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Effect of NaCl stress on pigment composition, membrane integrity and proline metabolism of little millet (*Panicum sumatrense* L.) CO-4 variety

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Abstract
Salinity stress poses a great threat to a growing population that accounts for huge agricultural loss per annum. Millets are well-known crop plants having the ability to grow well in different soil profiles under changing climatic conditions. In this view, the present study was chosen to determine the effects of NaCl stress on *Panicum sumatrense* (L.) CO-4 variety exposed to 25 mM, 50 mM, 75 mM, and 100 mM NaCl doses imposed on 7th DAS (days after sowing). The result showed an enhancement in total chlorophyll (Chl) constituent and a decrease in carotenoids with increase in stress doses in *P. sumatrense* over control one. However, increased malonaldehyde and hydrogen peroxide contents have shown a reduction insoluble protein content. The proline content elevated significantly with increased salinity level than the control one. Thus, proline accumulation results in upsurge tolerance *in P. sumatrense* by enhancing photosynthetic rate when subjected to NaCl treatments.

Keywords: abiotic stress, proline, pigments, *Panicum sumatrense* (L.)

1. Introduction
Salinity stress has become a serious abiotic environmental concern worldwide. Since salt stress does not affect only plant growth and yield but yield quality as well. Globally, over 100 countries having salt-affected land around billion hectares (1-10 billion hectares) constitutes more than 7-10% of the total land area, with increasing potential of 10 -15% per annum (Panta *et al*., 2014; Jesus *et al*., 2015; Zhang *et al*., 2017) [33, 20, 50]. Salt stress leads to a reduction in plant growth by hindering cell wall extensionality due to osmotic stress followed by oxidative stress, a consequence of drought like situation created by NaCl stress that restricts water movement to roots. Saltiness led osmotic stress, ion toxicities, and oxidative stress exert a threefold effect in crop plants, as a consequence reduces growth, yield, and quality of yield (Deinlein *et al*., 2014; Khare *et al*., 2015) [9, 23]. Unlike osmotic stress, the ionic-stress results in a changed redox ratio showed a long-lasting adverse effect of salt toxicity in plants. The harmful effect of ion (s) toxicity is expressed on the whole plant and is visualized in all growing stages, viz., seed germination, seedling emergence, vegetative to reproductive stages. In addition, increased ion toxicity lead by salinity causes a tremendous change in the rate of photosynthesis by declining photopigment constituents (Kaya *et al*., 2013; Lin *et al*., 2017) [21, 26].

The formation of reactive oxygen species (ROS) under environmental stresses is a primary and vital biochemical response. Since, they regulate metabolism, cell functions, and signaling networks at an optimum level. However, when the concentrations of ROS production exceed the defense mechanism, it imposes damage to the cell membrane and other vital biomolecules, as a consequence of which apoptosis triggers that leads to cell demise.

Interestingly, as the ROS concentration increases beyond their optimal level, plant cells tackle it by triggering two essential mechanisms. The activation of antioxidant systems like enzymatic antioxidants (catalase, superoxide dismutase, peroxidase, and polyphenol oxidase), play a key part in neutralizing ROS accumulation during abiotic stress. While the other mechanism known as an osmotic adjustment, maintained by osmolytes accumulation such as proline, glycine betaine, and proteins in the cell cytosol, provides potential to crop plants to face harsh environmental conditions, for instance, under saline stress. (Munns and Tester, 2008; Farhangi-Abriz and Torabian, 2017) [30, 11]. According to Chen and Dickman (2005) [6], among osmoprotectants, proline is the primary and major osmolyte that plants and other organisms required to counteract the inhibitory effects of ROS.

Millets are suitable crops for farming in arid and semi-arid parts of the world, where other crops cease to grow under harsh environmental conditions. Moreover, millets are useful in exploring the genetic variations in improving food quality and nutritional security towards the growing population. Millets serve as a staple food in millet producing areas and are employed in making common foods like idli, porridges, bread, and chakli, etc. (Singh & Sarita, 2016)[41]. Besides, their nutritional benefits, these grains also possess various phytochemical compounds, which help in the surveillance of lifelong disorders like cancer, diabetes, and heart-related diseases (Chandrasekara & Shahidi, 2011a) [5]. Little millet (*Panicum sumatrense* L.) Roth., a self-pollinating crop (3.5% cross-pollination), belong to genera Panicum, is extensively used as landraces. Because of their short lifespan, high nutritional benefits and remarkable ability to survive under various abiotic conditions like drought, salinity, and high temperature, etc. The study was chosen with the prime objective to find out the influence of salinity stress and determining the change in the physio-biochemical properties of *Panicum sumatrense* (L.) CO-4 variety by exposing to increasing doses of NaCl stress.

