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SHORT COMMUNICATION

EFFECT OF NaCl, $CaCl_2$ AND THEIR INTERACTION ON PROLINE, GLYCINE BETAINE CONTENTS AND LIPOXYGENASE ACTIVITY DURING SEEDLING DEVELOPMENT OF GROUNDNUT (*ARACHIS HYPOGEA* L.)

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The present study was carried out to study the proline, glycine betaine (GB) contents and lipoxygenase (LOX) activity under NaCl salinity and its amelioration by $CaCl_2$ during early seedling growth of groundnut (*Arachis hypogaea* L.). Proline and glycine betaine contents were found to be increased more by addition of $CaCl_2$ to the NaCl stressed seedlings than NaCl alone. NaCl treatment caused decrease in the level of LOX activity when compared to the seedlings treated with either $CaCl_2$ or its combination with NaCl. LOX showed a single peak with an UV absorption maximum at 235 nm on HPLC.

Key words: **Glycine betaine, lipoxygenase, proline, salinity stress**

Salinity affects the yield of agricultural crops by decreasing both the growth and photosynthesis (Murugan and Kishor Mohan 2007). Improving plant tolerance to salinity may provide field stability in subsistence agriculture and limit salinisation due to irrigation by reducing inputs (Flowers and Yeo 1995). Ca^{2+} is essential for K^+/Na^+ selectivity and the membrane integrity (Hanson 1984). Plants posses antioxidant system to endure the oxidative damage caused by reactive oxygen species under oxidative stress such as high temperature, drought and salinity (Madhumita *et al.* 2007). The production of reactive oxygen species is one of the biochemical changes that takes place during salt stress (Vaidyanathan *et al.* 2003). In germinating cucumber seeds, a specific LOX associated with lipid bodies is capable of adding oxygen to the esterified fatty acids (Feussner *et al.* 2001). Free proline is known to accumulate in response to biotic and abiotic stresses and has been shown to protect plants against free radical induced damage (Matysik *et al.* 2002). Groundnut is one of the important oil seed crops grown in various parts of India and is known to be sensitive to salinity. Hence, the present study was aimed to investigate the adverse effects of NaCl salinity and its amelioration by $CaCl_2$ on LOX activity, proline and GB content of groundnut cultivar TPT-2.

Seeds of groundnut cultivar TPT-2 were obtained from the Regional Agricultural Research Centre (Tirupati, Andhra Pradesh, India). The seeds were surface sterilised with 0.02% $HgCl_2$ solution and divided into four batches and germinated in bread boxes containing fluted filter paper towels. Seedlings were subjected to the following treatments: irrigation with (1) Distilled water (control) (2) 100 mM NaCl, (3) 30 mM $CaCl_2$, (4) 100 mM NaCl + 30 mM $CaCl_2$. Seedlings were illuminated continuously with fluorescent lamps in a growth room. The temperature was maintained at

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25±2°C. The seedlings were harvested randomly from the bread boxes on 3, 6, 9 and 12th day from the bread boxes. Measurements were carried out separately in the cotyledons and the embryonic axis.

Proline content was determined by the method of Bates *et al.* (1973) and GB was extracted following the method of Grieve and Grattan (1983). Data was analysed by ANOVA. The values are mean ± SE of five replications and means compared by the least significant difference test at the 0.05 level.

LOX activity was assayed by determining O_2 consumption using Clark's Oxygen Electrode (Model 5300). 5 gm each of the cotyledons and embryonic axis of the seedlings were homogenised with 10 ml of 200 mM of phosphate buffer (pH 7.4). The homogenate was filtered through muslin cloth and centrifuged at 25,000 Xg for 60 min. The supernatant was used as a source of enzyme. The assay mixture contained 2 ml of 200 mM phosphate buffer (pH 7.4), 100 µl of enzyme solution and 0.2 µl of 40 mM linoleic acid (in ethanol). The enzyme activity was measured at 25°C. HPLC was carried out on reverse phase column equipped with a pump model 6000A and an injector model U 6K. A reaction was carried out in an oxygenated buffer, 15 mM potassium phosphate (pH 7.0) with an excess of enzyme. The reaction was initiated by the addition of Arachidonic acid (133 µM) and incubated for 2 min at 30°C linoleic acid and the products were extracted immediately twice with hexane: ether (1:1v/v). The organic extracts were pooled, dried over anhydrous sodium sulphate and evaporated to dryness. The residue was reconstituted in the HPLC solvent system of hexane: 2-propanol: acetic acid (1000:4:1). The HPETE was determined on RP-8 column at a flow rate of 2 µl min^{-1}.

