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Salt stress mitigation by calcium chloride in *Phyllanthus amarus*

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A pot culture experiment was carried out in *Phyllanthus amarus* plants in order to study the effect of calcium chloride ($CaCl_2$) as an agent ameliorating sodium chloride (NaCl) stress. The plants were raised in pots and salinity stress was imposed by 80 mM of NaCl. The ameliorating effect of $CaCl_2$ was studied by giving 5 mM $CaCl_2$ to the stressed plants. NaCl-stressed plants showed decreased proteins, total sugars, polyphenol oxidase and catalase activity with increased free amino acids, proline and peroxidase activities. When $CaCl_2$ was combined with NaCl, the $CaCl_2$ altered overall plant metabolism and paved the way for partial amelioration of the oxidative stress caused by salinity.

Key words: antioxidant enzymes, calcium chloride, *Phyllanthus amarus*, proline, protein, sodium chloride.

Abbreviations: POX – peroxidase activity, PPO – polyphenol activity, DAS – days after sowing, CAT – catalase

Introduction

Salt accumulation in irrigated soils is one of the main factors that diminish crop productivity, since most plants are not halophytic (HOSHIDA et al. 2000). Salt stress induces various biochemical and physiological responses in plants and affects almost all plant processes (NEMOTO and SASAKUMA 2002). Stresses associated with temperature, salinity and drought singly or in combination are likely to enhance the severity of problems to which plants will be exposed in the coming decades (DUNCAN 2000). Salinity affects plant growth and germination in a variety of ways i.e., reducing water uptake and nutrient availability, causing toxic accumulation of sodium chloride and other ions. Salinity 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 (SAIRAM and SRIVASTAVA 2002). Excessive sodium (Na^+) inhibits the growth of many salt-sensitive plants and glycophytes, which include most crop plants. The typical first response of all plants to salt stress is osmotic adjustment. Compatible solute accumulation in the cytoplasm is considered a mechanism to contribute salt tolerance (JALEEL et al. 2007a).

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An important consequence of salinity stress in plants is the excessive generation of ROS such as the superoxide anion (O_2^-), H_2O_2 and the hydroxyl radicals, particularly in chloroplast and mitochondria (MITTLER 2002). In order to survive under stress conditions, plants are equipped with oxygen radical detoxifying enzymes such as superoxide dismutase, ascorbate peroxidase, catalase (and antioxidant molecules like ascorbic acid), α-tocopherol and reduced glutathione (JALEEL et al. 2007b). Antioxidant mechanisms may provide a strategy to enhance salt tolerance in plants.

One possible approach to reducing the effect of salinity on plant productivity is through the addition of calcium supplements to irrigation water (SOHAN et al. 1999). Supplementing the medium with Ca^{2+} alleviates growth inhibition by salt in glycophyte plants. 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 (RENGEL 1992). The effect of salt stress on nutrition is particularly interesting because Ca^{2+} is one of the important factors involved in the resistance of plants to salt stress. Calcium deficiency symptoms generally arise from differences in its allocation in the growing regions of plants (GREENWAY and MUNNS 1980). Calcium is known to increase salinity tolerance in many crop plants. Under saline conditions root growth has been found to be regulated by calcium and found to mitigate the adverse effect in barley (CRAMER et al. 1990).

Phyllanthus amarus (Family: Euphorbiaceae), although considered from the farm point of view a a weed, is a valuable medicinal plant used by herbalists (OUDHIA and TRIPATHI 2002). The roots, leaves, fruits, milky juice and whole plants are used in medicinal preparations. The bark yields a bitter principle called phyllanthin (SHAKILA and RAJESWARI 2006). The ameliorative effect of $CaCl_2$ during growth under NaCl stress requires detailed physiological studies in *P. amarus* plants. This study aims at an understanding of the effect of salinity on the physiological parameters of *P. amarus* under NaCl stress and also of the ameliorative effect of $CaCl_2$ on NaCl stressed plants.

