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Bipin KB
Department of Plant Biotechnology, Centre for Plant Molecular Biology and Biotechnology, TNAU, Coimbatore, Tamil Nadu, India

Uma D
Department of Biochemistry, Centre for Plant Molecular Biology and Biotechnology, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India

Rajesh S
Department of Plant Biotechnology, Centre for Plant Molecular Biology and Biotechnology, TNAU, Coimbatore, Tamil Nadu, India

Raghu R
Department of Plant Biotechnology, Centre for Plant Molecular Biology and Biotechnology, TNAU, Coimbatore, Tamil Nadu, India

Jeevanandhan K
Department of Plant Biotechnology, Centre for Plant Molecular Biology and Biotechnology, TNAU, Coimbatore, Tamil Nadu, India

Corresponding Author:
Uma D
Department of Biochemistry, Centre for Plant Molecular Biology and Biotechnology, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India

Salinity stress tolerance mechanism in Proso millet (*Panicum miliaceum* L.) revealed by physio-biochemical analysis under different salt concentration

Bipin KB, Uma D, Rajesh S, Raghu R and Jeevanandhan K

Abstract
Proso millet (*Panicum miliaceum* L.) is one of the commercially most important millet crop. Different concentrations (50mM, 100mM, 150mM and 200mM) of NaCl salt stress were imposed to proso millet plants in order to study the tolerance of proso millet towards salinity stress. To understand the response of proso millet plants towards the salinity stress, studies were conducted on the synthesis of proline, malondialdehyde (MDA) and total soluble sugars. Salt stress-imposed plants expressed increased level of these metabolites. In addition to this, decrease in chlorophyll content, relative water content (RWC) and chlorophyll stability index was observed under salt stress conditions. In case of water saturation deficit (WSD), it showed indirect relationship with RWC. Based on these results, the potential salinity-related candidate genes or quantitative traits may be isolated and characterized from proso millet.

Keywords: Salinity stress, Proso millet, physio-biochemical analysis, chlorophyll content

1. Introduction
Growth of the plants has been greatly affected by abiotic stresses such as high salinity, drought and low temperature or chilling stress. The productivity of the crop plants might be decreased in a large quantity as severe salt stress will lead to suppression of plant growth and development. Soil salinization is one of the most significant threat to sustainable agricultural production and ecological balance world-wide (Litalien & Zeeb, 2020) [15]. Soil salinity is one of the worst factors which prevent the yield of crops, by significantly effecting the plant growth and development. One of the most important factors that is contributing to the crop loss across the globe is salt stress. Approximately 6.5% of the world's arable and marginal soils are either saline or sodic was estimated based on the soil surveys conducted by the Food and Agriculture Organization of the United Nations (FAO) between 1970 and 1980 (FAO, 2016) [7].

Proso millet (*Panicum miliaceum* L.) which has been also known as broom corn millet, white millet, common millet or hog millet is one of the most important minor millet crop. It is an allotetraploid plant (2n=4x=36), self-pollinated (sometimes, cross-pollination may surpass 10%), belonging to the Panicoideae subfamily, with an estimated genome size of 1020.5 Mbp (Kubešová *et al*., 2010) [14].

Large number of physiological and biochemical strategies are developed by plants to cope up with the stresses (Pastori & Foyer, 2002) [16]. During the imposition of salinity stress morphological, physiological, and biological responses of the plants are altered (Amirjani, 2010) [3]. The present study was undertaken to understand the impact of salinity stress on Proso millet in terms of physiological and biochemical components.

2. Material and Methods
2.1 Plant material
Proso millet seeds [*Panicum miliaceum* L. variety ATL-1] were collected from Department of millets, Tamil Nadu Agricultural University, Coimbatore (11.0231° N, 76.9286° E). Seeds were sown in paper cups containing compost: coir pith in 1:1 ratio and maintained in greenhouse condition. As proso millet seeds will be in dormant stage, these seeds are treated with 10% sodium hypochlorite (NaOCl) for 10 minutes to improve germination before sowing. After 2 weeks, proso millet plants were transferred to the pots containing Yoshida media (pH-6.8). Salt stress study was conducted in 6 weeks old proso millet plants at 0 hour, 3 hours, 6 hours, 9 hours and 12 hours after imposition of stress under different salt concentrations *i.e.*,

50mM NaCl, 100mM NaCl, 150mM NaCl and 200mM NaCl. One pot was maintained as control. Three replicated samples were collected in both control and salt stressed plants for each treatment. With these samples physiological and biochemical parameters such as relative water content (RWC), water saturation deficit (WSD), chlorophyll stability index (CSI), chlorophyll content, proline content, malondialdehyde (MDA) content were determined.

