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Physio-biochemical analysis reveals drought stress tolerance mechanism in Proso millet (*Panicum miliaceum* L.)

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Abstract
Proso millet (*Panicum miliaceum* L.) is one of the important millet crops grown in India and other parts of the world. Proso millet withstands drought by its short life cycle and high water use efficiency mechanism. During drought conditions, some of the osmolytes are accumulated in plants to acclimate the stress. To understand the drought stress response in proso millet, studies were conducted on the activities of antioxidant enzymes ascorbate peroxidase (APX) and peroxidase (POX) in addition to proline, malondialdehyde (MDA) and total soluble sugars. The drought induced plants showed elevated levels of these antioxidant enzymes and metabolites. Also, a decrease in chlorophyll content, relative water content (RWC) and chlorophyll stability index (CSI) was observed under stress conditions. Water saturation deficit showed the indirect relationship with RWC. These results provide a base for isolation and characterization of potential drought-related candidate genes or quantitative traits from proso millet. This can be further used for molecular breeding and/or engineering programs for improving abiotic stress tolerance in crops.

Keywords: Proso millet, antioxidant enzymes, chlorophyll content, drought stress

1. Introduction
Drought stress is the most devastating environmental stress among the abiotic stresses and it decreases the yield upto 50-60% in major crops (Shinozaki *et al*., 2015) [33]. It is multidimensional complex stress that affects the morphological, physiological, biochemical and molecular states of plants that influence their growth and development in terms of both quality and crop yield. This is exacerbated by changes in rainfall patterns, limited water resources and rapid changes in global climatic circumstances (Fahad *et al*., 2017; Seleiman *et al*., 2021) [8, 32]. It is a major limitation of agricultural production. With the increase in world population and global warming, drought stress will aggravate in the future due to limited water resources. Therefore, to improve agricultural production, the drought tolerance mechanism must be understood. To overcome abiotic stresses, plants have developed several genetic and molecular mechanisms. Under stress conditions, plants produce regulatory compounds such as mannitol, proline and a large number of soluble oligosaccharides (trehalose, raffinose and stachyose) to protect the cell against stresses (Cuin and Shabala, 2008) [7]. However, its complex drought-responsive mechanism remains unclear (Zhang *et al*., 2019) [40].
Proso millet (*Panicum miliaceum*), alternatively known as the common millet, white millet, broom corn millet, hog millet or Hershey millet is an important minor millet crop which is rich in protein than other cereals and millets. It is a short duration millet widely grown in India which is used for both food and feed purpose. It is a self-pollinated (sometimes cross-pollination may exceed upto 10%) allotetraploid plant (2n=4x=36) that comes under Panicoideae subfamily with an estimated genome size of 1020.5 Mbp (Kubešová *et al*., 2010) [17]. It is rich in amino acids and has anti-cancerous properties (Tadele, 2016) [35]. Proso millet performs C_4 type of photosynthesis, most of the C_4 plants are efficient in carbon fixation and the use of water and nitrogen compared to their C_3 relatives (Vitkauskaitė G and Venskaitytė L., 2011) [37]. It is adapted to hot summers in tropics and high altitudes, where the growing season is short and poor marginal soil is present. Among grain crops, the common millet has the lowest water and nutrient requirement. It can grow on any kind of soil except course sand. This crop can bypass the drought by its quick maturity. Among all cultivated cereals, proso millet has the highest water use efficiency (WUE), because of its low respiration rate, short life cycle (60-90 days) and harvestable index (Hunt *et al*., 2014; Zhang *et al*., 2021; Kumar *et al*., 2021) [14, 39, 18]. With this backdrop, the present study was performed to analyse the physiological and biochemical parameters to understand the drought stress mechanism in proso millet at vegetative and reproductive stages.

2. Materials and Methods

2.1 Plant material

Proso millet seeds [*Panicum miliaceum* L. variety CO (PV) 5] were collected from the Department of millets, Tamil Nadu Agricultural University, Coimbatore (11.0231° N, 76.9286° E). Plants were grown in plastic pots containing potting mixture red soil: sand: compost in 2:1:1 ratio and maintained in greenhouse conditions. Before sowing, the seeds were incubated in a hot air oven at 37^0 C for 2 days for improve germination. Drought stress study was conducted by withholding water in 3-weeks and 7-weeks old proso millet plants for 0, 2, 4, 6, 8 and 10 days respectively (T0, T1, T2, T3, T4 and T5). For each treatment, three replicate samples were collected in control and water stressed plants. Physiological and biochemical parameters such as relative water content (RWC), water saturation deficit (WSD), chlorophyll stability index (CSI), chlorophyll content, proline content, malondialdehyde (MDA), total soluble sugars (TSS) were carried out. And also, antioxidant enzyme activities such as peroxidase (POX) and ascorbate peroxidase (APX) were done.