Materials and methods

The seeds of *Phyllanthus amarus* were obtained from the Herbal Folklore Research Centre, Andhra Pradesh, India. The seeds were surface sterilized with 0.2% (w/w) $HgCl_2$ solution for 5 min with frequent shaking and then thoroughly washed with deionised water. The seeds were sown in plastic pots (300 mm diameter) filled with 3 kg of soil mixture containing red soil, sand and farmyard manure at a 1:1:1 ratio. Ten seeds were sown per pot and all the pots were watered to field capacity with ground water up to 19 days after sowing. On 20 days after sowing the pots were irrigated with groundwater (control), 80 mM NaCl, 80 mM NaCl with 5 mM $CaCl_2$ and 5 mM $CaCl_2$ solution. The plants were harvested randomly 30, 40 and 50 days after sowing and used for estimating the biochemical markers and antioxidant enzyme activities.

The protein content was determined by the method of BRADFORD (1976). Total free amino acids were extracted and estimated using the method of MOORE and STEIN (1948).

The proline content was estimated by the method of BATES et al. (1973). The plant material was homogenized in 3 % aqueous sulfosalicylic acid and the homogenate was centrifuged at 10000 g. Supernatant was used for estimation of proline content. The reaction mixture consisted of 2 ml acid ninhydrin and 2 ml of glacial acetic acid, which was boiled

at 100 °C for 1 h. After termination of the reaction in an ice bath, the reaction mixture was extracted with 4 ml of toluene and absorbance was read at 520 nm.

Soluble sugars were estimated from the samples by the method of DUBOIS et al. (1956). Plant samples were homogenized in 80% ethanol (v/v). The homogenate was refluxed over a water bath and centrifuged. The residue was extracted twice more with 80% ethanol. The supernatant liquids were combined and used for the estimation of soluble sugars.

Peroxidase activity of (POX) was assayed by the method of KUMAR and KHAN (1982). The assay mixture contained 2 ml of 0.1 M phosphate buffer (pH 6.8), 1 ml of 0.01 M pyrogallol, 1 ml of 0.005 M H_2O_2 and 0.5 ml of enzyme extract. The solution was incubated for 5 min at 25 °C after which the reaction was terminated by adding 1 ml of 1.25 M H_2SO_4. The amount of purpurogallin formed was determined by measuring the absorbance at 420 nm against a blank prepared by adding the extract after the addition of 1.25 M H_2SO_4 at zero time. The activity was expressed in U mg^{-1} protein. One U is defined as the change in the absorbance by 0.1 min^{-1} mg^{-1} protein.

The assay of polyphenol oxidase (PPO) was carried out by the method of KUMAR and KHAN (1982). The assay mixture contained 2 ml of 0.1 M phosphate buffer (pH 6.0), 1 ml of 0.1 M catechol and 0.5 ml of enzyme extract. This was incubated for 5 min at 25 °C, after which the reaction was stopped by adding 1 ml of 1.25 M H_2SO_4. The absorbance of the quinone formed was read at 495 nm. To the blank 1.25 M H_2SO_4 was added of the zero time of the same assay mixture. PPO activity is expressed in U mg^{-1} protein (U = Change in 0.1 absorbance min^{-1} mg^{-1} protein).

Catalase was measured according to the method of CHANDLEE and SCANDALIOS (1984) 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 U mg^{-1} protein (U = 1 mM of H_2O_2 reduction min^{-1} mg^{-1} protein).

Statistical analysis was performed using one way analysis of variance (ANOVA) followed by Duncan's Multiple Range Test. The values are mean ± SD for six samples in each group. P values ≤ 0.05 were considered as significant.

Results

The NaCl lowered the protein content and $CaCl_2$ stress in root, stem and leaves of the *P. amarus* plants when compared with control plants. Addition of $CaCl_2$ with NaCl treatments increased the protein content when compared with NaCl-stressed plants (Tab.1).

NaCl and $CaCl_2$ treatments increased the free amino acids and proline content in all parts of the plants as compared to the control. $CaCl_2$ treatment to the NaCl stressed plants lowered the free amino acid and proline content in all parts when compared with NaCl treated plants (Tab.1). Among the organs the leaves showed a higher free amino acid and proline content followed by the root and stem in all the treatments.