The proline content increased in the presence of NaCl in both cotyledons and the embryonic axis of groundnut seedlings. Accumulation was high in embryonic axis whereas cotyledons showed lower accumulation of proline under salt stress. On the other hand proline content decreased by the addition of $CaCl_2$, while $CaCl_2$ alone was similar in the effect of the control (Table 1) ($p<0.05$). As seedlings grew, the specific proline content of the cotyledons decreased in all the treatments possibly due to a dilution effect while in the embryonic tissue, it increased with advanced seedling age in the presence of NaCl. Our results are supported by Misra and Gupta (2005). Increase in proline content under NaCl + $CaCl_2$ treatment was much less than NaCl treatment. Increased proline may be an adaptation to overcome the salinity (Girija *et al.* 2002).

Table 1. Effect of NaCl, $CaCl_2$ and their interaction on changes in proline content (mg g^{-1} fw) of cotyledons (COTY) and embryonic axis (EA) of groundnut cultivar during seedling growth (Values are mean ± SE of 3 replications) ($p< 0.01$). (T1 - control; T2 - 100 mM NaCl; T3 - 30 mM $CaCl_2$; T4 - 100 mM NaCl +30 mM NaCl)

Days after treatment	Seedling parts	TPT-2			
		Treatments			
		T1	T2	T3	T4
3	COTY	154.5 (±1.304)	181.5 (±0.512)	129.5 (±1.647)	118.5 (±6.212)
	EA	162.2 (±2.290)	185.6 (±2.460)	151.25 (±2.229)	142.5 (±1.435)
6	COTY	262.4 (±1.128)	332.5 (±3.315)	212.1 (±1.476)	191.21 (±2.807)
	EA	249.1 (±1.621)	276.2 (±2.227)	329.1 (±1.763)	369.2 (±1.340)
9	COTY	149.5 (±0.587)	186.5 (±0.946)	134.5 (±1.869)	109.2 (±0.931)
	EA	174.54 (±1.829)	194.4 (±2.439)	166.25 (±2.720)	141.5 (±1.085)
12	COTY	114.7 (±2.331)	264.5 (±1.306)	182.5 (±1.023)	156.21 (±2.333)
	EA	201.5 (±2.463)	215.62 (±1.886)	224.19 (±1.338)	274.54 (±1.572)

GB content was higher in the embryonic axis in comparison with cotyledons. GB content increased in addition of $CaCl_2$ to NaCl stressed seedlings than NaCl alone (Table 2) ($p < 0.05$). For all treatments there was consistently more GB in embryonic axis than the cotyledons. $CaCl_2$ alone also promoted GB synthesis significantly above control levels especially in the cotyledons. An increase in the GB levels under stress conditions was found to increase the sodium flux from the cytoplasm to the vacuole and was also known to modify the membrane behaviour in water stressed barley leaves (Hitz *et al*. 1981). Sodium chloride stress increased the level of GB in groundnut cotyledonds and embryonic axis, addition of $CaCl_2$ to the NaCl stressed seedlings increased the GB to a greater extent than in unstressed seedlings. The findings suggested that the cultivar TPT-2 is sensitive to NaCl stress and $CaCl_2$ treatment alleviated NaCl stress.

The level of LOX activity of the cotyledons increased upto 6th day of sowing followed by continuous decline in all treatments (Table 1), and similar trend was observed by Tappel *et al.* (1963). NaCl treatment caused decrease in the level of LOX activity when compared to the seedlings treated with $CaCl_2$ and its combination with NaCl. In $CaCl_2$ treated seedlings LOX activity was found to be higher compared to the NaCl treatment and NaCl + $CaCl_2$ treatments. The level of LOX activity of embryonic axis of the control seedlings showed a gradual decrease from 3rd day to 12th day after sowing. NaCl treatment caused a gradual decline in the LOX activity of the embryonic axis of the seedlings upto 9th day followed by a sudden decrease by 12th day after sowing. On the other hand in $CaCl_2$ treated seedlings LOX activity increased steadily upto 9th day followed by a decline. The LOX enzyme is widely distributed in plant tissue being particularly abundant in leguminous plants and its activities needed to initiate lipid peroxidation (Funk *et al*. 1986). During the progressive germination of groundnut cotyledons $CaCl_2$ prevents the formation of linoleic and linolenic acid from Sn^2 acyl-side chain may be by maintaining cytosolic Ca^{2+} at homeostatic level and because of the decrease in substrate level LOX activity declines. This may be the reason for the decreased LOX activity under $CaCl_2$ treatment and its combination with NaCl. The biological implication of the $CaCl_2$ in lowering endogenous activity of the LOX enzyme include decleration of production of the free radicals and possibly preventing oxidation of phospholipids (Grossman and Leshem 1978) in *Pisum*. The pH optima of the groundnut LOX was found to be 6.0 (Fig. 1a). When arachidonic acid was incubated with the groundnut LOX, a single major peak with an UV absorption maximum at 235 nm (Fig. 1b), characteristic of a conjugated diene system was resolved on HPLC with standard 15-HPETE (Fig. 1c). This data suggests that groundnut LOX is rather