2.2 Physio-biochemical analysis of drought stress tolerance

2.2.1 Chlorophyll content

Chlorophyll content from both control and stressed plant samples were estimated by using the method of Arnon, 1949 [3]. About 250 mg of leaf material was homogenised with 10 ml of 80% (v/v) acetone using pestle and mortar. The extract was centrifuged at 3000 rpm for 15 min and the supernatant was transferred to a 25 ml volumetric flask. The total volume was made up to 25 ml using 80% (v/v) acetone. The clear solution was transferred to a cuvette and optical density was measured at 645 nm and 663 nm against a blank (80% (v/v) acetone).

Chlorophyll 'a' = [(12.7 x A663-2.69 x A645) x V / (W in g x 1000)]
Chlorophyll 'b' = [(22.9 x A645-4.48 x A663) x V / (W in g x 1000)]
Total chlorophyll = [(20.2 x A645+8.02 x A663) x V / (W in g x 1000)]

Where, V denotes the volume taken for chlorophyll content estimation; W denotes weight of sample taken for analysis.

2.2.2 Relative water content (RWC)

Leaf samples were collected from control and drought stressed plants at different days interval. The FW (FW) of each leaf sample was measured. After that leaf samples were immersed in double-distilled water for 4 h of incubation at room temperature. Then the samples were taken out and wiped with tissue to remove water to take the turgid weight (TW). Finally, the dry weight (DW) was measured after 48 h of incubation of leaves in a hot air oven at 70°C. The relative water content was calculated using the formula:

RWC (%) = [(FW-DW) / (TW-DW)] ×100

The water saturation deficit (WSD) was calculated using the formula,

WSD= 100-RWC (%)

2.2.3 Chlorophyll stability index (CSI)

Chlorophyll stability index was measured based on protocol described by Kaloyereas 1958 [15] and it was expressed in percentage (%).

$$\text{Chlorophyll stability index (\%)} = \frac{\text{Total chlorophyll content (Treated)}}{\text{Total chlorophyll content (Control)}} \times 100$$

2.2.4 Proline

Estimation of proline content was carried out according to Bates *et al*., 1973 [6]. Leaf samples (250 mg) from each stage were homogenized in 3% (w/v) sulphosalicylic acid and the homogenate was filtered through what man filter paper. After the addition of Orthophosphoric acid, glacial acetic acid and acid ninhydrin to the filtrate, the resultant mixture was heated for 1h at 100°C in the heating mantle. The reaction was then stopped by kept in an ice bath. The mixture was extracted with toluene, and the absorbance of the fraction with toluene accomplished from the liquid phase was read at 520 nm. Proline concentration was determined using a graph and expressed as µmol proline g^{-1}.

$$\text{Proline} = \frac{\text{g proline/ml} \times \text{ml tolune} \times 5}{115.5\ \text{/mole} \times \text{g sample}}\ \mu\text{moles g}^{-1}$$

2.2.5 Total soluble sugars

Estimation of total soluble sugars (TSS) was carried out based on Yemm and Willis, (1954) [38] method. About 250 mg of control and drought treated leaf samples were ground with 10 ml of 80% ethanol and centrifuged at 4000 rpm for 20 minutes. The pellet was then re-extracted with 10 ml of 80% ethanol. The supernatant was mixed and the test tube was incubated at 60° C for dryness and allowed to cool at ambient temperature. One ml of distilled water was added and mixed well. To each tube, 4 ml of anthrone reagent was added and kept in a waterbath at boiling temperature for 10 minutes. It was then cooled down and the OD was measured at 630 nm. Total soluble sugars were calculated based on the standard curve prepared from graded concentration of glucose and expressed as mg g^{-1} of FW.