NaCl and $CaCl_2$ treatments decreased the total sugar content in all organs when compared with control plants (Tab.1). When $CaCl_2$ was combined with NaCl treatments the plants showed increased the total sugar content when compared with NaCl-treated plants.

Tab. 1.

Parameters	DAS	Root				Stem				Leaf			
		Control	80 mM NaCl	80 mM NaCl + 5 mM $CaCl_2$	5 mM $CaCl_2$	Control	80 mM NaCl	80 mM NaCl + 5 mM $CaCl_2$	5 mM $CaCl_2$	Control	80 mM NaCl	80 mM NaCl + 5 mM $CaCl_2$	5 mM $CaCl_2$
Proteins mg g^{-1} DW	30	29.24 ± 0.69	23.43 ± 0.57*	29.98 ± 0.69	30.20 ± 0.68*	21.90 ± 0.53	17.92 ± 0.44*	20.88 ± 0.52	20.31 ± 0.50	26.41 ± 0.66	20.30 ± 0.50*	25.47 ± 0.63	24.62 ± 0.61*
	40	35.73 ± 0.74	30.24 ± 0.80*	35.37 ± 0.73	33.11 ± 0.73*	31.83 ± 0.77	26.23 ± 0.55*	30.40 ± 0.76*	30.07 ± 0.75	32.65 ± 0.81	28.24 ± 0.70*	32.28 ± 0.80	31.57 ± 0.78
	50	37.74 ± 0.92	31.82 ± 0.77*	36.97 ± 0.89	35.24 ± 0.85*	33.07 ± 0.80	27.26 ± 0.68*	31.46 ± 0.78	30.31 ± 0.75	35.10 ± 0.87	30.21 ± 0.75*	34.88 ± 0.87	33.20 ± 0.83
Free amino acids mg g^{-1} DW	30	6.53 ± 0.28	6.80 ± 0.19	6.51 ± 0.17	6.62 ± 0.17*	4.92 ± 0.11	4.11 ± 0.12*	3.89 ± 0.11*	3.99 ± 0.12	7.44 ± 0.24	8.62 ± 0.28*	7.40 ± 0.23	8.09 ± 0.26*
	40	8.09 ± 0.24	8.87 ± 0.24	8.03 ± 0.23	8.66 ± 0.25*	6.32 ± 0.16	6.76 ± 0.25	5.28 ± 0.16*	5.98 ± 0.18*	8.00 ± 0.26	8.66 ± 0.28	7.99 ± 0.26	8.45 ± 0.27
	50	9.66 ± 0.25	10.35 ± 0.32**	9.52 ± 0.27	9.96 ± 0.29	8.63 ± 0.24	7.87 ± 0.25	7.60 ± 0.25*	7.68 ± 0.24*	9.40 ± 0.30	9.98 ± 0.34	9.38 ± 0.32	9.54 ± 0.33
Proline mg g^{-1} DW	30	0.41 ± 0.01	0.51 ± 0.01*	0.44 ± 0.01	0.48 ± 0.01	0.45 ± 0.01	0.49 ± 0.01	0.39 ± 0.01*	0.40 ± 0.01	0.39 ± 0.01	0.57 ± 0.02*	0.42 ± 0.01	0.49 ± 0.01
	40	0.81 ± 0.03	1.17 ± 0.03*	1.57 ± 0.03*	1.10 ± 0.03*	0.98 ± 0.03	1.10 ± 0.03*	0.90 ± 0.03	0.99 ± 0.03	0.88 ± 0.03	1.13 ± 0.03	0.92 ± 0.03	1.02 ± 0.03
	50	0.96 ± 0.03	1.32 ± 0.04	1.15 ± 0.03	1.19 ± 0.03	0.99 ± 0.03	1.39 ± 0.04*	1.07 ± 0.03	1.18 ± 0.03	0.96 ± 0.03	1.13 ± 0.04*	0.99 ± 0.03	1.21 ± 0.04*
Total sugars mg g^{-1} DW	30	1.85 ± 0.04	1.62 ± 0.03	1.80 ± 0.04	1.72 ± 0.04	1.81 ± 0.02	1.24 ± 0.02*	1.41 ± 0.02	1.30 ± 0.02*	1.53 ± 0.03	1.23 ± 0.02	1.48 ± 0.03	1.36 ± 0.02
	40	3.94 ±0.12	3.67 ± 0.10	3.89 ± 0.11	3.77 ± 0.11	3.18 ± 0.08	2.82 ± 0.07	3.03 ± 0.08	2.93 ± 0.08	3.48 ± 0.10	3.24 ± 0.09	3.39 ± 0.09	3.34 ± 0.09
	50	4.33 ± 0.13	3.93 ± 0.11*	4.18 ± 0.12	4.14 ± 0.12	3.89 ± 0.11	3.45 ± 0.10*	3.68 ± 0.10	3.62 ± 0.10	4.03 ± 0.12	3.71 ± 0.11*	3.89 ± 0.11	3.89 ± 0.11

