

## Interactive Effects of Calcium Chloride on Salinity-Induced Oxidative Stress in *Pennisetum typoides*

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**Abstract:** *Pennisetum* plants were grown with NaCl and CaCl<sub>2</sub> in order to study the effect of CaCl<sub>2</sub> on NaCl induced oxidative stress in terms of lipid peroxidation (TBARS content), H<sub>2</sub>O<sub>2</sub> content and antioxidant enzyme activities. The plants were treated with solutions of 100 mM NaCl, 100 mM NaCl with 5 mM CaCl<sub>2</sub> and 5 mM CaCl<sub>2</sub> alone. Groundwater was used for irrigation of control plants. Plants were uprooted randomly on 40 days after sowing (DAS). NaCl-stressed plants showed increased TBARS, H<sub>2</sub>O<sub>2</sub>, when compared to control. The antioxidant enzymes superoxide dismutase (SOD), peroxidase (POX) and catalase (CAT) were increased under salinity and further enhanced due to CaCl<sub>2</sub> treatment.

**Key words:** Salinity • Antioxidants • *Pennisetum* • Oxidative stress

### INTRODUCTION

Plant growth and productivity is adversely affected by various abiotic and biotic stress factors. Plants are frequently exposed to many stress conditions such as low temperature, salt, drought, flooding, heat, oxidative stress and heavy metal toxicity. Various anthropogenic activities have accentuated the existing stress factor [1]. Calcium is a divalent cation that is extremely important in maintaining the strength of stems and stalks of plants. This mineral also regulates the absorption of nutrients across plasma cell membranes. Calcium functions in plant cell elongation and division, structure and permeability of cell membranes, nitrogen metabolism and carbohydrate translocation [2]. Soil salinity is one among the several environmental stresses causing drastic changes in the growth, physiology and metabolism of plants and threatening the cultivation of plants around the globe. Salt accumulation in irrigated soils is one of the main factors that diminish crop productivity, since most of the plants are not halophytic [3]. Salt stress induces various biochemical and physiological responses in plants and affects almost all plant processes [4]. Salinity also induces water deficit, even in well-watered soils by decreasing the osmotic potential of soil solutes, thus making it difficult for roots to extract water from their surrounding media [6]. Salinity can cause hyperionic and

hyperosmotic effects on plants, leading to membrane disorganization, increase in reactive oxygen species (ROS) levels and metabolic toxicity [5].

Excessive sodium (Na<sup>+</sup>) inhibits the growth of many salt-sensitive plants and glycophytes, which include most crop plants. Mechanisms of salt tolerance, not yet clear, can be to some extent explained by stress-adaptation effectors that mediate ion homeostasis, osmolyte biosynthesis, toxic radical scavenging, water transport and long distance response coordination [7]. Chemical treatment and agronomical crop management practices have been tried to alleviate the salt stress, but the application of CaCl<sub>2</sub> to stressed plants attracted little attention. One possible approach to reducing the effect of NaCl stress on plant productivity is through the addition of calcium supplements to irrigation in the case of salt stress [4]. Supplementing the medium with Ca<sup>2+</sup> alleviates growth inhibition by salt in glycophyte plants [8]. Ca<sup>2+</sup> sustains K<sup>+</sup> transport and K<sup>+</sup>/Na<sup>+</sup> selectivity in Na<sup>+</sup> challenged plants. The interaction of Na<sup>+</sup> and Ca<sup>2+</sup> on plant growth and ion relations is well established [9].