2.2 Physio-biochemical analysis of salt stress tolerance

2.2.1 Chlorophyll content

Chlorophyll content in salt stressed plant samples were estimated by DMSO method using the procedure given by Hiscox and Israelston (1979) [11], About 100 mg of leaf sample was cut into small pieces and transferred to test tubes containing 10 ml of DMSO solvent. The test tubes were incubated at 60 °C in a water bath for one hour. The tissues were fully decolorised in this time interval. Following this, the tubes were cooled at room temperature for 30 minutes. Later it was filtered and the absorption of the filtrate was measured at 665 and 648 nm against a blank (DMSO solvent) using SHIMADZU UV Spectrophotometer-1800.

Chlorophyll a, b and total chlorophyll concentration was determined by the following formulae (Barnes *et al*., 1992) and expressed as mg/g fresh weight.

Chlorophyll a (mg/g F.W) = (14.85 A_{665} - 5.14 A_{648}).

Chlorophyll b (mg/g F.W) = (25.48A_{648} - 7.36 A_{665}).

Total Chlorophyll (mg/g F.W) = (7.49 A_{665} + 20.34 A_{648}).

Where, the absorbance at 665 nm is expressed as $A_{665}$and the absorbance at 648 nm is expressed as A_{648}.

2.2.1 Relative water content (RWC)

Leaf samples were collected from both control and salt stressed plants at different time intervals for RWC analysis. Fresh weight (FW) of leaf samples was recorded and then these samples were immersed in double distilled water for 8 h at room temperature. Later these leaf samples were taken out and wiped with tissue paper to remove extra water and the turgid weight (TW) of these leaf samples were measured. Finally, the dry weight (DW) of leaves was measured after 24h of incubation in a hot air oven at 80 °C. The formula to calculate the relative water content:

$$RWC(\%) = [(FW-DW)/(TW-DW)] \times 100$$

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 the protocol described by Kaloyereas (1958) [12] and expressed in percentage (%).

$$\text{Chlorophyll stability index} = \frac{\text{Total Chlorophyll Content (Stressed)}}{\text{Total Chlorophyll Content (Control)}} \times 100$$

2.2.4 Proline: Proline content of the leaf samples was estimated according to the method developed by Bates *et al*., (1973) [5]. Proline concentration was determined from the standard graph and expressed as µmol proline g^{-1}.

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

2.2.5 Malondialdehyde content

The level of lipid peroxidation was quantified by measurement of malondialdehyde (MDA) in both control and stressed leaves as described by Heath and Packer, (1968) [10] as determined by the reaction of thiobarbituric acid.

2.2.6 Total Soluble Sugars

The estimation of total soluble sugars (TSS) was carried out based on the method described by Yemm and Willis, (1954) [18]. Based on a standard curve generated from a graded concentration of glucose, the total soluble sugars were determined and expressed as mg g-1 of FW.

2.3 Statistical analysis: Using complete randomized design, the statistical analysis of physiological and biochemical analysis was performed. The results were interpreted as mean ± S.E. Duncan's multiple range test (DMRT) was performed with the help of WASP computer software 1.0.

3. Results and Discussion

3.1 Physiological analysis of salt stress tolerance

In the present study conducted, salinity stress of different concentrations has been induced to 6 weeks old plants to create a water deficit stress, in order to know how the plants will react under stress. As the salinity stress level increased, the reduction in the chlorophyll content has been observed. The reduction in chlorophyll concentration was possibly accompanied by the oxidative stress that damages the thylakoids' membrane and leads to degradation of chlorophyll (Pinto *et al*., 2016) [17]. In the present study, chlorophyll a, chlorophyll b and total chlorophyll content recorded 1.25,1.22 and 1.26 fold reduction in 50mM NaCl, 1.33, 1.44 and 1.36 fold reduction in 100mM NaCl, 1.39,1.66 and 1.45 fold reduction in 150mM NaCl and 1.43, 2.09 and 1.55 fold reduction in 200mM NaCl respectively.