Among the organs, the roots showed a higher total sugar content, followed by a leaf and stem in the treatment.

NaCl treatment decreased the protein, sugar content and increased the free amino acid and proline content. $CaCl_2$ treatment improved all these solutes examined except for the free amino acids, which were decreased to the level of control by $CaCl_2$ treatments. Addition of calcium increased the protein and decreased the free amino acid in the salt-stressed plants (Tab.1).

NaCl- and $CaCl_2$-treated plants showed increased peroxidase activity as compared with the control. Combining $CaCl_2$ with the NaCl treatment decreased peroxidase activity as compared with NaCl-treated plants (Fig. 1). Under NaCl and $CaCl_2$ stress, the activities of antioxidant enzymes like polyphenol oxidase (Fig. 2) and catalase (Fig. 3) decreased in all parts of the plants as compared to the control. When $CaCl_2$ was combined with NaCl, the treatment increased the polyphenol oxidase and catalase activity as compa-

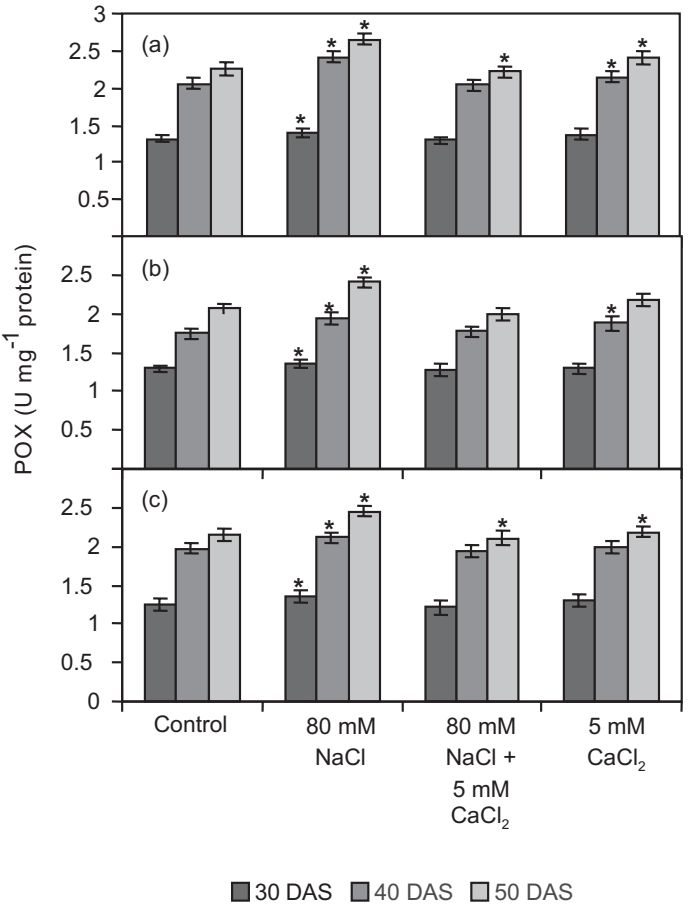

Fig. 1. Effect of NaCl, $CaCl_2$ and their combinations on peroxidase (POX) activity in the (a) root, (b) stem and (c) leaf of *P. amarus*. Values are given as mean ± SD of six samples in each group. * – significant difference at $p \leq 0.05$; DAS – days after sowing.