Table 2. Effect of NaCl, $CaCl_2$ and their interaction on changes in glycine betaine content (mg g^{-1} fw) of cotyledons (COTY) and embryonic axis (EA) of ground nut cultivars during seedling growth. (Values are mean ± SE of 3 replications) ($p<0.01$). (T1 - control; T2 - 100 mM NaCl; T3 - 30 mM $CaCl_2$; T4 - 100 mM NaCl +30 mM NaCl)

Days after treatment	Seedling parts	TPT-2			
		Treatments			
		T1	T2	T3	T4
3	COTY	0.038 (±0.002)	0.049 (±0.004)	0.074 (±0.002)	0.096 (±0.003)
	EA	0.132 (±0.003)	0.151(±0.003)	0.179 (±0.004)	0.192 (±0.006)
6	COTY	0.132 (±0.003)	0.146 (±0.007)	0.172 (±0.001)	0.181 (±0.003)
	EA	0.284 (±0.003)	0.300 (±0.005)	0.332 (±0.010)	0.356 (±0.005)
9	COTY	0.112 (±0.003)	0.132 (±0.003)	0.146 (±0.003)	0.159 (±0.006)
	EA	0.261 (±0.002)	0.274 (±0.002)	0.304 (±0.002)	0.311 (±0.005)
12	COTY	0.142 (±0.005)	0.161 (±0.001)	0.189 (±0.001)	0.198 (±0.004)
	EA	0.296 (±0.003)	0.311 (±0.004)	0.352 (±0.006)	0.376 (±0.005)