2. Materials and Methods

2.1 Experimental design

The seeds of *Panicum sumatrense* (L.) CO (Samai) 4 variety were purchased from Tamil Nadu Agricultural University (TNAU), Coimbatore, Tamil Nadu, India. The pot experiment and procedures were performed in the botanical garden and stress physiology laboratory, department of Botany, Annamalai University. The two factors (one *P. Sumatrense* and another NaCl treatment) and three replicates were maintained in a randomized block design (RBD). The seeds of *P. Sumatrense* were disinfected with 0.2 % (V/V) sodium hypochlorite solution for 3 minutes with frequent shaking and then washed with sterile water to clean traces of sodium hypochlorite. Afterwards, earlier to sowing these sterilized seeds were soaked in sterile water for 6 hours in a beaker. Subsequently, blotted dry hydrated seeds were transferred to plastic pots containing sand, soil, and manure in the ratio of 1:1:1 for sowing. The seeds (3 replicates of 4 seeds each pot) were allowed to germinate for a week. Subsequently, various doses of NaCl stress treatment viz., 25mM, 50mM, 75mM, and 100mM were given to seedlings on 7th DAS (days after sowing) up to 18th DAS. Salinity concentrations were maintained through EC. However, control (0mM) seedlings were irrigated with normal tap water up to 18th DAS. Then the 18days old seedlings were picked up carefully and used for further analysis.

2.2. Determination of Morphological parameters

Panicum sumatrense L. seedlings uprooted from the pots were rinsed with tap water followed by sterile water to remove soil particles. Then the samples were blot dried with tissue paper and made ready for analysis.

2.2.1. Estimation of Fresh weight and Dry Weight

To quantify the fresh weight (FW) and dry weight (DW), plant samples collected were washed with normal water followed by sterile water to clean salt traces present on the surfaces. Then fresh weight was taken using a digital balance. For dry weight (DW), plants were dried in an aerated oven at 70 ºC for next 24 hours, values were noted and expressed in g plant^{-1}.

2.3. Biochemical contents

2.3.1. Determination of Photosynthetic pigments

Leaf chlorophyll and carotenoid contents were analyzed by following the Arnon method (1949)[2]. Briefly, fresh leaf tissue (200mg) was grinded with 10ml 80% acetone/water (v/v). The extract was centrifuged at 800xg for 10 min at 4ºC temperature and this step was repeated once again. Later, the extract volume was made up to 10 ml with 80 % ice-cooled acetone. The optical density (O.D.) of the aliquot (3ml) was measured against a blank of pure 80% acetone at three different wavelengths of 645, 663 nm (chlorophyll) and 475nm carotenoids (Kirk and Allen, 1965) [25] respectively, using UV-VIS spectrophotometer (Model-118, Systronic India Limited), and expressed in mg g^{-1} fresh weight (FW).

2.3.2. Determination of Hydrogen Peroxide

Hydrogen peroxide levels were quantified by following the protocol of Velikova *et al*., (2000) [48]. Fresh leaf tissue (200mg) was ground with 5 ml 0.1% trichloroacetic acid (TCA) (w/v) in an ice bath. The extract was then centrifuged at 12,000xg for 15 minutes. From this supernatant collected, 0.5ml was taken, and to this 0.5ml of 10 mM potassium phosphate buffer (pH 7.0) and 1ml of 1M KI were added. The absorbance of the supernatant was recorded spectrophotometrically at 390 nm against known H_2O_2 as a standard. The H_2O_2 concentration was expressed in µmolg^{-1} FW.