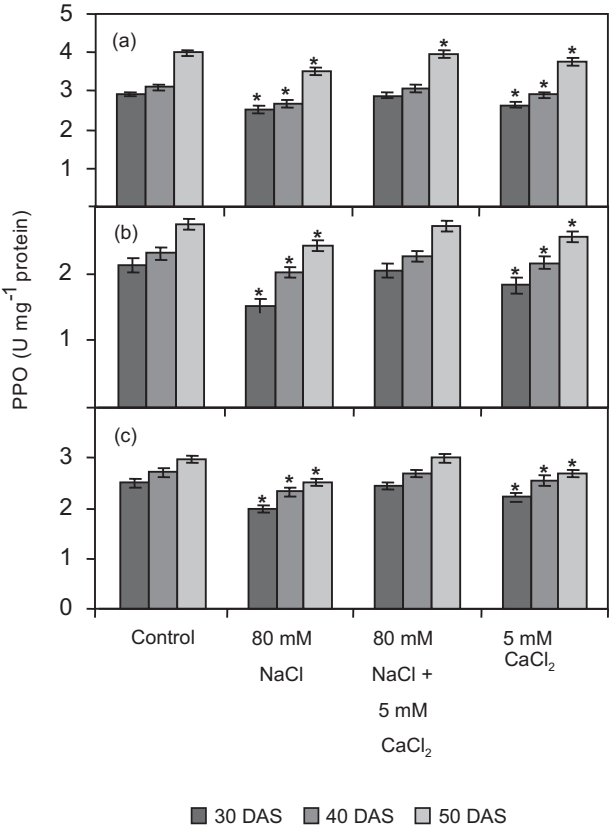

Fig. 2. Effect of NaCl, $CaCl_2$ and their combinations on polyphenol oxidase (PPO) activity in the (a) root, (b) stem and (c) leaf of *P. amarus*. Values are given as mean ± SD of six samples in each group. * – significant difference at $p \leq 0.05$; DAS – days after sowing.

red with NaCl-stressed plants. Among the organs, the roots showed a higher polyphenol oxidsae and catalase activity followed by the leaf and stem in all the treatments.

Discussion

The NaCl lowered the protein content in sorghum (AZOOZ et al. 2004) and mungbean (DHINGRA and SHARMA 1993). Addition of $CaCl_2$ with NaCl treatments increased the protein content when compared with NaCl-stressed plants. Similar results were observed in wheat (ABDLESAMAD 1993). Protein degradation in a saline environment has been attributed to the decrease in protein synthesis, accelerated proteolysis, decrease in the availability of amino acid and denaturation of enzymes involved in protein synthesis (LEVITT 1980).

Free amino acids increased with an increase in NaCl salinity in *Catharanthus roseus* (JALEEL et al. 2007a) and $CaCl_2$ in wheat (ABDELASMAD 1993). $CaCl_2$ treatment of the NaCl-stressed plants lowered the free amino acids and the proline content in all parts as compared with NaCl-treated plants. Similar results were observed in the groundnut (GIRIJA et

