, proline and soluble sugars which helps them in drought resistance (Delatorre et al., 2010; An et al., 2013; Filippou et al., 2014; Ejaz et al., 2020). They are potent osmoprotectants that help to establish suitable osmotic pressure through involvement in osmotic adjustment which protects various sensitive cellular components and mitigates oxidation inside the cells by maintaining the cell turgor pressure (Silva et al. 2014). It has been suggested that compatible solutes do not affect normal biochemical reactions taking place in a cell and act as osmoprotectants during stress. Proline is one such osmolyte which is accumulated in water-stressed plants (Balibrea et al., 1999; Zhang et al., 2014). In this study, it was observed that the amount of proline and soluble sugars increased significantly in plants under water stress (Figure 4A-B). Delatorre-Herrera et al. (2010) and Hazrati et al. (2017) observed similar patterns and demonstrated that increased synthesis of these compounds, improves the efficiency of water usage in. Furthermore, the studies also suggested that proline and soluble sugars enable plants to overcome drought-induced stress through osmotic adjustment (Delatorre-Herrera et al., 2010; Filippou et al., 2014; Hazrati et al., 2017).

Plants are also known to collect big amounts of potassium, a chief solute related to cellular osmotic water absorption and the preservation of cellular turgor (Hsiao and Läuchli, 1986). Likewise, a critical defensive function of carotenoids, a lipophilic pigment in chloroplasts, has been anticipated in the photosynthetic organism under stress conditions (Miller et al., 1996). Even though they're accessory pigment,

carotenoids play a pivotal role in protective photochemical procedures and sustaining them (Asada, 1994). A primary defensive role of carotenoids in photosynthetic tissue can be via direct quenching of triplet chlorophyll, which prevents the generation of singlet oxygen and protects from oxidative damage (Mozzo et al., 2008). This study confirmed that leaf carotenoid content can expanded in stressed plant of *C. ciliaris* and *D. commutata* (Figure 2b). Those findings propose the viable implication of carotenoids in plant protection in opposition to drought.

CONCLUSION

Water stress decreased total chlorophyll content in plants of *C. ciliaris* and *D. commutata*. Interestingly, these species demonstrated a higher ability to preserve the PSII functional integrity when exposed to water stress. Our results show that increased SOD activity synced with changes in CAT and POD activities plays a critical defensive role in the ROS-scavenging process. The active participation of these enzymes is linked, at least in part, to drought-induced oxidative stress tolerance in *C. ciliaris* and *D. commutata* plants. The enhanced carotenoids, proline and soluble sugars accumulation in tissues support their possible implication in the osmotic adjustment which can help the plant to overcome drought stress.

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