2.2.6 Malondialdehyde content (MDA)

The rates of lipid peroxidation levels in control and stressed plant leaves were determined by the method of Heath and Packer, 1968 [13] by measuring the amount of its ability to inhibit the photochemical malondialdehyde (MDA) formed by the thiobarbituric reduction. Leaves were ground with mortar and pestle in 1% TCA and centrifuged at 10000 rpm for 10 minutes at room temperature. To the 1 ml of supernatant add 4 ml of 20% TBA-TCA solution. The mixture was heated at boiling temperature for 30 min. Absorbance was measured at 532 nm and corrected for unspecific turbidity by subtracting the value at 600 nm. The blank contained 20% TBA-TCA solution. MDA content was calculated using an extinction coefficient of 155 mM^{-1} cm^1 and the results were expressed as µmol MDA g^{-1} of FW.

2.3 Antioxidant enzyme activity

2.3.1 Peroxidase (POX)

Peroxidase activity was determined as described by Pütter, (1974) [29]. 250 mg of leaf samples were homogenized with 10 ml of phosphate buffer and the contents were centrifuged at 4000 rpm for 20 minutes. In a test tube, 1 ml of supernatant

was taken and 3 ml of pyrogallol was added. This acts as a blank and to this content H_2O_2 (substrate) was added and OD measured at 430 nm for 2 minutes. The results are expressed as µmol min^{-1} g^{-1} FW.

2.3.2 Ascorbate peroxidase (APX)
Ascorbate peroxidase activity was determined as described by Nakano and Asada (1981) [26]. All enzyme activities were expressed as µmol of H_2O_2 min^{-1}.

2.4 Statistical analysis
The physiological and biochemical analysis was performed in a completely randomized design. The results were interpreted as mean ± S.E. the enzyme activity was studied using one-way analysis of variance (ANOVA). Duncan's multiple range test (DMRT) was performed using WASP computer software version 2.0.

3. Results and Discussion
Climate changes increase the rate of occurrence of abiotic stress conditions in the crops and this would reduce the yield and quality of food crops. The development of stress-tolerant crops is necessary to overcome these problems in future (Zougmoré, 2018) [41]. Proso millet is one of the well suited crop under drought which consumes less water than other cereals. It is more drought-tolerant and nutritious than other cereals. Physiological and biochemical parameters *viz.*, chlorophyll content, chlorophyll stability index (CSI) and relative water content (RWC) were significantly reduced by drought stress at both vegetative and reproductive stages of the crop whereas proline, total soluble sugars (TSS) and malondialdehyde (MDA) contents were significantly increased by the drought stress at both stages when compared to control plants. These change in biochemical parameters exhibit adaptive mechanism of the plant under drought stress situations.

3.1 Physiological analysis of drought stress tolerance
In the present study, water deficit stress was established by with-holding water during vegetative and reproductive stages. As the level of drought increased, chlorophyll content gradually decreased. The decline in chlorophyll content was probably coupled with the oxidative stress that causes the injury in the membrane of the thylakoids that leads to chlorophyll degradation (Samuel *et al*., 2015) [31]. In the present, the chlorophyll a, chlorophyll b and total chlorophyll content in vegetative stage shows respectively 1.5, 1.6 and 1.4 fold reduction compared to control. The contents of the above decreased 1.4, 1.5 and 1.76 times in reproductive stage (Table 1). There was an inverse relationship between drought stress and chlorophyll content. In rice, decreased chlorophyll a and chlorophyll a/b ratio under drought conditions was reported, by Maisura *et al*., (2014) [20]. Decreased chlorophyll a, b and total chlorophyll content was observed in our study as that of previous studies.

Drought stress significantly reduces the relative water content in plants. Under control conditions, all plants show a higher RWC. But increased water saturation deficit leads to greater degrees of plants drought stress. In our study, under control condition RWC ranges from 84 to 92% whereas in drought stressed condition RWC ranges from 81% to 92% (Fig. 1). From the two stages studied, leaf RWC content reduction was higher in vegetative stage followed by reproductive stage. At the same time, water saturation deficit of vegetative and reproductive stage drought stressed plants showed the increase of 15.4% and 41.5% respectively (Fig.2). Tasmina *et al*., 2016 [36] reported that under well watered condition RWC content was higher and under stressed condition water saturation deficit (WSD) was higher. Maize plants under drought stress condition showed decrease in RWC and chlorophyll content, after rewatering the plants growth and development was rapidly regained (Goodarzian *et al*., 2015) [10]. Also, moderate level of decrease in RWC and chlorophyll content was observed in our study as that of previous reports by Nadeem *et al*., (2020) [24].