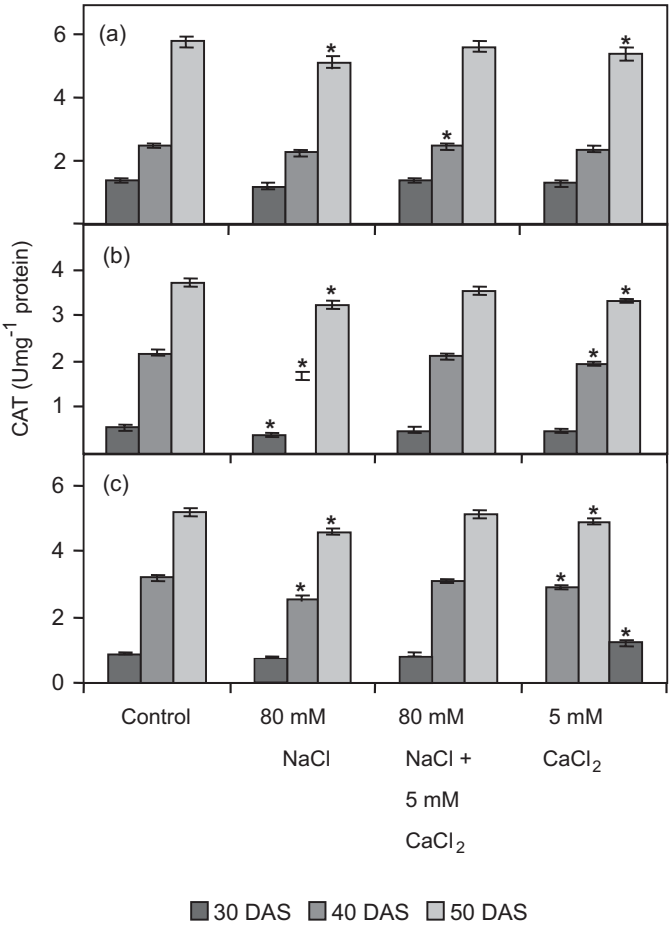

Fig. 3. Effect of NaCl, $CaCl_2$ and their combinations on catalase (CAT) activity in the (a) root, (b) stem and (c) leaf of *P. amarus*. Values are given as mean ± SD of six samples in each group. * – significant difference at $p \leq 0.05$; DAS – days after sowing.

al. 2002). Among the organs, the leaves showed a higher free amino acid and proline content followed by the root and stem in all the treatments.

NaCl and $CaCl_2$ treatments decreased the total sugar content in *Cajanus cajan* (GILL and SHARMA 1993). When $CaCl_2$ was combined with NaCl treatments, the plants showed an increase of the total sugar content as compared with NaCl-treated plants. Among the organs, the roots showed a higher total sugar content followed by the leaf and stem in the treatment. Addition of calcium may increase the alpha-amylase activity which leads to the degradation of starch into sugars. Alpha-amylase requires Ca^{2+} for its activity (BILDERBACK 1973). Addition of calcium increased the protein and decreased the free amino acid in the salt-stressed plants. So calcium treatment may reduce protein degradation or increase protein synthesis in salt-stressed plants.

Under salt stress, the antioxidant enzymes like polyphenol oxidase and catalase activities decreased in all parts of *Catharanthus roseus* (JALEEL et al. 2007a). When $CaCl_2$ was combined with NaCl, treatment increased the polyphenol oxidase and catalase activity as compared with NaCl-stressed plants. Among the organs, the roots showed a higher polyphenol oxidase and catalase activity, followed by the leaf and stem in all the treatments. The results obtained in this study were in accordance with those found in the roots of rice seedlings (LIN and KAO 1999). The inhibition of POX activity by salinity may interfere with the regulation of auxin levels and also with cell-wall biosynthesis (MITTOVA et al. 2002). Low basal rate and decreased POX activity seem to indicate that this enzyme does not take a crucial part in defense mechanisms against oxidative stress or that, suffering POX for salt toxicity, cooperation is activated between different antioxidant enzymes for the establishment of a proper H_2O_2 homeostasis (CHAPARZADEH et al. 2004). NaCl- and $CaCl_2$-treated plants showed increased activity of peroxidase as compared with the control. Similar results were observed in spinach leaves (OZTURK and DEMIR 2003). Antioxidant enzymes play a significant role in plants to protect them against the damaging effect of reactive oxygen generated during salinity stress (ASADA 1992). Reduction in catalase activity under salt stress may result in H_2O_2 accumulation and may be associated with its tolerant mechanism through signal transduction (JALEEL et al. 2007c). An increase in catalase activity was previously reported in triadimefon, a fungicide treated *Catharanthus roseus* plants (JALEEL et al. 2006).

Conclusion

NaCl was shown to have greater toxic effects than CaCl2 on the growth and metabolism of *P. amarus* plants. The addition of $CaCl_2$ to NaCl treatments had a varied effect on proteins, total sugars, free amino acids, proline and the activity of antioxidant enzymes in the growth of *P. amarus* plants. In general Ca^{2+} was shown to have an ameliorative effect on NaCl-stressed plant growth by modulating the overall metabolism.

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