Relative water content has been significantly reduced in plants which are affected with salt stress. Higher RWC was observed in all plants under control conditions. But the increased water saturation deficit has led to greater degree of plant salt stress. In the study we have conducted, it shows that, under control condition RWC ranges from 85% to 90% whereas in salt stressed condition at different concentrations it ranged from 78% to 84% for 50mM NaCl, 76% to 81% for 100mM NaCl, 76% to 82% for 150mM NaCl and 70% to 85% for 200mM NaCl (Fig 1.). The water saturation deficit (WSD) was lower in control plants and higher in salt stressed plants (Fig 2.). In rice plants, salinity stress was reduced by relative water content. When plants are exposed to salinity, they initially confront an osmotic challenge, which lowers root water uptake. Low relative water content in the cells has been entailed when there is low/no water uptake by roots due to transpiration pull induced by ABA mediated stomata closure (Blatt & Armstrong 1993) [6].

Chlorophyll stability index (CSI) is one of the important indication of plants' tolerance capacity against salinity stress. If the CSI value is high, it indicates that plants' chlorophyll content has not been much affected by salt stress. As NaCl level increased, the CSI percentage showed a decreasing trend in *Lasiurus scindicus* Henrard (Gadi and Goswami, 2016) [8]. Decrease in CSI percentage in *Pisum sativum* was also recorded and suggested that it is an effective technique or marker for identifying stress-tolerant genotypes under ideal environments (Ahmad *et al*., 2008) [1].

Table 1: Effect of salt stress on the chlorophyll content in 6-week-old proso millet leaves

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)				
	Control	50 mM	100 mM	150 mM	200 mM	Control	50 mM	100 mM	150 mM	200 mM	Control	50 mM	100 mM	150 mM	200 mM
0^{th} hour	2.36± 0.024	2.37± 0.028^{a}	2.33± 0.031^{a}	2.37± 0.025^{a}	2.35± 0.026^{a}	0.71± 0.028	0.66± 0.020^{a}	0.64± 0.017^{a}	0.62± 0.023^{a}	0.59± 0.023^{a}	3.07± 0.02	3.03± 0.041^{a}	2.99± 0.055^{a}	3.01± 0.052^{a}	2.98± 0.078^{a}
3^{rd} hour	2.34± 0.035	2.23± 0.029^{b}	2.14± 0.02^{b}	2.09± 0.023^{b}	2.05± 0.026^{b}	0.68± 0.034	0.61± 0.017^{ab}	0.56± 0.023^{b}	0.54± 0.023^{ab}	0.49± 0.024^{b}	3.05± 0.072	2.85± 0.029^{b}	2.69± 0.058^{b}	2.64± 0.057^{b}	2.54± 0.043^{b}
6^{th} hour	2.35± 0.014	1.95± 0.02^{c}	1.93± 0.017^{c}	1.85± 0.017^{c}	1.78± 0.020^{c}	0.63± 0.029	0.58± 0.020^{bc}	0.52± 0.023^{b}	0.49± 0.023^{bc}	0.48± 0.026^{b}	3.01± 0.029	2.54± 0.04^{c}	2.45± 0.041^{c}	2.36± 0.052^{c}	2.26± 0.078^{bc}
9^{th} hour	2.33± 0.008	1.89± 0.018^{cd}	1.82± 0.014^{d}	1.75± 0.023^{d}	1.69± 0.026^{d}	0.67± 0.029	0.57± 0.024^{bc}	0.45± 0.020^{c}	0.42± 0.032^{cd}	0.41± 0.020^{b}	3.02± 0.026	2.48± 0.036^{cd}	2.28± 0.041^{d}	2.17± 0.037^{d}	2.1± 0.011^{c}
12^{th} hour	2.27± 0.014	1.87± 0.017^{d}	1.76± 0.023^{d}	1.68± 0.023^{e}	1.63± 0.028^{d}	0.63± 0.023	0.53± 0.020^{c}	0.45± 0.014^{c}	0.39± 0.032^{d}	0.31± 0.035^{c}	2.91± 0.017	2.4± 0.036^{d}	2.22± 0.027^{d}	2.08± 0.026^{d}	1.95± 0.058^{c}