2.3.4. Determination of Lipid Peroxidation

The lipid peroxidation level was estimated by measuring the Malondialdehyde (MDA) content following the thiobarbituric acid (TBARS) reaction. Briefly, fresh leaf material (200mg) was homogenized in 5 ml of 0.1 % (w:v) TCA. The homogenate was centrifuged at 10,000xg for 5 minutes. From this 1ml of the supernatant was taken and blended with 4 ml of 20 % TCA containing 0.5 % (w:v) TBA. Subsequently, the mixture was heated at 95ºC for 30 min and then quickly cooled on an ice bath to stop the reaction. The contents were again centrifuged at 10,000 xg for 15 min. to remove precipitates and absorbance values were read at differences 600nm and 532 nm in a spectrophotometer. The MDA content was calculated using an extinction coefficient of 155mM/1 cm/1 and expressed as nmol g^{-1} FW (Heath and Packer, 1968) [16].

2.3.5. Measurement of protein and proline

Compatible Solutes like soluble protein and proline contents were quantified from the samples by following standard methods.

Soluble protein content was quantified following the method of Bradford (1976) [4]. Briefly, 0.2g of plant leaf tissue was ground in a mortar with 4ml of phosphate buffer (pH 7.0) and then centrifuged @ 10,000xg at 4℃.To an aliquot (50µl) of the supernatant diluted to 1ml with extraction buffer, 5ml of Coomassie brilliant blue (CBB) G-250 was added and mixed thoroughly. The absorbance was read at 595nm in a UV- spectrophotometer against a reagent blank. The amount of protein was calculated using standard prepared with different concentrations of bovine serum albumin (BSA) ranging from 10µg/ml to 100µg/ml and expressed in mg g^{-1} fresh weight (FW).

Proline content was quantified according to Bates *et al*., method (1973) [3]. In this method, fresh leaf material weighing (200 mg) was homogenized in 3% (w/v) aqueous 5- sulphosalicylic acid. The homogenate was shifted to a centrifuge tube, and then centrifuged at 10,000 rpm and the supernatant was collected. 2ml of supernatant was taken in a test tube, and to this, an equal volume of 2 ml acid ninhydrin and 2 ml glacial acetic acid were added. The whole mixture was boiled at 100 ºC for 1 h. Then, the tubes were immediately cooled in an ice bath. To this reaction mixture, 4 ml of toluene was added and vortexed for 3 min at 30 rpm. After phase formation and separation, the upper organic layer was used for measuring the absorbance at 520 nm using a spectrophotometer (Model-118, Systronic India Limited, Gujarat, India UV–VIS). The toluene was used as a blank reagent. The free proline was calculated from the standard curve, obtained using L-proline as standard and denoted in mg g^{-1} FW.

2.4 Statistical analysis

All the data collected were statistically analyzed by one-way (ANOVA) using SPSS (version 16.0) statistical software for windows. The obtained data represented in bars are mean values of three replicates(n=3), and (±) standard error (SE) of mean values.

3. Results and Discussion

Salinity effects on plants include osmotic stress, and oxidative stress (caused by ion toxicity) leads to physio-biochemical variations and thus, significantly reduces crop yield and productivity. To increase the resistance of crops to abiotic stress, it is essential to tackle the difficulties caused by salinity stress (Golldack *et al*., 2011) [14]. The present study was investigated to find out the tolerance range and metabolic adaptation changes in *P. sumetrense* at varied levels of NaCl stress. Undoubtedly, millets show tolerance to abiotic stresses. However, their range of tolerance varies and depends upon species, their growth stages, and their level of stress.

Growth-related characteristics, including fresh and dry biomass of *P. sumatrense* L. seedlings, were negatively affected by salt-stress treatment (Fig. 1-2). However, with increasing NaCl doses, a profound reduction was noticed in both fresh and dry biomass particularly, for 75mM and 100mM NaCl, and were 0.51, 0.37 FW, and 0.05, 0.041 DW g plant^{-1} respectively, relative to control.

Plant growth reduction and accumulation of dry-matter under saline conditions have been well addressed in several grain legumes, and other crops including *P. vulgaris* (Tejera *et al*., 2006; Taïbi *et al*., 2012, 2013a, 2013b) [47,44-46], on *Bruguiera gymnorrhiza* L. (Rui *et al*., 2009)[37], and on *Brassica campestris* L. (Memon *et al*., 2010)[28]. A similar result has been determined by AbdElgawad *et al*., (2016) [1] on maize (*Zea mays* L.), found that salinity at 150 mM caused leaf biomass reduction by 20%. Fresh mass and dry mass (represented in Fig.1&2) of *P. sumetrense* seedlings decreased significantly with increasing salt stress doses compared to control. The decline in plant biomass could be largely due to a reduction in photosynthetic capacity, the consequences of osmotic and oxidative damages in plants.