In order to survive under stress conditions, plants are equipped with oxygen radical detoxifying enzymes, such as superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT) and antioxidant molecules, like ascorbic acid (AA) and  $\alpha$ -tocopherol, [10-12]. Antioxidant mechanisms may provide a strategy

to enhance salt tolerance in plants. Chemical treatment and agronomical crop management practices have been tried to alleviate the salinity effects without much success and a widespread practice to reduce salt content in soils is leaching. With the rising cost of water, this method may not continue to be a feasible method for the future. In addition, economic pressures on water supplies may force agriculture to use greater amounts of water of lesser quality with respect to salt concentration level [13]. Improving plant resistance to salt, although not a final solution, may provide field stability in subsistence agriculture. A possible alternative is to induce the capability within plants to face successfully the detrimental situation by treatment with growth regulators. Application of growth regulators has been reported to mitigate the adverse effects of salinity [14]. The use of plant growth regulators results in a significant increase in the growth and yield of many crops under stress conditions [15].

The purpose of this study was to provide additional information on the oxidative damage (LPO and  $H_2O_2$  contents) and antioxidant enzymes (SOD, APX and CAT) activities in *Pennisetum* under individual and combined NaCl and  $CaCl_2$  treatments.

## MATERIALS AND METHODS

**Plant Materials and Growth:** The seeds of *Pennisetum* were collected from the Department of Horticulture, Annamalai University, Tamil Nadu, India. Seeds were then surface sterilized in an aqueous solution of 0.1%  $HgCl_2$  for 60 s to prevent fungal attack and rinsed in several changes of sterile water. The seeds were pre-soaked in 500 ml of deionized water (control), 100 mM NaCl, 100 mM NaCl + 5 mM  $CaCl_2$  and 5 mM  $CaCl_2$  solutions for 12 h. Seeds were sown in plastic pots filled with soil mixture containing red soil, sand and farmyard manure (FYM) at 1:1:1 ratio. Before sowing the seeds, the pots were irrigated with the respective treatment solutions and the electrical conductivity (EC) of the soil mixture was measured. Ten seeds were sown per pot and the pots were watered to the field capacity with deionized water up to 40 days after sowing (DAS) and every care was taken to avoid leaching. The initial EC level of the soil was maintained by flushing each pot with the required volume of corresponding treatment solution on 10, 20 and 30 DAS. The position of each pot was randomized at five-day intervals to minimize spatial effects in the greenhouse, where the temperature was 28°C during the day and 22°C at night and the relative humidity (RH) varied between 60 and 70%. The seedlings were thinned

to three per pot on 10 DAS. Plants were uprooted randomly on 40 and 50 DAS and analysed for estimating the oxidative damage, osmolyte concentration, PRO metabolism.

### Oxidative Damage

**$H_2O_2$  Content:** The hydrogen peroxide content was determined according to Velikova et al. [16]. 0.5 g of fresh plant material was homogenized in an ice bath with 5 ml of 0.1% (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 12,000 rpm for 15 min and 0.5 ml of the supernatant was added to 0.5 ml of 10 mM potassium phosphate buffer (pH 7.0) and 1 ml of 1 M potassium iodide (KI). The absorbance of the supernatant was measured at 390 nm in a spectrophotometer (U-2001-Hitachi). The content of  $H_2O_2$  was calculated by comparison with a standard calibration curve, previously plotted by using different concentrations of  $H_2O_2$ .

**Lipid Peroxidation:** LPO was estimated as thiobarbituric acid reactive substances (TBARS) [17]. A fresh sample (0.5 g) was homogenized in 10 ml of 0.1% TCA and the homogenate was centrifuged at 15,000 rpm for 15 min. To 1.0 ml aliquot of the supernatant, 4.0 ml of 0.5% thiobarbituric acid (TBA) in 20% TCA were added. The mixture was heated at 95 °C for 30 min in the laboratory electric oven and then cooled in an ice bath. After centrifugation at 10,000 rpm for 10 min, the absorbance of the supernatant was recorded at 532 nm in our spectrophotometer. The TBARS content was calculated according to its extinction coefficient of  $155 \text{ mM}^{-1}\text{cm}^{-1}$  and expressed in units (U). One 'U' is defined as imole of MDA formed  $\text{min}^{-1} \text{mg}^{-1}$  protein.