The chlorophyll stability index is an important factor that reflects the ability of the plants to sustain photosynthesis under drought conditions. Chlorophyll stability index showed a 13.5% and 15% decrease in the vegetative and reproductive stages of stressed plants, respectively (Fig. 3). Sampath Kumar *et al*., 2014 [30] reported that increases drought stress leads to a reduction in CSI in cotton and maize. Higher the chlorophyll stability index irrespective of chlorophyll content is the more dependent parameter for water-deficit stress tolerance (Nahakpam, 2017) [25]. As that of earlier reports, proso millet shows a significant decrease in stressed plants.

Table 1: Effect of drought stress on the chlorophyll content in proso millet leaves at vegetative and reproductive stages Chlorophyll content

Treatment	Chlorophyll a (mg g^{-1} of fresh tissue)				Chlorophyll b (mg g^{-1} of fresh tissue)				Total chlorophyll (mg g^{-1} of fresh tissue)			
	Developmental stage				Developmental stage				Developmental stage			
	Vegetative		Reproductive		Vegetative		Reproductive		Vegetative		Reproductive	
	Control	Stress	Control	Stress	Control	Stress	Control	Stress	Control	Stress	Control	Stress
T0	2.21±0.07	2.20±0.12^{a}	1.71±0.03	1.72±0.05^{a}	0.85±0.05	0.82±0.04^{a}	0.65±0.02	0.65±0.02^{a}	3.01±0.13	3.03±0.16^{a}	2.37±0.02	2.34±0.08^{a}
T1	2.28±0.05	2.17±0.12^{a}	1.73±0.03	1.73±0.03^{a}	0.79±0.04	0.75±0.04^{a}	0.64±0.03	0.59±0.02^{b}	2.96±0.13	2.92±0.15^{ab}	2.33±0.02	2.26±0.07^{a}
T2	2.24±0.03	2.01±0.11^{ab}	1.70±0.03	1.61±0.02^{b}	0.80±0.03	0.61±0.03^{b}	0.59±0.04	0.48±0.01^{c}	2.87±0.10	2.61±0.13^{bc}	2.34±0.05	2.02±0.06^{b}
T3	2.20±0.07	1.85±0.10^{b}	1.73±0.04	1.48±0.02^{c}	0.84±0.02	0.51±0.03^{bc}	0.62±0.03	0.39±0.01^{d}	2.86±0.15	2.35±0.12^{cd}	2.33±0.08	1.82±0.06^{bc}
T4	2.17±0.01	1.72±0.09^{bc}	1.71±0.07	1.38±0.01^{d}	0.86±0.06	0.49±0.02^{c}	0.63±0.02	0.39±0.01^{d}	2.82±0.18	2.21±0.11^{d}	2.38±0.06	1.71±0.05^{cd}
T5	2.28±0.02	1.50±0.08^{c}	1.72±0.02	1.25±0.04^{e}	0.84±0.03	0.52±0.03^{c}	0.63±0.03	0.41±0.01^{d}	2.81±0.17	2.02±0.10^{d}	2.42±0.02	1.56±0.05^{d}

