Journal of Environmental Biology
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November 2010, 31(6) 939-944 (2010)
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Effect of calcium against salinity-induced inhibition in growth, ion accumulation and proline contents in *Cichorium intybus* L.

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(Received: September 07, 2009; Revised received: February 15, 2010; Accepted: February 26, 2010)

Abstract: *This study assesses the effect of NaCl (80 and 160 mM) and $CaCl_2$ (10 mM) solutions, alone and in combination, to 30-day-old seedlings of Cichorium intybus L. Observations were made at 30 day intervals from the time of treatment till harvest (180 days after sowing). Application of NaCl resulted in significant decreases in lengths of root and stem, in dry weights of root, stem and leaves and in the leaf area, as compared with control. The reduction was less with the combined application of NaCl and $CaCl_2$ than with the NaCl treatment alone. On the contrary, treatment of $CaCl_2$ alone promoted the above variables. Proline content in the leaves was enhanced with NaCl and $CaCl_2$ alone as well as with the NaCl + $CaCl_2$ treatments; the maximum (six-fold) enhancement was observed with the combined treatments, compared with NaCl (four-fold increase) and $CaCl_2$ (two-fold increase) alone. The sodium (Na^+) and Chloride (Cl) contents in different plant parts increased both with NaCl and with NaCl + $CaCl_2$ treatments. The maximum accumulation was observed in leaves, followed by that in stem and root. The potassium (K^+) and calcium (Ca^{2+}) contents decreased under NaCl stress, but increased with $CaCl_2$ treatment. Thus, calcium ameliorated the deleterious effects of NaCl stress and stimulated the plant metabolism and growth.*

Key words: *Calcium chloride, Cichorium intybus L., Growth regulation, Sodium chloride, Salinity stress*
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Introduction

Soil salinization is one of the major factors operative in soil degradation. It has affected 19.5% of the irrigated land and 2.1% of the dry-land agriculture existing on the globe (FAO, 2000). Salinity effects are more conspicuous in arid and semi-arid areas where 25% of irrigated land is salt affected. Salinity hampers growth and yield of plants (Demiral and Turkan, 2006). Salinity-induced inhibition of plant growth results from osmotic and ionic effects, and different plant species have developed different mechanisms to cope with these effects (Munns, 2002; Sumithra *et al.,* 2006). Osmotic adjustment, *i.e.* reduction of osmotic potential due to net solute accumulation, is an important mechanism of salt tolerance in plants (Sairam and Tyagi, 2004). The reduction in osmotic potential in salt-stressed plants may stem from accumulation of inorganic ions (Na^+, Cl^-, Ca^{2+} and K^+) and compatible solutes (soluble carbohydrates, amino acids, proline) (Hasegawa *et al.,* 2000; Kavi Kishore *et al.,* 2005). The osmotic adjustment in leaves contributes to the maintenance of water uptake and cell turgor, allowing for physiological processes like stomatal conductance, photosynthesis and cell expansion (Serraj and Sinclair, 2002). In addition, organic solute accumulation may also help in maintaining ionic homeostasis and C/N ratio, removal of free radicals, and stabilization of such macromolecules and organelles as proteins, protein complexes and membranes (Bray *et al.,* 2000).

Calcium has a role in building salt tolerance in plants. Externally supplied Ca^{2+} reduces the toxic effects of NaCl, presumably by facilitating a high K^+/Na^+ selectivity (Liu and Zhu, 1998). High salinity results in increased cytosolic Ca^{2+}, which is transported from the apoplast and the intracellular compartments (Knight *et al.,* 1997). This transient increase in the cytosolic Ca^{2+} initiates stress-signal transduction leading to salt adaptation. Adequate levels of calcium are necessary for the membrane to function normally (Jaleel *et al.,* 2007b). Most of the interest in calcium in plants has centered on its role in the cytoplasm in controlling the developmental processes.

The deleterious effects of salinity on plant growth are attributed to the decrease in osmotic potential of the growing plant, specific ion toxicity and nutrient ion deficiency (Luo *et al.,* 2005). Addition of calcium salt might restore adequate levels of potassium and calcium (Kent and Lauchli, 1985). Supplemental calcium also enhances accumulation of proline in saline conditions (Shah *et al.,* 1990).

Cichorium intybus is an erect perennial herb, 30-90 cm in height at rosette stage, with taproot up to 7.5 cm in length. It has a rosette type growth at early stages and the stem formation starts about 120 days after sowing. The plant is known in the Indian medicine as a powerful hepatic stimulant. It forms a component of several hepatoprotective formulations such as Liv-52 and Geriforte.The present study investigates the effect of calcium against salinity-induced inhibitions in plant growth, ionic accumulation and proline levels in *Cichorium intybus* L., a popular medicinal plant in the Indian system of medicine.