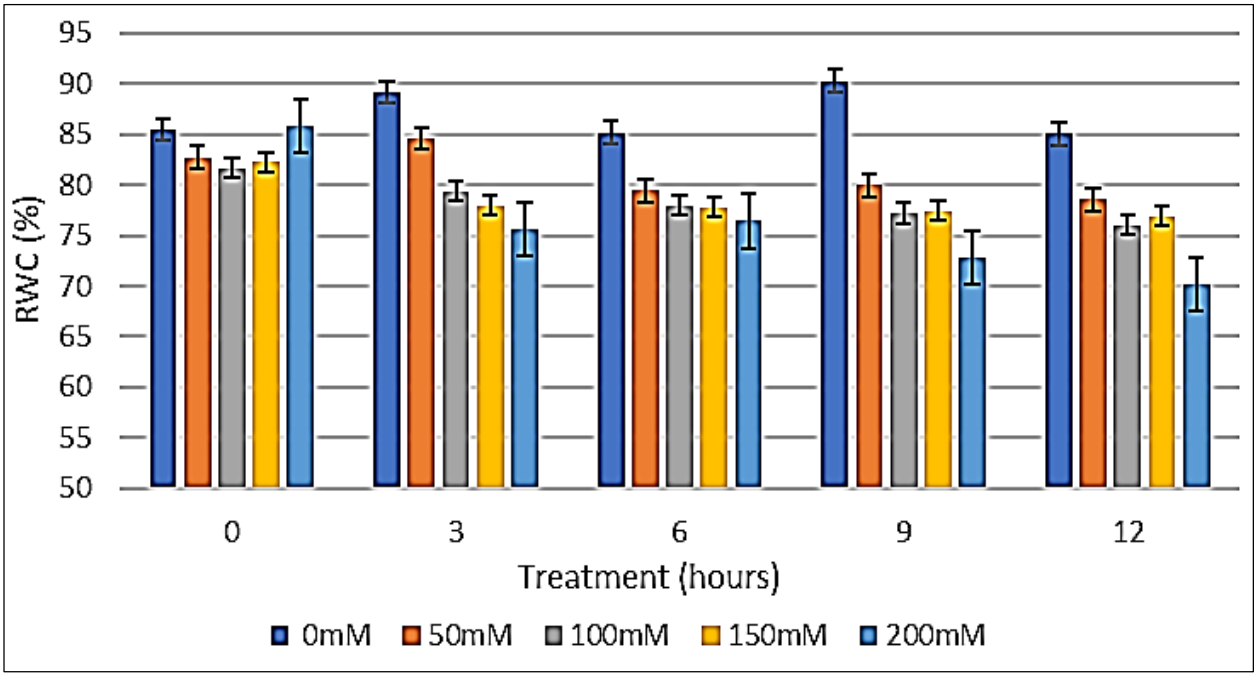

Fig 1: Effect of salt stress on relative water content in proso millet leaves of 6week old plants.
Data are presented as mean ± standard error (n=5)