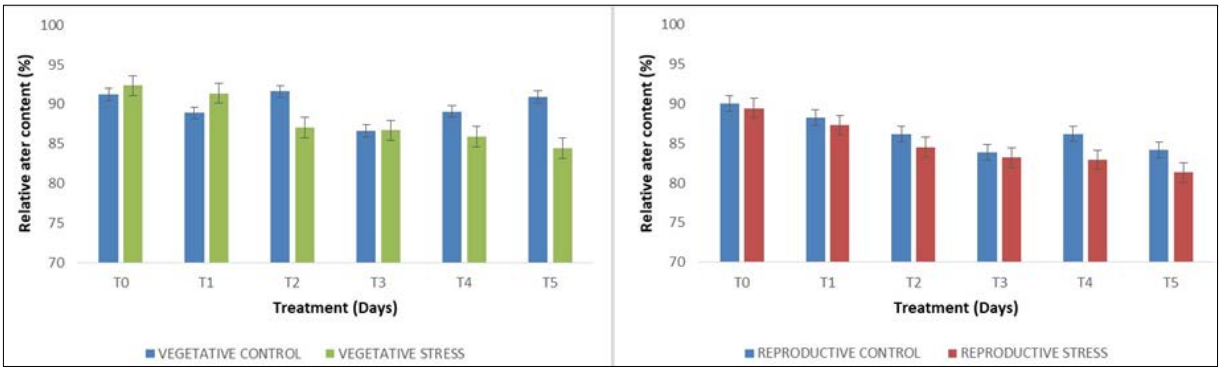

Fig 1: Effect of drought stress on relative water content in proso millet leaves at vegetative and reproductive stages. Data are presented as mean ± standard error (n=6).

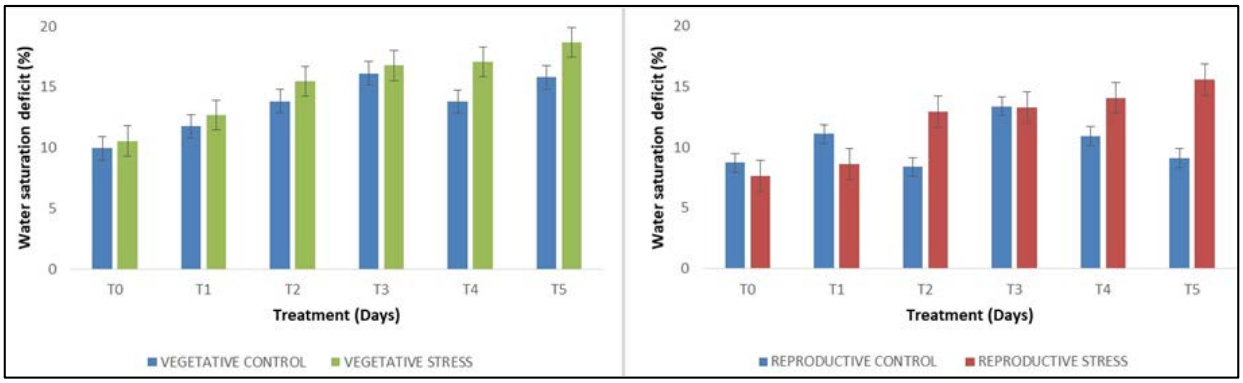

Fig 2: Effect of drought stress on water saturation deficit in proso millet leaves at vegetative and reproductive stages. Data are presented as mean ± standard error (n=6).

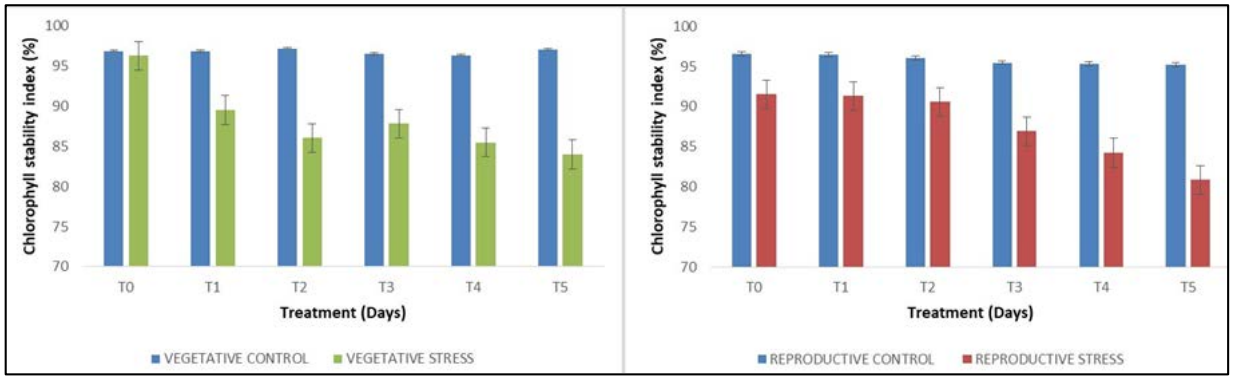

Fig 3: Effect of drought stress on chlorophyll stability index in proso millet leaves at vegetative and reproductive stages. Data are presented as mean ± standard error (n=6)

Biochemical analysis of drought stress tolerance

The drought tolerance mechanism can be triggered by the increasing level of antioxidant enzymes and osmolytes in stressed plants. This will reduce the adverse effect caused by the drought stress and works as an osmoprotectant (Gurumurthy *et al*., 2019) [11]. Osmoprotectants are known compatible solutes that includes amino acids (e.g., proline), sugars (e.g., sucrose, trehalose, Raffinose), sugar alcohols (e.g., Myo-inositols) and betaines (e.g., glycine betaine).