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Materials and Methods

Experimental setup: Seeds of chicory (*Cichorium intybus* L.) were obtained from the Herbal Garden, Hamdard University, New Delhi. Pot culture experiments were conducted at the experimental site of the Botany Department in Hamdard University. The seeds were sown in earthen pots (12'X 12") in the second week of October at 25-30°C temperature, 50% relative humidity and 450 µmol m^{-2} s^{-1} irradiance. The pots were filled with 12 kg of sandy loam soil (with pH 7.2 and electrical conductivity 0.207 m mhos cm^{-1}) and farmyard manure (FYM) in a ratio of 2:1. The basal dose of NPK was applied, as recommended for chicory crop, at the time of pot filling and 20 days after sowing. The crop lasted for 180-190 days. For maintaining proper growth of the plant, one plant per pot was kept after thinning. Solutions of NaCl (80 and 160 mM) and $CaCl_2$ (10 mM) were prepared in double distilled water and applied to the growing plants singly as 80 mM (T_1) and 160 mM (T_2) of NaCl and 10 mM (T_3) of $CaCl_2$, and collectively as the 80 mM+10 mM (T_4) and 160 mM+10 mM (T_5) of NaCl+$CaCl_2$ combination. These solutions (500 ml), were applied to the growing plants at 30 days after sowing (DAS). The control (without NaCl and $CaCl_2$ treatment) and the treated plants were provided a uniform water supply. Sampling was done at 30 days interval from the time of application of salts till harvest (*i.e.* 60, 90, 120 150 and 180 DAS). The sampling times were fixed in view of the vegetative stage (60 and 90 days), flowering (120 days) and post-flowering stage (150 and 180 days). Fifteen replicates (15 pots in randomized block design) were taken for growth parameters and five for biochemical parameters for each treatment in each sampling.

Growth measurement: Fifteen plants taken from each of the treatments and the control were separated into root, stem and leaves. The length of root and stem was measured with the help of a meter scale and expressed in cm. Samples of root, stem and leaves were kept in oven at 65°C for drying. After 48 hr, dry weights of the samples were recorded using an electronic top pan balance (Eagle) and expressed in g per plant. The total green leaf area $plant^{-1}$ was measured with the help of a Leaf Area Meter (LICOR 3000, Lincoln, USA) and expressed in cm^2 $plant^{-1}$.

Estimation of proline content: Proline content in the leaf samples was estimated by the method of Bates *et al.* (1973). A 0.5 g of sample was homogenized in 10 ml of 3% sulphosalicylic acid and centrifuged at 10,000 rpm for 10 min. The mixture of 2 ml of supernatant, 2 ml of acid ninhydrin and 2 ml of glacial acetic acid was incubated at 100°C in a water bath for 60 min. Reaction was stopped by keeping the test tubes in an ice bath and then a 4 ml of toluene was added to each sample and mixed vigorously on a cyclomixer for 10-15 sec. The toluene layer was separated from the mixture and the absorbance was read at 520 nm on a spectrophotometer (Lambda 20, England) using toluene as blank. The corresponding concentration of proline was determined against the standard curve of L-proline and expressed in µg g^{-1} fresh wt.

Estimation of sodium(Na^+) and calcium (Ca^{2+}): The Na^+ and Ca^{2+} ion contents were estimated following the method described by Tondon (1995).100 mg of plant samples (root, stem and leaves) were digested in the Kjeldahl digestion unit with 10 ml of acid mixture (nitric acid and perchloric acid in a ratio of 9:4). The ion contents were estimated photometrically using a flame photometer (Systronic 125, India). Standard curves were drawn and the content of each element was determined and expressed in µ mol g^{-1} dry wt.

Statistical analysis: Data were analysed statistically and given as Mean ± SE. Analysis of variance and least significant differences between treatments and plant growth stages ($p \leq 0.05$) were performed by SPSS version 10 Inc., USA. Mean was separated using the Duncan's multiple range test (DMRT). The experiment was conducted in randomized block design.

Results and Discussion

The roots and shoots increased in length with age of the plant. Compared with the control, they were shorter in NaCl-treated plants at different time periods. The maximum difference was 35% in the root length and 29% in the shoot length with 160 mM NaCl treatment (Table 1). The root length was reduced under salinity stress, showing that salinity possibly affects the external water potential, ion toxicity and/or ion imbalances (Jaleel *et al.,* 2007a) and can impose biochemical restraints on cell wall expansion, which in turn can inhibit root growth (Hernandez and Almansa, 2002). Similar reductions in the root and shoot lengths was observed in *Cassia angustifolia* plants growing under salt stress (Arshi *et al.,* 2002).