### Antioxidant Enzyme Extractions and Assays

**Superoxide Dismutase (SOD, EC 1.15.1.1):** The activity of SOD was assayed as described by Beauchamp and Fridovich [18]. The reaction mixture contained  $1.17 \times 10^{-6}$  M riboflavin, 0.1 M methionine,  $2 \times 10^{-5}$  M KCN and  $5.6 \times 10^{-5}$  M nitroblue tetrazolium (NBT) salt dissolved in 3 ml of 0.05 M sodium phosphate buffer (pH 7.8). Three millilitres of the reaction medium were added to 1 ml of enzyme extract. The mixtures were illuminated in glass test tubes by two sets of Philips 40-W fluorescent tubes in a single row. Illumination was started to initiate the reaction at 30°C for 1 h; identical solutions that were kept under dark served as blanks. The absorbance was read at 560 nm in the spectrophotometer against the blank. SOD activity is expressed in  $\text{Umg}^{-1}$  protein (U = change in 0.1 absorbance  $\text{h}^{-1} \text{mg}^{-1}$  protein).

#### Ascorbate Peroxidase (APX, EC 1.11.1.1) Activity:

APX activity was determined according to Asada and Takahashi [19]. The reaction mixture (1 ml) contained 50mM potassium phosphate buffer (pH 7.0), 0.5mM ascorbic acid, 0.1mM  $H_2O_2$  and 200 $\mu$ l of enzyme extract. The absorbance was read as decrease at 290 nm against the blank, correction was done for the low, non-enzymatic oxidation of ascorbic acid by  $H_2O_2$  (extinction coefficient  $2.9\text{mM}^{-1}\text{cm}^{-1}$ ). The enzyme activity was expressed in  $\text{Umg}^{-1}\text{ protein}$  ( $\text{U} = \text{change in } 0.1 \text{ absorbance min}^{-1}\text{mg}^{-1}\text{ protein}$ ).

**Catalase (CAT, 1.11.1.):** The activity of CAT was measured according the method of Chandlee and Scandalios [20] with small modifications. The assay mixture contained 2.6 ml of 50 mM potassium phosphate buffer (pH 7.0), 0.4 ml of 15 mM  $H_2O_2$  and 0.04 ml of enzyme extract. The decomposition of  $H_2O_2$  was followed by the decline in absorbance at 240 nm. The enzyme activity was expressed in units  $\text{mg}^{-1}\text{ protein}$  ( $\text{U} = 1 \text{ mM of } H_2O_2 \text{ reduction min}^{-1}\text{mg}^{-1}\text{ protein}$ ).

**Statistical Analysis:** Each treatment was analysed with at least seven replicates and a standard deviation (S.D.) was calculated and data are expressed in mean  $\pm$  S.D. of seven replicates.

## RESULTS

**$H_2O_2$  Content:**  $H_2O_2$  content (Fig. 1) increased with salinity treatment in shoot and root, the highest content being 40 DAS in shoot. Under  $\text{CaCl}_2$  treatment in combination with NaCl slightly reduced the  $H_2O_2$  content, but higher than in unstressed plants.  $\text{CaCl}_2$  alone also increased the  $H_2O_2$  content.

**Lipid Peroxidation (TBARS Content):** Oxidative damage to tissue lipid was estimated by the content of total TBARS. The plants under treatments of NaCl showed a trend of increasing content of TBARS (Fig.2). The  $\text{CaCl}_2$  treatment reduced the TBARS content in both root and shoot.

**Superoxide Dismutase:** The SOD activity has been increased in all parts of the NaCl- and  $\text{CaCl}_2$ -stressed plants when compared to control. Addition of  $\text{CaCl}_2$  to NaCl-treated plant increased SOD activity when compared to NaCl-stressed plants (Fig. 3).