A positive trend has been recorded between chlorophyll content and enhanced salt concentrations. The levels of total chlorophyll content (a+b) increase during the stress period generated by 75 and 100 mM NaCl, and were 1.23 and 1.43 mg g^{-1} fresh weight when compared to control (Fig.3).

Our results regarding the increment in total chlorophyll content (Fig.3) with increasing NaCl concentration doses (25, 50, 75 and 100mM) agreed with results reported by Misra *et al*., (1997) [29], indicated increased chlorophyll content significantly in 15days old rice seedlings (Oryza sativa L.), when exposed with sodium chloride stress. Similarly, Jamil *et al*., (2007)[19] on sugar beat (*Beta vulgaris* L.), mentioned increased total chlorophyll content with increased doses of sodium chloride treatment (0, 50, 150 mM). Since, when plants are subjected to NaCl stress, Chl-b degradation takes place at a higher rate than Chl-a in leaves; possibly, the first step in Chl-b degradation involves its conversion to Chl-a, as a consequence of which the chlorophyll a/b ratio increases (Hortensteiner, and Krautler, 2011) [18]. In addition to this, increased chlorophyll content might be due to enhanced osmolyte that assists to stabilize the turgor potential of cells and prevents osmotic shock and thus, declines ROS level and chlorophyllase activity.

While, increased salt concentration causes a tremendous decline in carotenoids for 75 and 100 mM NaCl, and were recorded 0.17 and 0.13 mg g^{-1} fresh weight respectively, relative to control plants and 0.35 mg/g fresh weight (Fig.4). Our results represented (Fig.4) coincide with the results obtained by Mustard and Renault (2006) [31], on dogwood (*Cornus sericea* L.) seedlings, reported reduction in carotene content upon exposure to salt stress. Taibi *et al*., (2016)[43] also found decreased carotenoids in two Phaseolus vulgaris varieties, with increased salt treatment doses (50, 150, and 200mM). Carotenoids are the antioxidants formed under stress conditions in the chloroplasts and prevent the photosynthetic apparatus against harmful environmental factors by scavenging ROS (Ramel *et al*., 2012) [35]. However, a decline in the carotenoids leads to breakdown of β-carotene and synthesis of zeaxanthins, which are involved in protection of photosynthetic apparatus against photo-inhibition (Sharma and Hall, 1991) [38].

A sharp peak in H_2O_2 contents were noticed, when *P. sumatrense* seedlings were exposed to different doses of NaCl stress, and maximum values recorded for 75mM and 100mM are 0.43 and 0.49 µmol mg^{-1} FW respectively, compared to control plants (Fig.5).

Hydrogen peroxide is the primary ROS responsible for the major plant injuries, for instance, damage to cell membranes and other essential biomolecules under salt stress (Parvaiz and Satyawati, 2008) [34]. Formation of oxygen radicals, like superoxide anion (O_2^-) and H_2O_2, can adversely affect plants under salt stress. While as H_2O_2 may led to enzyme inactivation through oxidation of SH groups (Gill and Tuteja, 2010) [13]. Hirofumi Saneokaa *et al*., (2018) [17] found increased (H_2O_2) content with increment in salt concentrations in two barley varieties.

A tremendous increase in the values of MDA content was noticed with raise in NaCl stress level relative to the control plants and were recorded 0.75 and 0.95 nmol mg^{-1} FW for 75 and 100 mM NaCl in *P. sumatrense* (Fig.6).

