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# Interactive Effects of Calcium Chloride on Salinity-Induced Proline Metabolism in *Pennisetum typoidies*

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**Abstract:** *Pennisetum* plants were grown with NaCl and $CaCl_2$ in order to study the effect of $CaCl_2$ on NaCl induced oxidative stress in terms of osmolyte concentration, proline (PRO)-metabolizing enzymes. The plants were treated with solutions of 100 mM NaCl, 100 mM NaCl with 5 mM $CaCl_2$ and 5 mM $CaCl_2$ alone. Groundwater was used for irrigation of control plants. Plants were uprooted randomly on 40 days after sowing (DAS). NaCl-stressed plants showed increased glycine betaine (GB) and PRO contents, decreased proline oxidase (PROX) activity and increased γ-glutamyl kinase (γ-GK) activity when compared to control. Addition of $CaCl_2$ to NaCl-stressed plants lowered the PRO concentration by increasing the level of PROX and decreasing the γ-GK activities. Calcium ions increased the GB contents. $CaCl_2$ appears to confer greater osmoprotection by the additive role with NaCl in GB accumulation.

**Key words:** Osmolytes · Proline oxidase · γ-Glutamyl kinase · Salinity · *Pennisetum*

## INTRODUCTION

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 [1]. 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 [5,6].

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 [7]. Salinity can cause hyperionic and hyperosmotic effects on plants, leading to membrane disorganization, increase in reactive oxygen species (ROS) levels and metabolic toxicity [8]. Excessive sodium ($Na^+$) 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 [9]. Chemical treatment and agronomical crop management practices have been tried to alleviate the salt stress, but the application of $CaCl_2$ 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 [10]. Supplementing the medium with $Ca^{2+}$ alleviates growth inhibition by salt in glycophyte plants [11]. $Ca^{2+}$ sustains $K^+$ transport and $K^+/Na^+$ selectivity in $Na^+$ challenged plants. The interaction of Na+ and $Ca^{2+}$ on plant growth and ion relations is well established [12]. The typical first response of all plants to salt stress is osmotic adjustment. Compatible solutes accumulation in the cytoplasm is considered as a mechanism to contribute salt tolerance [13]. To counter with salt stress, plants increase the osmotic potential of their cells by synthesizing and accumulating compatible osmolytes such as proline (PRO) and glycine betaine (GB), which participates in the

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osmotic adjustment [14]. PRO and GB are thought to function as osmoprotectants for proteins [15]. Accumulation of PRO and GB provide an environment compatible with the macromolecular structure and function and helps to adapt the salinity injury [16]. Proline oxidase (PROX) and γ-glutamyl kinase (γ-GK) play an important role in controlling the level of PRO, PROX catalyzes the conversion of PRO to glutamate and γ-GK plays an important role in the synthesis of PRO [16]. The enzymes γ-GK and γ-glutamyl phosphate reductase (γ-GPR) are regarded as an enzyme complex called pyrroline-5-carboxylate (P5C) synthetase because the resulting product, glutamic γ-semialdehyde is non-enzymatically converted to P5C. From there, the P5C is converted into PRO by -pyrroline-5-carboxylate reductase (P5CR). The regulation of PRO synthesis is probably controlled by the activity of P5C synthase [14]. In addition to this, an important consequence of salinity stress in plants is the excessive generation of ROS such as superoxide anion ($O_2$-), $H_2O_2$ and the hydroxyl radicals, particularly in chloroplasts and mitochondria [12, 13]. Generation of ROS causes rapid cell damage by triggering off a chain reaction [14].

The purpose of this study was to provide additional information on the osmolyte concentration (GB and PRO contents) and PRO metabolizing enzymes (γ-GK and PROX) 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 osmolyte concentration, PRO metabolism.