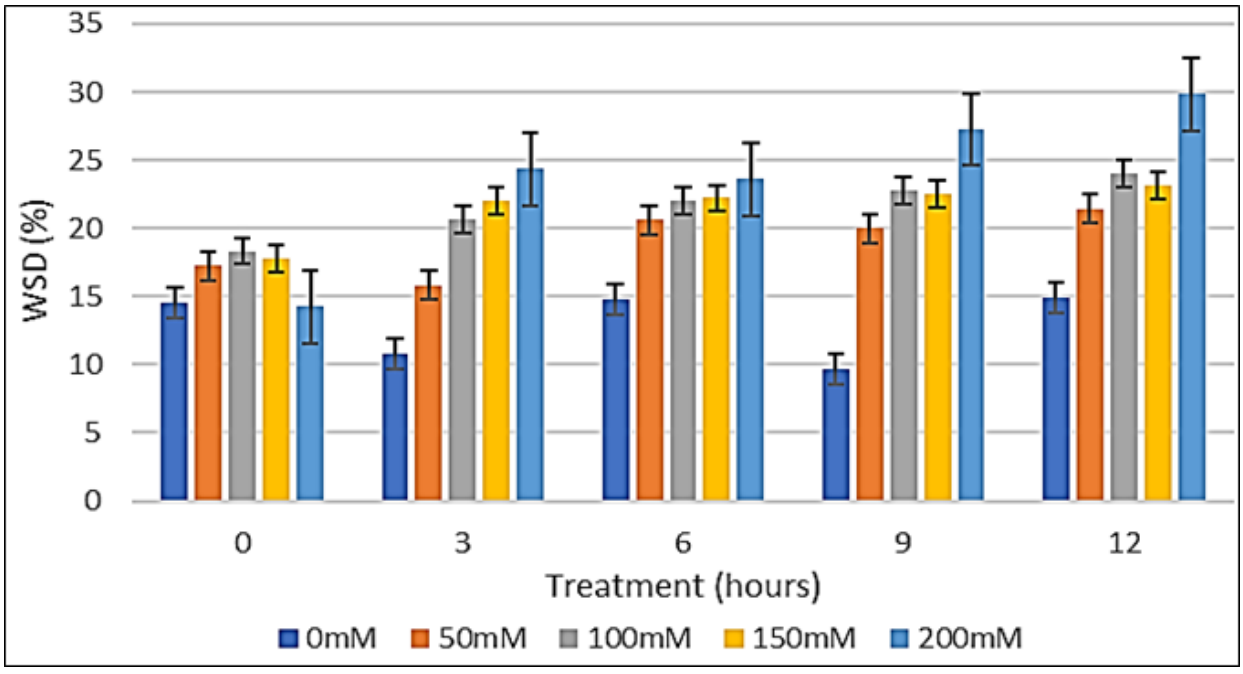

Fig 2: Effect of salt stress on water saturation deficit in proso millet leaves of 6 week old plants.
Data are presented as ± standard error (n=5)

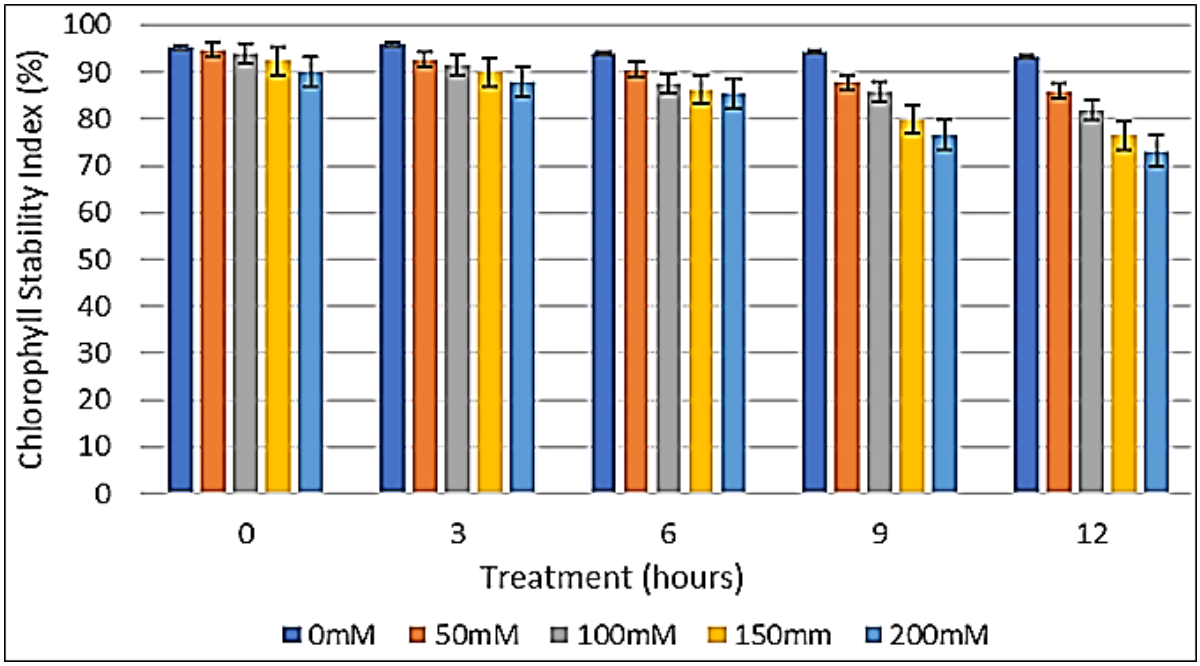

Fig 3: Effect of salt stress on chlorophyll stability index in proso millet leaves of 6 week old plants. Data are presented as mean ± standard error (n=5)

3.2 Biochemical analysis of salt stress tolerance

In salinity stress, proline accumulation is a primary defence in order to maintain osmotic pressure in a cell. Compared to control plants, the proline content was high in salt stressed plants. It is one of the most crucial biochemical index for the response of plants to salinity stress. It supports the growth of plants and also survival under stress conditions. Osmotic adjustment at the cellular level may be facilitated with proline accumulation. Proline acts as the molecular chaperone, ROS scavenger, osmoprotectant, an oxidative defence molecule, metal chelator and a signalling molecule during stress (Hayat *et al*., 2012) [9].