In our study, MDA concentration in *P. sumetrense* was enhanced with increased saline concentrations (Fig.6). Our results correlated with the findings of Xu, *et al*., (2013) [49] on turfgrass species; Khare *et al*., (2015) **[23]** on rice cultivars, who suggested that MDA content increased with the increase in NaCl concentrations. AbdElgawad *et al*., (2016) [1] also reported that salt stress at 75 and 150mM increased MDA content in 3-weeks old corn seedlings. Similarly, Sheidollah *et al*., (2018) [39], investigated MDA concentration increased in four rice varieties under varied saline levels (0, 30, 60, and 90 mmol/L NaCl). The MDA content, a product of lipid peroxidation is considered an essential biomarker of cell membrane damage in plant tissues subjected to salt stress (Silambarasan *et al*., 2019) [40]. The oxygen-free radicals result in lipid peroxidation by reacting with unsaturated fatty acids such as linoleic acid and linolenic acid of cell membranes. The higher the MDA content, the more severe cell membrane damage under salt stress.

In regard to soluble protein content of *P. sumatrense* plants exposed to NaCl salt stress, a sharp decline in protein content was observed with rise in NaCl stress doses related to control plants. The maximum reduced protein level recorded in *P. sumetrense* were 0.41 (mg g^{-1} fw) and 0.28(mg g^{-1} fw) for 75 and 100 mM NaCl than to control one (Fig.7).

The soluble protein contents decreased in response to salt stress and effect was determined higher at increased stress conditions (Fig.7). The decline in protein content under salinity results from less availability of amino acids and the denaturation of enzymes required for protein synthesis

(Hassanpour, *et al*., 2013) [15]. Chen *et al*., (2007) [7] found reduced soluble protein content in 14 days old cowpea (*Vigna unguiculata* L.) plants, exposed to 75 mM of NaCl treatment. Similarly, Khosravinejad *et al*. (2009) [24] with their study on barley (*Hordeum vulgare* L.) seedlings, observed reduced protein content when subjected with sodium chloride stress.

Interestingly, *P. sumatrense* seedlings subjected to NaCl stress at different concentrations, show an increase in proline content and maximum values recorded in 75mM and 100mM NaCl, were 0.68 and 0.90 mg g^{-1} FW respectively, relative to control (Fig.8).

Our results (Fig.8) agreed with previous studies done by Mansour *et al*., (2005)[27], and Kaya *et al*., (2010) [22] who reported an enhanced proline content under salt stress. Recently, Chiconato *et al*., (2019) [8] reported an increment in proline content with enhanced salt concentration doses (0, 40, 80, and 160mM) in sugarcane under different salinity conditions. Osmolytes accumulation, especially proline and glycine betaine, play a key part to maintain cellular turgor under various abiotic stresses (Negrao, *et al*., 2017) [32]. Since, their hydrophilic nature helps to replace water by bonding at the surface of proteins and membranes, which clearly explains their role as osmoprotectants and as chaperones (heat shock proteins). Among osmolytes, proline is known as a vital osmolyte, maintaining low osmotic potential in stressed plants (Farooq, *et al*., 2015) [12]. Several authors reported that, besides its osmotic adjustment, proline also plays a vital role in ROS detoxification by non-enzymatic antioxidant activity (Dolatabadian *et al*., 2008, Szabados and Savoure, 2010; Rejeb, *et al*., 2014) [10,42,36,]. This study also determined that the proline level elevated gradually as NaCl concentration increases from 0 to 145 mM, but takes peak sharply when the NaCl concentration increased from 145 mM to 195 mM. This change in proline content in return to salt stress is accompanied by a change in MDA levels and antioxidant enzyme activity.

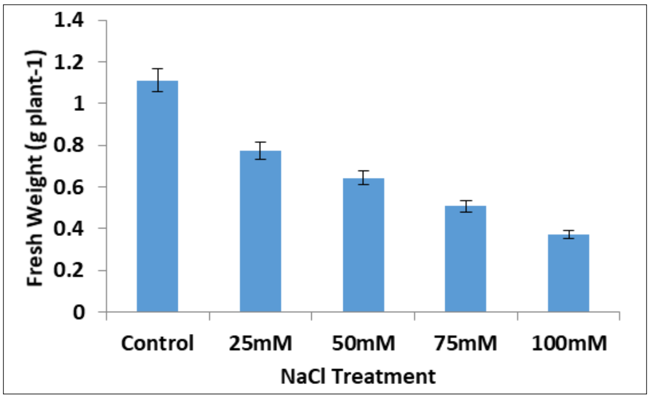

Fig 1: Effect of NaCl on Fresh weight in seedlings of *P. Sumatrense* L. CO-4 Variety. Values are mean ± SE based on three replicates (n=3).