**Osmolyte Concentration**

**Glycine Betaine Content:** The amount of GB was estimated according to the method of Grieve and Grattan [17]. The plant tissue was finely ground, mechanically shaken with 20 ml deionized water for 24 h at 25°C. The samples were then filtered and filtrates were diluted to 1:1 with 2 N $H_2SO_4$. Aliquots were kept in centrifuge tubes and cooled in ice water for 1 h. Cold KI–$I_2$ reagent was added and the reactants were gently stirred with a vortex mixture. The tubes were stored at 4°C for 16 h and then centrifuged at 10,000 rpm for 15 min at 0°C. The supernatant was carefully aspirated with a fine glass tube. The periodide crystals were dissolved in 9 ml of 1,2-dichloroethane. After 2 h, the absorbance was measured at 365 nm using GB as standard.

**Proline Content:** The PRO content was estimated by the method of Bates *et al.* [18]. The plant material was homogenized in 3% aqueous sulfosalicylic acid and the homogenate was centrifuged at 10,000 rpm. The supernatant was used for the estimation of the PRO content. The reaction mixture consisted of 2 ml of acid ninhydrin and 2 ml of glacial acetic acid, which was boiled at 100°C for 1h. After termination of reaction in ice bath, the reaction mixture was extracted with 4 ml of toluene and absorbance was read at 520 nm.

**Proline Metabolizing Enzymes**

***γ-Glutamyl Kinase [ATP: L-glutamate 5-phosphotransferases (EC 2.7.2.11)] Activity*:** γ-GK activity was assayed by the method of Hayzer and Leisinger [19]. Plant samples (1 g) were extracted with 50 mM Tris-HCl buffer and centrifuged at 40,000 g for 30 min at 4°C. 0.1 ml reaction buffer was prepared by adding 0.1 ml 10 × ATP and 1.8 ml of extract and incubated at 37°C for 30 min, 2 ml of stop buffer was added. γ-GK activity was measured at 535 nm and expressed in units (U $mg^{-1}$ protein). One unit of enzyme activity is defined as μg of γ-glutamylhydroxamate formed $min^{-1}$ $mg^{-1}$ protein.

***Proline oxidase [L. Proline: O2 Oxidoreductase (EC 1.4.3.1)] Activity*:** PROX activity was determined according to the method outlined by Huang and Cavalieri [20]. Plant samples (1 g) were extracted with 5 ml of Tris-HCl buffer (pH 8.5) grinding medium and centrifuged at 10,000 *g* for 10 min at 4°C. The supernatant was again centrifuged at 25,000 *g* at 20 min at 4°C. A 3-ml assay mixture was prepared by taking 0.1 ml of extract, 1.2 ml of 50 mM Tris HCl buffer (pH 8.5), 1.2 ml of 5 mM $MgCl_2$, 0.1 ml of 0.5 mM NADP, 0.1 ml of 1 mM KCN, 0.1 ml of 1 mM phenazine methosulphate (PMS), 0.1 ml of 0.06 mM 2,6-dichlorophenol indophenol (DCPIP) and 0.1 ml distilled water instead of PRO. The reaction was monitored at 600 nm at 25°C using PRO to initiate reaction; the OD value's increase was noted at 0, 1, 2, 3, 4 and 5 min. PROX activity was expressed in $Umg^{-1}$ protein (one U = mM DCPIP reduced $min^{-1}$ $mg^{-1}$ 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 ± S.D. of seven replicates.

## RESULTS

**Glycine Betaine Content:** One of the most important mechanisms exerted byhigher plants under salt-stress conditions is the accumulation of compatible solutes such as GB. In the present study, the amount of GB content increased with individual and combined treatments of NaCl and $CaCl_2$ in *Pennisetum* plants. The GB accumulation was higher in $CaCl_2$ treatment when compared to unstressed plants (Fig. 1).

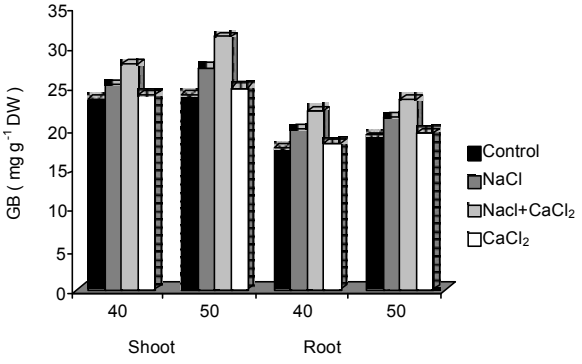


Fig. 1: Effect of NaCl (100mM), $CaCl_2$ (5mM) and their combination on GB accumulation in *pennisetum typhoides* on 40 and 50 DAS. Values are given as a mean ± SD of six samples in each group.