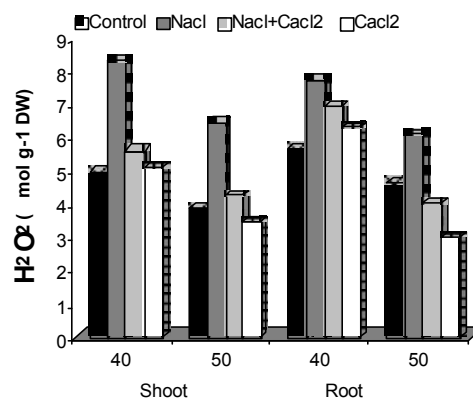


Fig. 1: Effect of NaCl (100mM),  $\text{CaCl}_2$  (5mM) and their combination on  $H_2O_2$  accumulation in *pennisetum typhoides* on 40 and 50 DAS. Values are given as a mean  $\pm$  SD of six samples in each group.

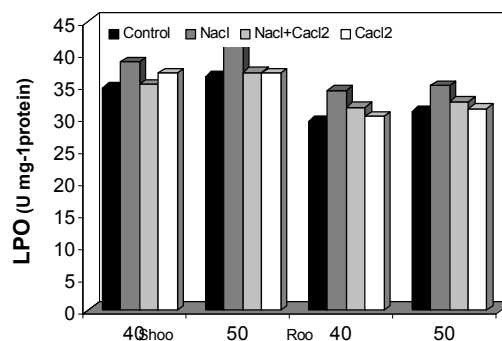


Fig. 2: Effect of NaCl (100mM),  $\text{CaCl}_2$  (5mM) and their combination on LPO accumulation in *pennisetum typhoides* on 40 and 50 DAS. Values are given as a mean  $\pm$  SD of six samples in each group.

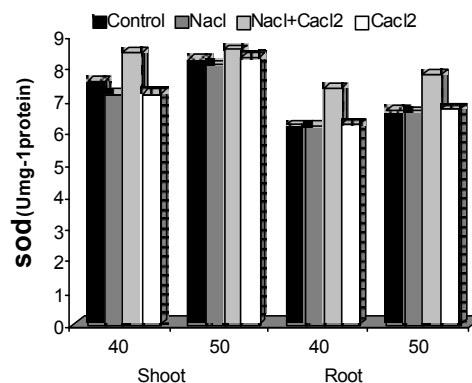


Fig. 3: Effect of NaCl (100mM),  $\text{CaCl}_2$  (5mM) and their combination on Super Oxidase Dismutase (SOD) accumulation in *pennisetum typhoides* on 40 and 50 DAS. Values are given as a mean  $\pm$  SD of six samples in each group.

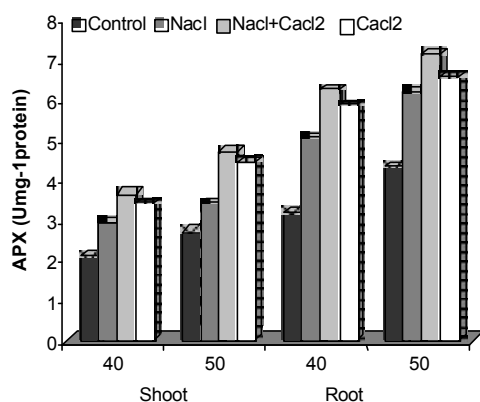


Fig. 4: Effect of NaCl (100mM), CaCl<sub>2</sub> (5mM) and their combination on Ascorbate peroxidase (APX) accumulation in *pennisetum typhoides* on 40 and 50 DAS. Values are given as a mean  $\pm$  SD of six samples in each group.