The peroxidation of the membrane's fatty acids by peroxy radicals, which produced the malondialdehyde (MDA), the end product of the lipid peroxidation (Kotchoni *et al*., 2006) [13].

In the present study, salt stressed plants showed increased proline and MDA content level 2.245 and 1.429 times in 50mM NaCl, 2.573 and 1.44 times in 100mM NaCl, 2.81 and 1.52 times in 150mM NaCl and 3.66 and 1.6 times in 200mM NaCl respectively (Figs. 4 & 5).

A typical osmoprotectant, soluble sugar can sustain turgor pressure and stabilize cellular membranes. In salt stressed leaves, total soluble sugar (TSS) content was 1.48 times high in 50mM NaCl, 2.066 times high in 100mM NaCl, 2.199 times high in 150mM NaCl and 2.76 times high in 200mM NaCl compared to the control plants.

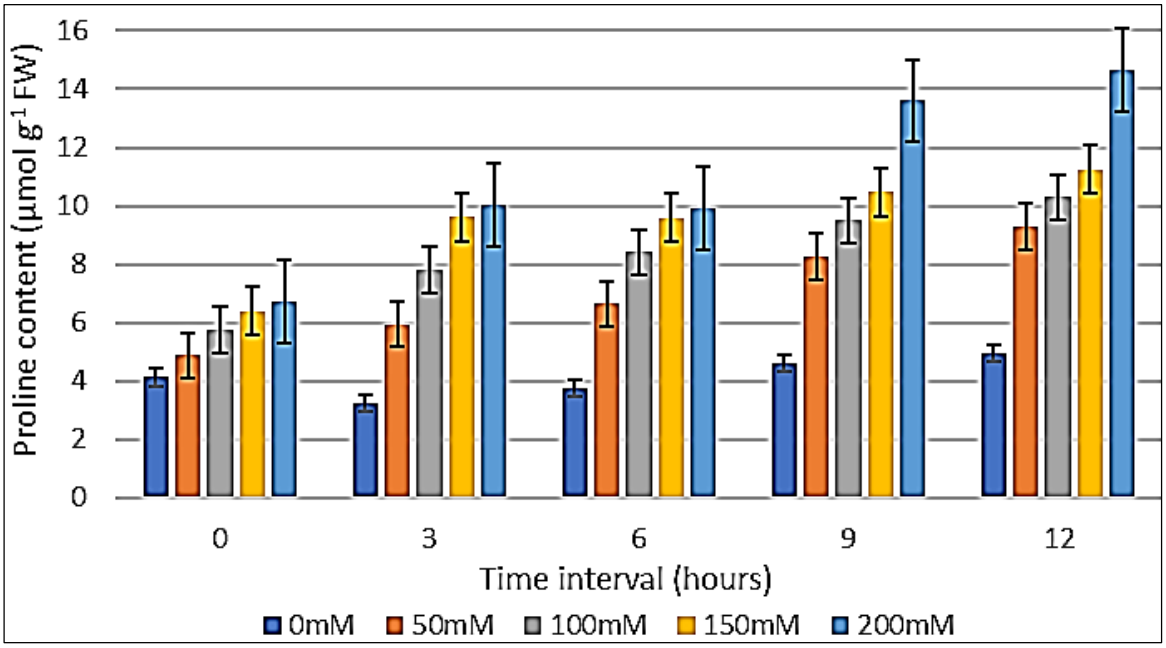

Fig 4: Effect of salt stress on proline content in proso millet leaves of 6 week old plants. Data are presented as mean ± standard error (n=5)