Proline accumulation is the most important physiological index for the plant's response to drought stress and gives the energy for plants growth and survival under stress conditions. Proline serves as a metal chelator, an antioxidative defence molecule and a signalling molecule during stress, in addition to being an effective osmolyte (Hayat *et al*., 2012) [12]. The MDA content was derived from the peroxidation of fatty acids present in the membrane caused by the peroxy radicals (Kotchoni *et al*., 2006) [16]. It shows the regulatory activity on plant defence mechanism of gene regulation. Nematpour *et al*., 2019 [27] reported that drought stress increased the H_2O_2 and MDA content in proso millet. Results from lipid peroxidation show that the MDA content was increased concerning with the drought withhold period.

In present study, stress at vegetative stage showed increased level of proline and MDA content respectively 1.87 and 2.02 times. Reproductive stage showed 3.24 and 1.97 times increased content of proline and MDA (Fig. 4 & 5). A vast amount of evidence implies that proline accumulation and plant stress are linked. In our study proline content was increased with respect to drought period and growth stages, showing similar results of proline content of wheat and finger millet (Akhkha *et al*., 2011; Mude *et al*., 2020) [2, 22]. MDA accumulation is the best drought stress tolerant index which is induced by environment in chick pea (Farooq *et al*., 2018). From this results, it revealed that the proso millet responses to drought is depends on the water deficit period and growth

stages. Soluble sugars may function as a typical osmoprotectant, stabilizing cellular membranes and maintaining turgor pressure. Soluble sugar content increased during the drought, especially at the reproductive stage (Singh *et al*., 2015) [34]. In leaves, total soluble sugar (TSS) content was 2.0 and 2.5 times greater in vegetative stage and reproductive stage respectively (Fig. 6). These elevated level of TSS in the reproductive stage corroborates with the previous reports in fingermillet (Mundada *et al*, 2020) [23].

Plant protect themselves from drought-induced oxidative stress, through anti-oxidative enzymes *viz.,* peroxidase and ascorbate peroxidase. ROS balance is maintained by the antioxidant defence system developed in the plants during abiotc stress (Ahanger *et al*., 2017) [1]. Several reports state that increase in antioxidant enzyme activity has an increased tolerance to environmental stresses (Liu *et al*., 2011) [19]. Duration of water deficit condition increases the antioxidants activities in elevated levels than the control plants that were watered regularly (Mir *et al*., 2019) [21]. The higher activity of H_2O_2 metabolizing enzymes could efficiently remove the H_2O_2 produced excessively. The ascorbate peroxidase metabolic enzyme detoxifies the H_2O_2 playing a key role in the management of ROS during oxidative stress in plants (Noctor and Foyer, 1998) [28]. In vegetative stage, 1.82 and 3.0 fold increase of peroxidase and APX enzyme activity was observed in stressed plants. In reproductive stage, 2.32 and 2.1 times of above antioxidant enzymes activity (Fig. 7 and 8). A significant increase in POX and APX content was observed in drought-stressed plants compared to control plants. Overexpression of cytosolic APX in arabidopsis results in both salinity and drought stress tolerance (Badawi *et al*., 2004) [4]. From the experimental results, its clear that antioxidant enzymes activity increases due to the drought period as reported in other crops such as finger millet (Mude *et al*., 2020; Bartwal *et al*., 2016) [22, 5].