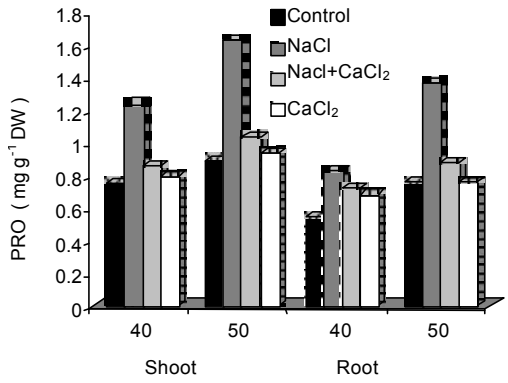


Fig. 2: Effect of NaCl (100 mM), $CaCl_2$ (5 mM) and their combination on PRO accumulation in *pennisetum typhoides* on 40 and 50 DAS. Values are given as a mean ± SD of six samples in each group.

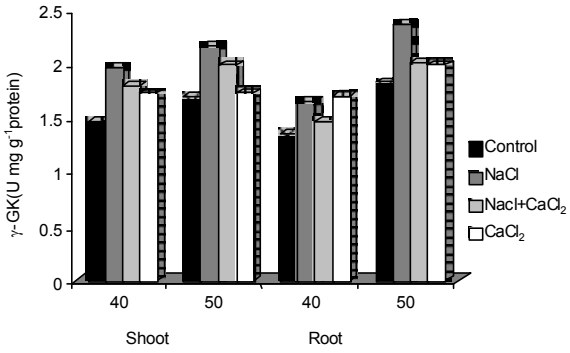


Fig. 3: Effect of NaCl (100 mM), $CaCl_2$ (5 mM) and their combination on γ-GK accumulation in *pennisetum typhoides* on 40 and 50 DAS. Values are given as a mean ± SD of six samples in each group.

**Proline Content:** Compatible solute, which accumulates under salt stress in plants, is PRO. In the present study, an increase in PRO accumulation in *Pennisetum* seedlings under salinity, with a concomitant increase in γ-GK (PRO synthesizing enzyme) and a decrease in PROX (PRO degrading enzyme) activities (Fig. 2) was observed. PRO content was diminished upon the addition of $CaCl_2$; anyhow, $CaCl_2$ alone increased PRO when compared to unstressed control.

**γ-Glutamyl Kinase Activity:** The γ-GK activity has been increased largely in shoot and root in the NaCl-stressed plants when compared with control (Fig. 3). NaCl-with-$CaCl_2$-treated plants showed decreased γ-GK activity when compared to NaCl-stressed and control plants. $CaCl_2$ alone also increased the level of γ-GK when compared to control, but less than in NaCl treatment.

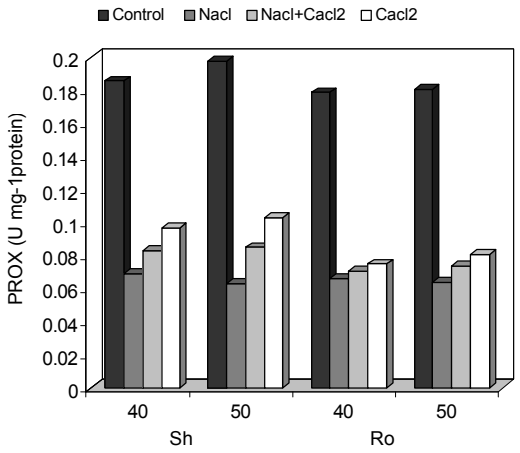


Fig. 4: Effect of NaCl (100 mM), $CaCl_2$ (5 mM) and their combination on PROX accumulation in *pennisetum typhoides* on 40 and 50 DAS. Values are given as a mean ± SD of six samples in each group.