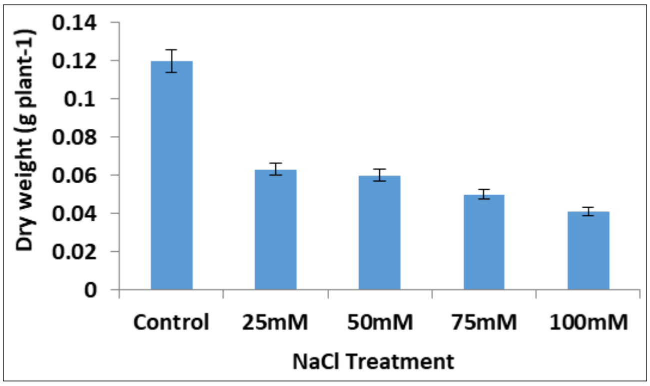

Fig 2: Effect of NaCl on Dry weight in seedlings of *P. Sumatrense* L. CO-4 Variety. Values are mean ± SE based on three replicates.

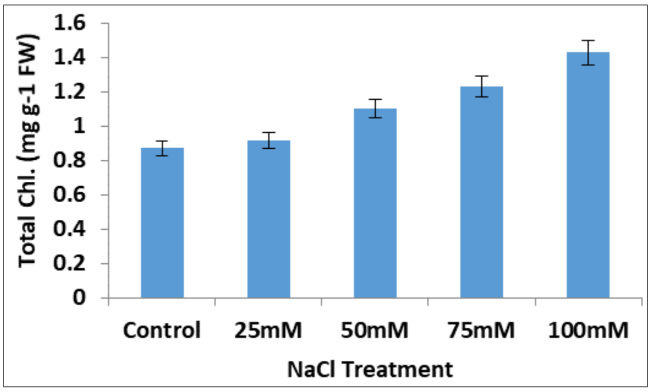

Fig 3: Effect of NaCl on Total Chlorophyll content of *P. Sumatrense* L.CO-4 Variety. Values are mean ± SE based on three replicates.

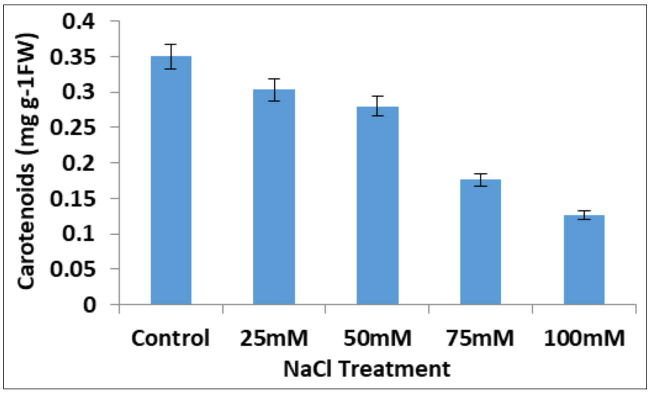

Fig 4: Effect of NaCl on carotenoids content of *P. Sumatrense* (L.) CO-4 Variety. Values are mean ± SE based on three replicates

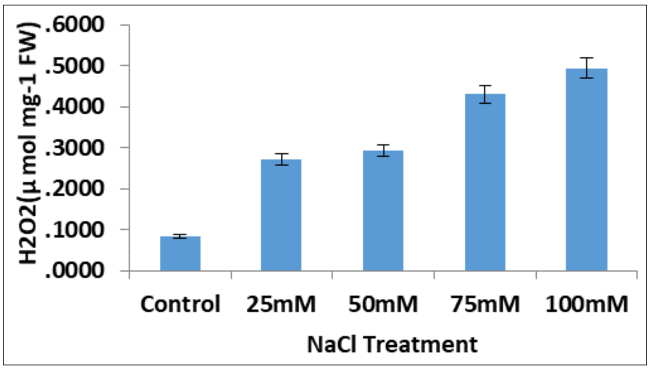

Fig 5: Effect of NaCl on H_2O_2 in seedlings of *P. Sumatrense* (L.) CO-4 variety. Values are mean ± SE based on three replicates.