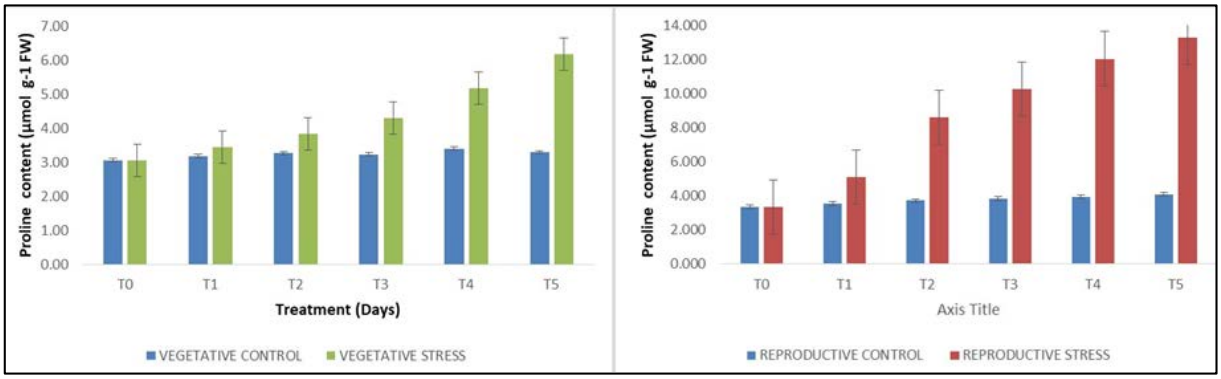

Fig 4: Effect of drought stress on proline content in proso millet leaves at vegetative and reproductive stages under stress. Data are presented as mean ± standard error.

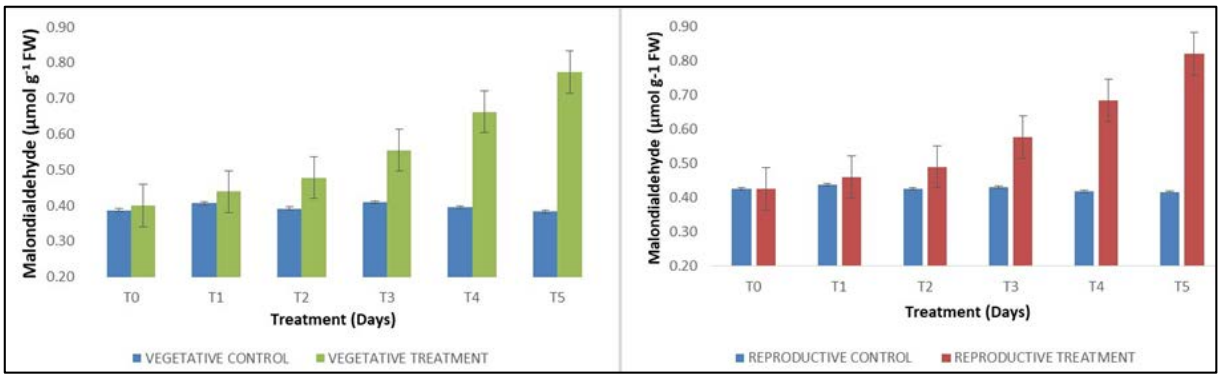

Fig 5: Effect of drought stress on malondialdehyde content in proso millet leaves at vegetative and reproductive stages under stress. Data are presented as mean ± standard error (n=6).

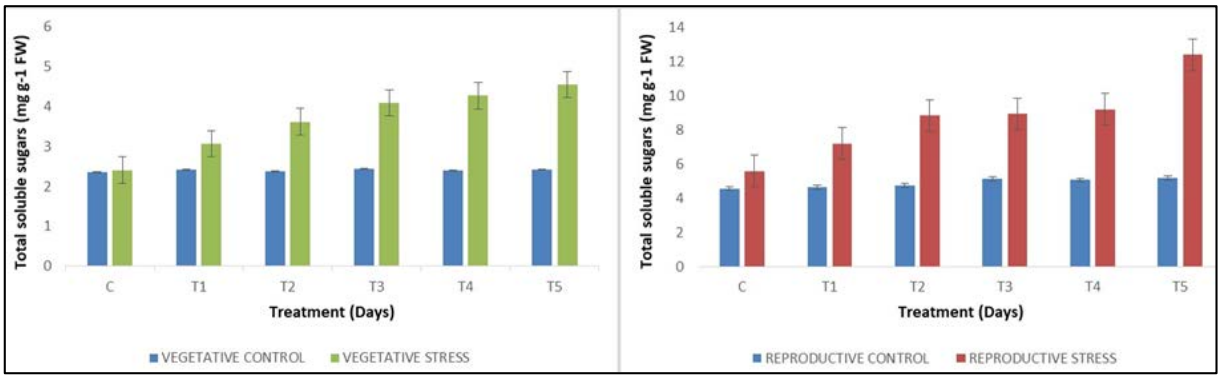

Fig 6: Effect of drought stress on of total soluble sugar content in proso millet leaves at vegetative and reproductive stages under stress. Data are presented as mean ± standard error (n=6).