**Proline Oxidase Activity:** PROX activity has been inhibited largely by NaCl, $CaCl_2$ and their combination in all parts of *Pennisetum* when compared with control. The lowest value was recorded in NaCl treatments. Addition of $CaCl_2$ to NaCl-treated plants increased the PROX activity when compared to NaCl-stressed plants, but less than in unstressed plants (Fig. 4).

## DISCUSSION

In a majority of plants, salt stress leads to changes in gene expression, leading to an increased synthesis of osmoprotectors and osmoregulators [11]. Osmotic adjustment is the main component of physiological machinery by which plants respond to soil-salt stress. In most plants, there is an increased accumulation of amino acids and amines (e.g., PRO, B-alanine, GB) in their tissues in response to salt stress. The way these compounds are accumulated differs between species and ranges from only one to several different compounds being accumulated. Generally, plant species that accumulate PRO usually have low amounts of this amino acid when grown in well-watered and non-saline soils, increasing its contents upon imposition of drought or salt stresses [12]. The GB accumulation that resulted from the NaCl-induced oxidative stress is helpful in the stimulation of salt-tolerance mechanisms [13, 14].

The induction of PRO accumulation may be due to an activation of PRO synthesis through glutamate pathway involving γ-GK, glutamyl phosphate reductase and P5CR activities. The PRO metabolizing enzyme, γ-GK, increased under the NaCl salinity in *Pennisetum* seedlings. This enzyme plays an important role in the synthesis of PRO. The γ-GK activity can be inversely correlated with PROX activity and protein content in salt-treated plants [13]. PRO accumulation in NaCl-stressed seedlings can be attributed in part to the increased level of γ-GK activity [15].

PROX activity decreased under NaCl stress in *Pennisetum* seedlings when compared to control. This enzyme converts free PRO into glutamate. Reduction in PROX activity and simultaneous increase in PRO level were reported in low-temperature-stressed wheat [14]. PROX oxidizes the PRO and converts it back to glutamate. This enzyme also influences the level of free PRO. The activities of PRO degrading enzymes, PROX and proline dehydrogenase (PDH), were significantly inhibited in the salt-stressed green gram seedlings [21]. PRO may act as a non-toxic osmotic solute preferentially located in the cytoplasm, or as an enzyme protectant, stabilizing the structure of macromolecules and organelles. Accumulated PRO may supply energy to increase salinity tolerance [22]. PRO, as an osmoprotectant compound, plays a major role in osomoregulation and osmotolerance [23]. However, its definite role in exerting salinity resistance continues to be a debate [24].

Several plants, including halophytes, accumulate high PRO levels in response to osmotic stress as a tolerance mechanism to high salinity and water deficit [25]. In plants, PRO is synthesized from either glutamate or ornithine. However, the glutamate pathway is primary route used under osmotic stress or nitrogen limitation conditions, whereas the ornithine pathway is prominent under high nitrogen input [26]. However, recent data suggest that glutamate is the major amino acid involved in PRO synthesis, since transgenic tobacco plants with reduced expression of cytosolic glutamine synthetase accumulated less PRO than non-transformed plants in response to salt stress. Accumulation of PRO in plants under stress is a result of the reciprocal regulation of two pathways: [(Δ-1-pyrroline-5-carboxylate synthetase (P5CS) and P5CR] and repressed activity of PRO degradation [27]. PRO catabolism is catalyzed by pyrroline-5-carboxylate dehydrogenase and PDH, a mitochondrial enzyme, whose activity had been shown to reduce during salt stress. The first two steps of PRO biosynthesis are catalyzed by P5CS by means of its γ-GK

and glutamic-γ-semialdehyde dehydrogenase activities. Subsequently, the P5C formed is reduced by P5CR to PRO [28].

Although the precise role of PRO accumulation is still debated, PRO is often considered as a compatible solute involved in osmotic adjustment [29]. Accumulation of PRO may occur through an increase in its synthesis constantly with inhibition of its catabolism [30] and may be a mechanism for stress tolerance. However, its role in imparting stress resistance under saline conditions is controversial. Anyway, understanding the biosynthesis, degradation, transport and role of PRO during stress and the signalling events that regulate stress-induced accumulation is vital in developing plants for stress tolerance [31].