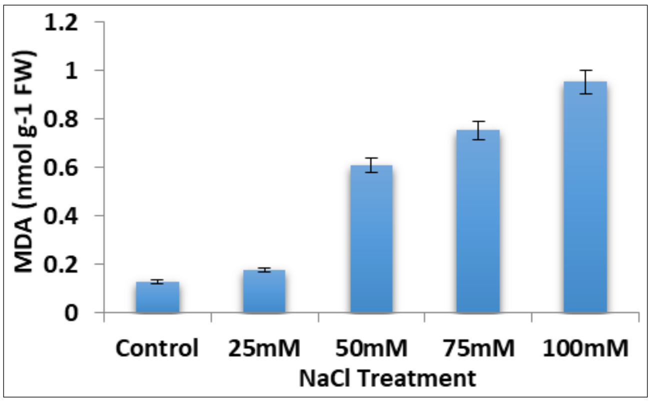

Fig 6: Effect of NaCl on lipid Peroxidation (MDA) of *P. Sumatrense* (L.) CO-4 variety. Values are mean ± SE based on three replicates.

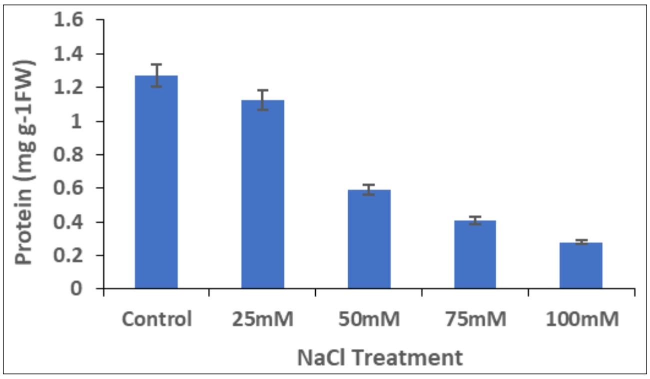

Fig 7: Effect of NaCl on Protein content of *P. Sumatrense* (L.) CO-4 Variety. Values are mean ± SE based on three replicates.

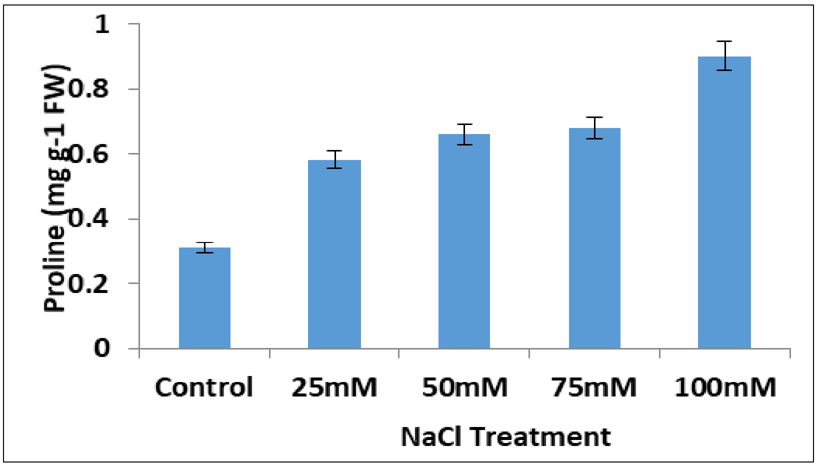

Fig 8: Effect of NaCl on Proline content of *P. Sumatrense* (L.) CO-4 Variety. Values are mean ± SE based on three replicates.

4. Conclusion

In our study, it was found that salinity stress causes a reduction in overall plant growth, results by a decline in the average biomass production, and photosynthetic pigments especially carotenoids, which play a significant role in the prevention of photosynthetic apparatus damage. Additionally, increased H_2O_2 and MDA content results increased proline level under salt stress, which serves as ROS detoxifier by acting as an antioxidant role. Furthermore, results evaluated from *P. sumatrense* to salt tolerance showed that *Panicum sumatrense* CO (Samai) 4 variety at seedling stage possesses resilience to salt stress, when exposed to mild stress and could be an efficient source to understand tolerance mechanisms in the tolerant plants and thus, can be used in the future investigation for plant breeding programs, to curb food security against the increasing population.

5. Acknowledgement

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