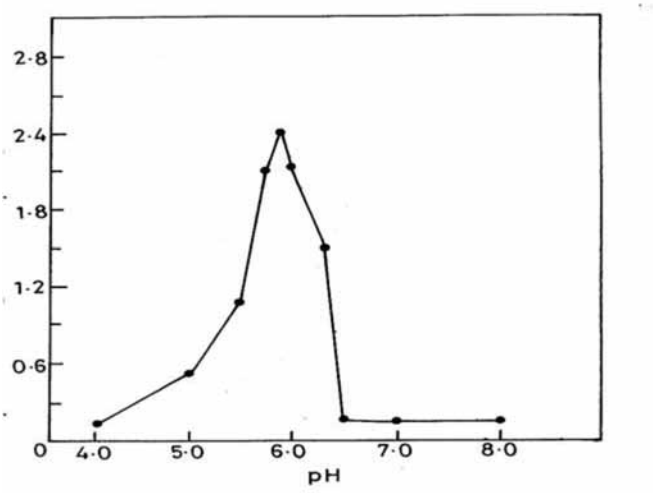

Fig. 1a. pH optima of lipoxygenase activity of groundnut seedlings

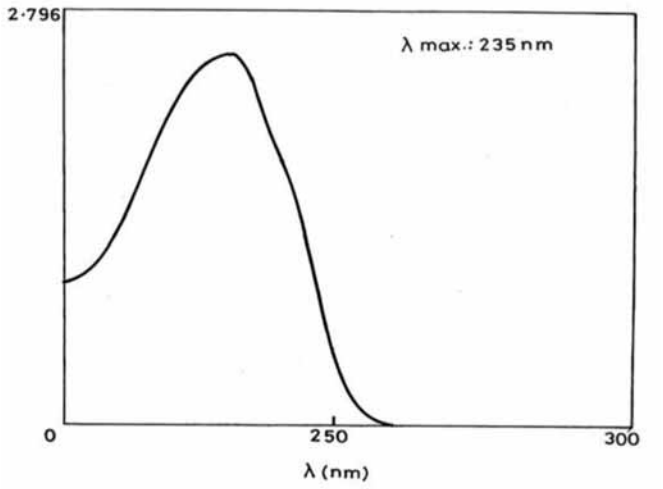

Fig. 1b. UV absorption spectra of LOX product of groundnut seedlings

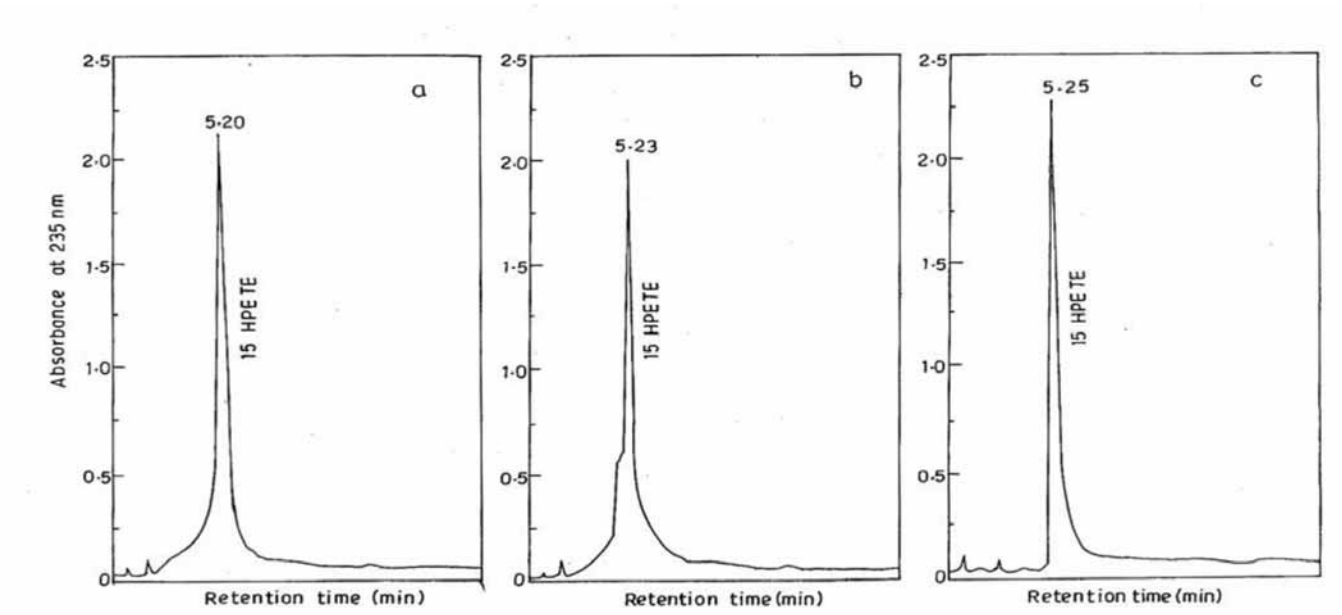

Fig. 1c. HPLC profiles at 235 nm of a) standard 15-HPETE b) products generated after incubation of lipoxygenase with arachidonic acid and c) mixture of 'a' and 'b'

specific for the insertion of O_2 and 15th carbon on acid molecule.

Both the glycine betaine and proline production were induced by NaCl in cotyledons and embryonic axis of groundnut seedlings. Concentrations of osmolytes were much greater in embryonic axis than in cotyledon tissue. $CaCl_2$ treatment promoted biosyntheses of both glycine betaine and proline and significantly reduced LOX enzyme activity in both cotyledons and embryonic axis of groundnut seedlings which indicated the reduced levels of free radical production and lesser tissue damage.

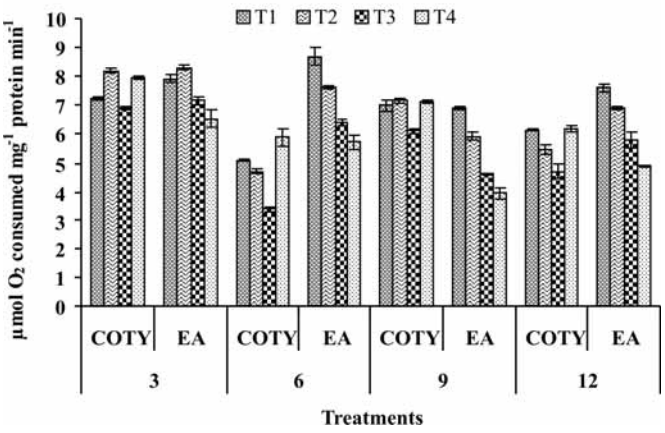

Fig. 2. Effect of NaCl, $CaCl_2$ and their interaction on changes in the total lipoxygenase activity (µmol O_2 consumed mg^{-1} protein min^{-1}) of cotyledons (COTY) and embryonic axis (EA) of groundnut cultivar during seedling growth. (Values are mean± SE of 3 replications) (p< 0.01). (T1 -control; T2 -100 mM NaCl; T3-30 mM $CaCl_2$; T4 -100 mM NaCl +30 mM NaCl)

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