## REFERENCES


1. Jaleel, C.A., P. Manivannan, A. Kishorekumar, B. Sankar, R. Gopi, R. Somasundaram and R. Panneerselvam, 2007. Alterations in osmoregulation, antioxidant enzymes and indole alkaloid levels in *Catharanthus roseus* exposed to water deficit, Colloids Surf. B: Biointerfaces, 59: 150-157.
2. Jaleel, C.A., P. Manivannan, B. Sankar, A. Kishorekumar, R. Gopi, R. Somasundaram and R. Panneerselvam, 2007. Induction of drought stress tolerance by ketoconazole in *Catharanthus roseus* is mediated by enhanced antioxidant potentials and secondary metabolite accumulation, Colloids Surf. B: Biointerfaces , 60: 201-206.
3. Jaleel, C.A., P. Manivannan, B. Sankar, A. Kishorekumar, R. Gopi, R. Somasundaram and R. Panneerselvam, 2007. Water deficit stress mitigation by calcium chloride in *Catharanthus roseus*; effects on oxidative stress, proline metabolism and indole alkaloid accumulation, Colloids Surf. B: Biointerfaces, 60: 110-116.
4. Jaleel, C.A., P. Manivannan, B. Sankar, A. Kishorekumar, R. Gopi, R. Somasundaram and R. Panneerselvam, 2007. *Pseudomonas fluorescens* enhances biomass yield and ajmalicine production in *Catharanthus roseus* under water deficit stress, Colloids Surf. B: Biointerfaces, 60: 7-11.
5. Uniyal, G.C., S. Bala, A.K. Mathur and R.N. Kulkarni, 2001. Symmetry C18 column: A better choice for the analysis of indole alkaloids of *Catharanthus roseus*, Phytochem. Anal., 12: 206-210.
6. Hernandez, J.A., M.A. Ferrer, A. Jimenez, A.R. Barcelo and F. Sevilla, 2001. Antioxidant system and $O_2$•-/$H_2O_2$ production in the apoplast of *Pisum sativum* L. leaves: its relation with NaCl-induced necrotic lesions in minor veins, Plant Physiol., 127: 817-831.
7. Bor, M., F. Ozdemir and I. Turkan, 2003. The effect of salt stress on lipid peroxidation and antioxidants in leaves of sugar beet *Beta vulgaris* L. and wild beet *Beta maritima* L, Plant Sci., 164: 77-84.
8. Hong-Bo, S., C. Xiao-Yan, C. Li-Ye, Z. Zi-Ning, W. Gang, Y. Yong-Bing, Z. Chang-Xing and H. Zan-Min, 2006. Investigation on the relationship of proline with wheat anti-drought under soil water deficits, Colloids Surf. B: Biointerfaces, 53: 113-119.
9. Teixeira, J. and S. Pereira, 2007. High salinity and drought act on an organdependent manner on potato glutamine synthetase expression and accumulation, Environ. Exp. Bot., 60(1): 121-126.
10. Girija, C., B.N. Smith and P.M. Swamy, 2002. Interactive effects of NaCl and CaCl2 on the accumulation of proline and glycine betaine in peanut (Arachis hypogaea L.), Environ. Exp. Bot., 47: 1-10.
11. Demiral, T., I. Turkan, 2006. Exogenous glycine betaine affects growth and proline accumulation and retards senescence in two rice cultivars under NaCl stress, Environ. Exp. Bot., 56: 72-79.
12. Sakamoto, A. and N. Murata, 1998. Metabolic engineering of rice leading to biosynthesis of glycine betaine and tolerance to salt and cold, Plant Mol. Biol., 38: 1011-1019.
13. Sulochana, C.H., T.J.V. Sreenivasa Rao and N. Savithramma, 2002. Effect of calcium on water stress amelioration through calmodulin and scavenging enzymes in groundnut, Indian J. Plant Physiol., 7: 151-158.
14. Prochazkova, D., R.K. Sairam, G.C. Srivastava and D.V. Singh, 2001. Oxidative stress and antioxidant activity as the basis of senescence in maize leaves, Plant Sci., 161: 765-771.
15. Misra, N. and A.K. Gupta, 2005. Effect of salt stress on proline metabolism in two high yielding genotypes of greengram, Plant Sci., 169: 331-339.
16. Charest, C.T. and Phan, 1990. Cold acclimation of wheat (Triticum aestivum): Properties of enzymes involved in proline metabolism, Physiol. Plant, 80: 159-168.