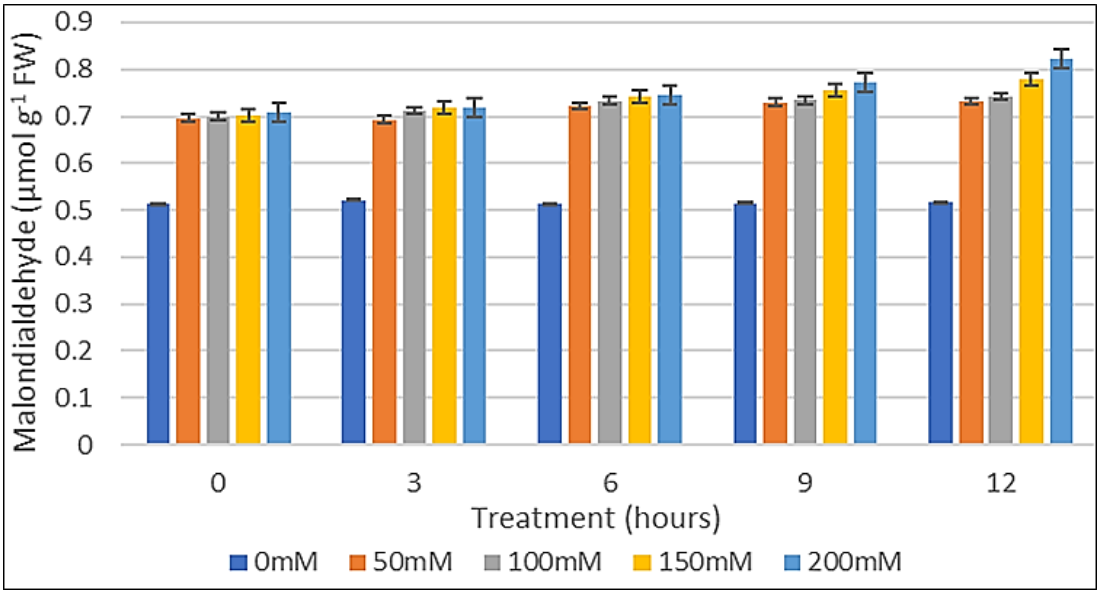

Fig 5: Effect of salt stress on malondialdehyde content in proso millet leaves of 6 week old plants. Data are presented as mean ± standard error (n=5)

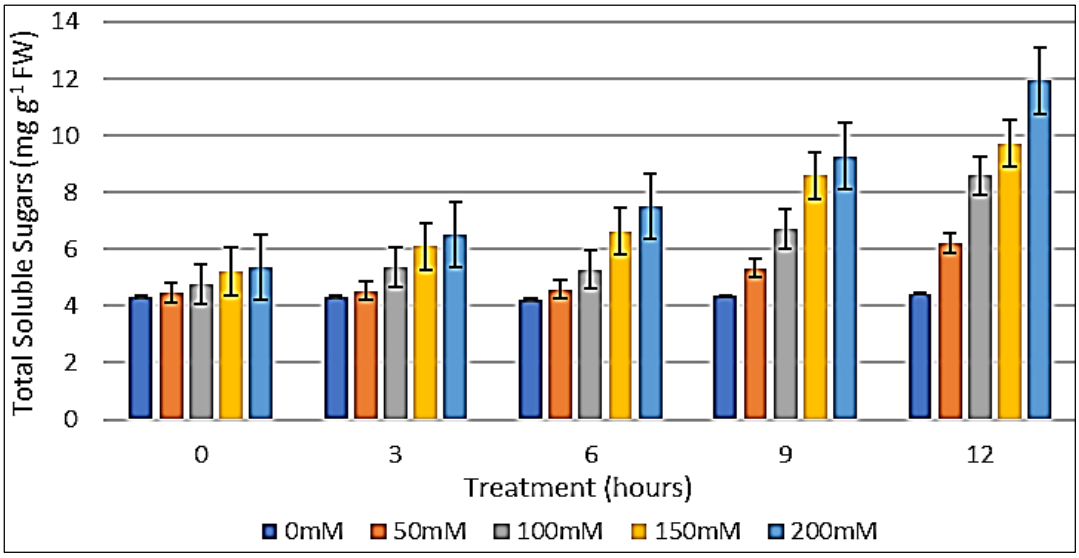

Fig 6: Effect of salt stress on total soluble sugar content in proso millet leaves of 6 week old plants. Data are presented as mean ± standard error (n=5)

4. Conclusion

Plants are impacted by salinity stress at every stage of their life cycles, from germination to maturity. Numerous physiological, biochemical, and molecular adaptations have been made by plants to help them cope with, avoid, or tolerate salinity stress. In our work, numerous physio-biochemical analyses showed the significance of the biochemical components in the proso millet's salinity stress tolerance mechanism. By identifying and characterizing prospective candidate genes implicated in salinity stress tolerance, this analysis can be further strengthened. These genes can then be expressed to create crops that can withstand stress.

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