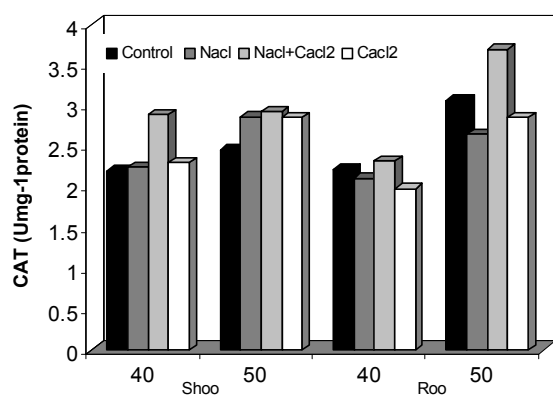


Fig. 5: Effect of NaCl (100mM), CaCl<sub>2</sub> (5mM) and their combination on Catalase (CAT) accumulation in *pennisetum typhoides* on 40 and 50 DAS. Values are given as a mean  $\pm$  SD of six samples in each group.

**Ascorbate Peroxidase:** APX activity increased in all the treatment when compared to control. The CaCl<sub>2</sub> treatment increased the APX activity in NaCl stressed as well as in control plants. CaCl<sub>2</sub> treatment alone increased the APX activity when compared to control and NaCl stress (Fig. 4).

**Catalase:** CAT activity has been increased in all parts of the NaCl- and CaCl<sub>2</sub>-stressed plants when compared to control. The NaCl- - with-CaCl<sub>2</sub> stressed plants increased CAT activity when compared to NaCl- and CaCl<sub>2</sub> stressed plants (Fig. 5).

## DISCUSSION

The individual NaCl (Salt stress) treatment increased the H<sub>2</sub>O<sub>2</sub> content in all parts of *Pennisetum* plants when compared to control. Nevertheless, the combination of NaCl decreased the increased H<sub>2</sub>O<sub>2</sub> content. The CaCl<sub>2</sub> treatment enhanced different H<sub>2</sub>O<sub>2</sub> scavenging enzymes, like SOD, APX and CAT and also non-enzymatic antioxidants. This enhancement would have helped in scavenging of ROS in *Pennisetum*. H<sub>2</sub>O<sub>2</sub> is an endogenous signaling molecule involved in plant responses to abiotic and biotic stresses such as extremes of temperature, light intensity, drought, pathogen, salinity as well as stimuli such as plant hormones and gravity [21]. Accumulation of H<sub>2</sub>O<sub>2</sub> will also lead to enhance potential for production of hydroxyl radicals, which leads to LPO and membrane deterioration [22].

LPO has been associated with damages provoked by a variety of environmental stresses. Poly-unsaturated fatty acids (PUFA) are the main membrane lipid components susceptible to peroxidation and degradation [23]. The increase in LPO can be correlated with the accumulation of ions and active oxygen species (AOS) production under salt stress [24].

SOD activity directly modulates the amount of ROS. Similar results were seen in the case of CAT activity also. The unique importance of Ca<sup>2+</sup> for stabilization of members is well known [25]. SOD and CAT activities have been reported to be negatively correlated with the degree of damage of plasmalemma, chloroplast and mitochondrial membrane systems and positively related to the indices of stress resistance [26]. CaCl<sub>2</sub>-treated seedlings maintain higher levels of SOD and CAT activities and lower levels of LPO and POX activity [27]. ROS scavenging depends on the detoxification mechanism provided by an integrated system of non-enzymatic reduced molecules, like AA and glutathione and enzymatic antioxidants [28]. As part of this, antioxidant enzymes play important roles in the defence mechanism against oxidative stress. Antioxidant mechanisms may provide a strategy to enhance salt tolerance in plants, though the detailed mechanisms are not yet clear [29].

## CONCLUSION

From these results, it can be concluded that the addition of CaCl<sub>2</sub> to NaCl-stressed *Penisetum* plants have a significant role in partial alleviation of salinity stress.

Thus, it is clear that plants under salt stress are highly regulated by components of the antioxidative system and secondary metabolite contents. Our results indicated that the cultivation of plants like *Pennisetum* in saline areas would increase its PRO metabolism, defence mechanisms and the level of active principles. However, the data presented here reflect the importance of a physiological analysis of plant response, which must accompany field experiments and evaluation. Further investigations are required to ascertain this conclusion.

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