17. Grieve, C.M. and S.R. Grattan, 1983. Rapid assay for determination of water-soluble quaternary ammonium compounds, Plant Soil, 70: 303-307.
18. Bates, L.S. R.P. Waldern and I.D. Teare, 1973. Rapid determination of free proline for water stress studies, Plant Soil, 39: 205-207.
19. Hayzer, D.J. and T.H. Leisinger, 1980. The gene-enzyme relationships of proline biosynthesis in *Escherichia coli*, J. Gen. Microbiol., 118: 287-293.
20. Huang, A.H.C. and A. Cavalieri, 1979. Proline oxidase and water stress induced proline accumulation in spinach leaves, Plant Physiol., 63: 531-535.
21. Sudhakar, P.S. Reddy and K. Veeranjaneyulu, 1993. Effect of salt stress on the enzymes of proline synthesis and oxidation of green gram seedlings, J. Plant Physiol., 141: 621-623.
22. Demir, Y., 2000. Growth and proline content of germinating wheat genotypes under ultraviolet light, Turk. J. Bot., 24: 67-70.
23. Delauney, A.J. and D.P.S. Verma, 1993. Proline biosynthesis and osmoregulation in plants, Plant J., 4: 215-223.
24. Hu, C.A., A.J. Delauney and D.P.S. Verma, 1992. A bifunctional enzyme ($\Delta^1$-Pyrroline-5-carboxylate synthetase) catalyzes the first two steps in proline biosynthesis in plants, Proc. Natl Acad. Sci. USA 89: 9354-9358.
25. Azooz, M.M., M.A. Shaddad and A.A. Abdel-Latef, 2004. The accumulation and compartmentation of proline in relation to salt tolerance of three sorghum cultivars, Indian J. Plant Physiol., 9: 1-8.
26. Yoshiba, Y., T. Kiyouse, K. Nakashima, K. Yamaguchi-Shinozaki and K. Shinozaki, 1997. Regulation of levels of proline as an osmolyte in plants under water stress, Plant Cell Physiol., 38: 1095-102.
27. Kavikishore, P.B., S. Sangam, R.N. Amrutha, P. Srilaxmi, K.R. Naidu, K.R.S.S. Rao, S. Rao, K.J. Reddy, P. Theriappan and N. Sreenivasulu, 2005. Regulation of proline biosynthesis, degradation, uptake and transport in higher plants: its implications in plant growth and abiotic stress tolerance, Curr. Sci., 8: 424-438.
28. Marinos, N.G., 1962. Studies on submicroscopic aspects of mineral deficiencies. I. Calcium deficiency in the shoot apex of barley, Am. J. Bot., 49: 834-841.
29. Wang, S.G. and Y. Liang, 1995. Protection of 6-benzyladine on cell membrane system of rice seedlings under chilling stress, J. Rice Sci., 9: 223-229.
30. Jaleel, C.A., P. Manivannan, B. Sankar, A. Kishorekumar, R. Gopi, R. Somasundaram and R. Panneerselvam, 2007. Water deficit stress mitigation by calcium chloride in *Catharanthus roseus*: Effects on oxidative stress, proline metabolism and indole alkaloid accumulation, Colloids Surf. B: Biointerfaces, 60: 110-116.
31. Asada, K. and M. Takahashi, 1987. Production and scavenging of active oxygen in photosynthesis, in: D.J. Kyle, B. Osmond and C.J. Arntzen (Eds.), Photoinhibition, Elsevier, Amsterdam, pp: 227-287.