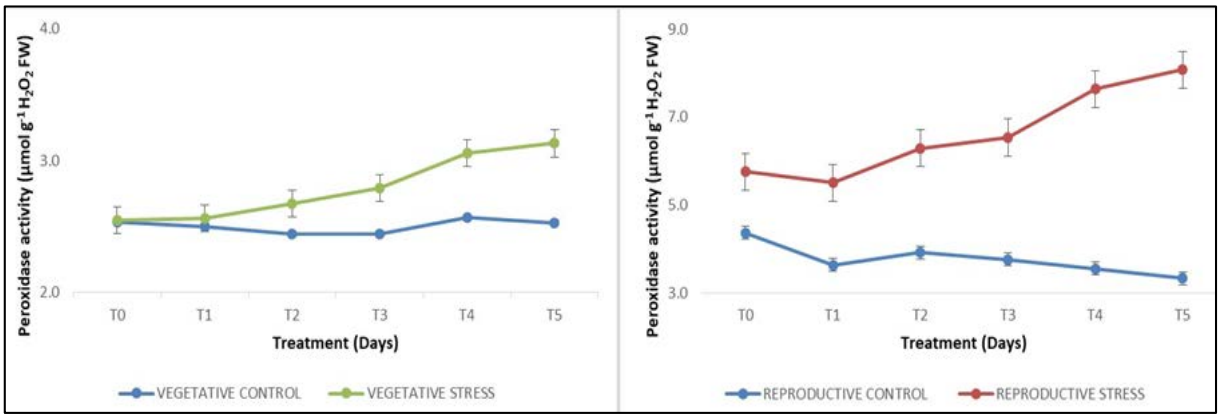

Fig 7: Effect of drought stress on peroxidase activity in proso millet leaves at vegetative and reproductive stages. Data are presented as mean ± standard error (n=6).

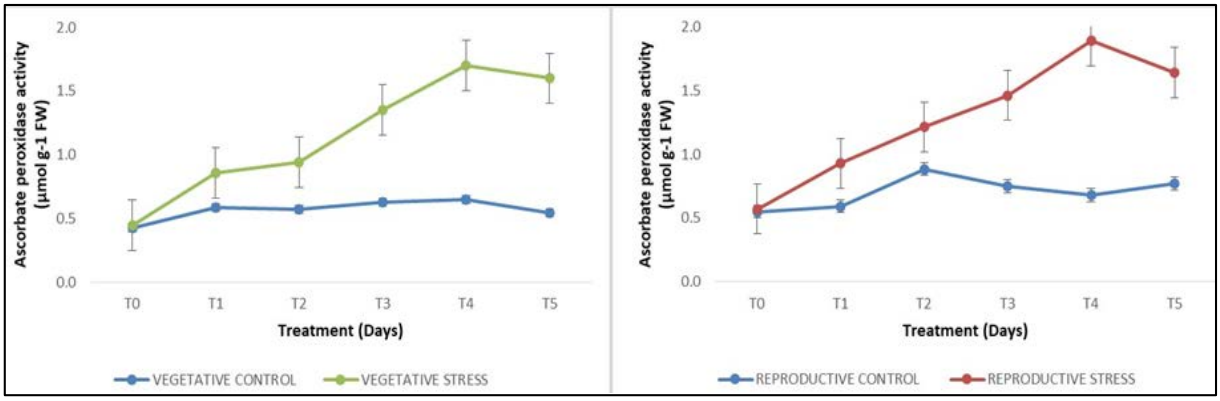

Fig 8: Effect of drought stress on ascorbate peroxidase activity in proso millet leaves at vegetative and reproductive stages. Data are presented as mean ± standard error (n=6).

4. Conclusion

Drought stress affects plants throughout their life cycle starting from germination to maturity. Plants have adapted many physiological, biochemical and molecular mechanisms to overcome, escape and/or tolerate drought stress. In our study various physio-biochemical analysis demonstrated the role of biochemical constituents involved in drought stress tolerance mechanism of proso millet. This analysis can be further validated by isolation and characterization of potential candidate genes involved in drought stress tolerance and can be further expressed to develop stress tolerance crops.

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