On the contrary, with $CaCl_2$ (10 mM) treatment the length of root and stem increased by 21 and 24%, respectively. The combination of NaCl + $CaCl_2$ (160 mM + 10 mM) resulted in a 30 and 24% shortening of root and shoot length respectively, as compared with the control, which was slightly less than one caused by the application of NaCl alone. Calcium ions are known to have a regulatory role in the metabolism, Ca^{2+} ions may compete with Na ions for membrane-binding sites and can protect the cell membrane from the adverse effect of salinity (Zidan *et al.*, 1990).

NaCl alone and in combination of $CaCl_2$ inhibited the root and shoot dry weights in comparison with the control at each growth stage. However, the combined salt treatment was less effective than NaCl treatment alone. The maximum reduction was 73% in the root dry weight and 46% in the stem dry weight against 160 mM NaCl treatment and 160 mM NaCl + 10 mM $CaCl_2$ treatment respectively (Table 2). Generally plant biomass is inhibited by an excess of the solute taken up by plants from the saline growth media (Arshi *et al.,* 2002, 2004).

Individual $CaCl_2$ (10 mM) treatment enhanced the root and stem dry weights by 24 and 18% respectively, as compared with their controls. The combined salts (160mM NaCl + 10mM $CaCl_2$) treatment also reduced the root dry weight by 65% and stem dry weight by 41%. Being important for cell wall extensibility, membrane stability and ion selective transport (Hanson, 1984), Ca acts as a secondary messenger for many biochemical processes within the

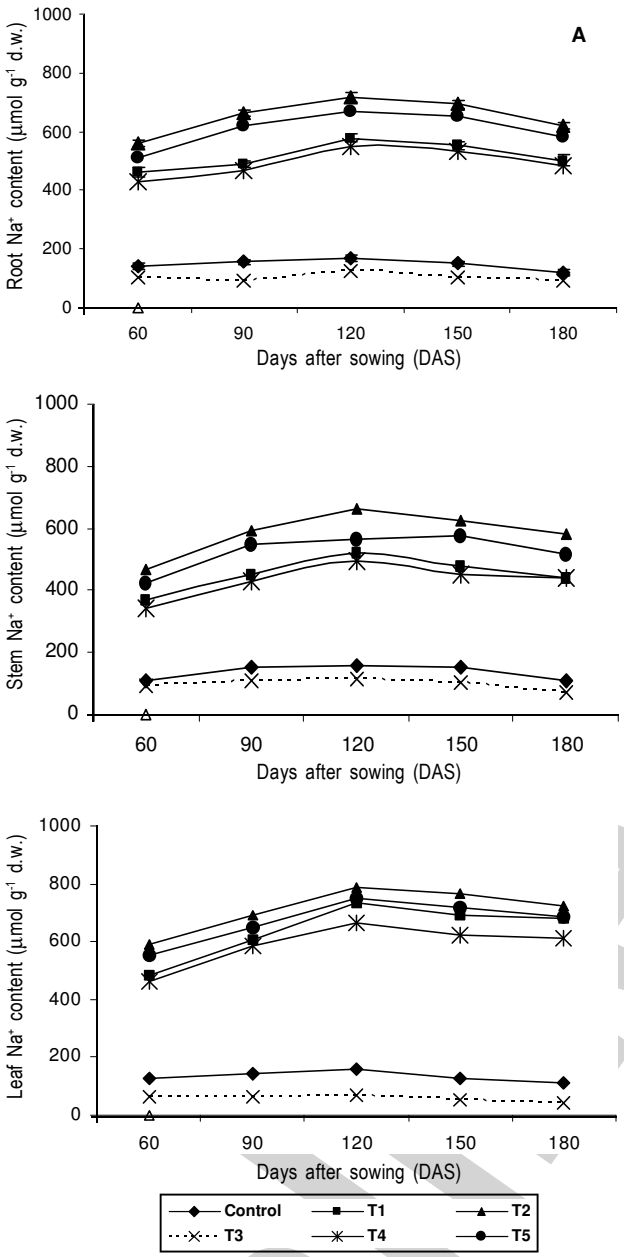

Fig. 1: Changes in sodium content (µmol g^{-1} d.w.) in the root (A), stem (B) and leaves (C) as induced by NaCl (T_1, T_2), $CaCl_2$ (T_3) and NaCl + $CaCl_2$ (T_4, T_5) treatments at different time intervals

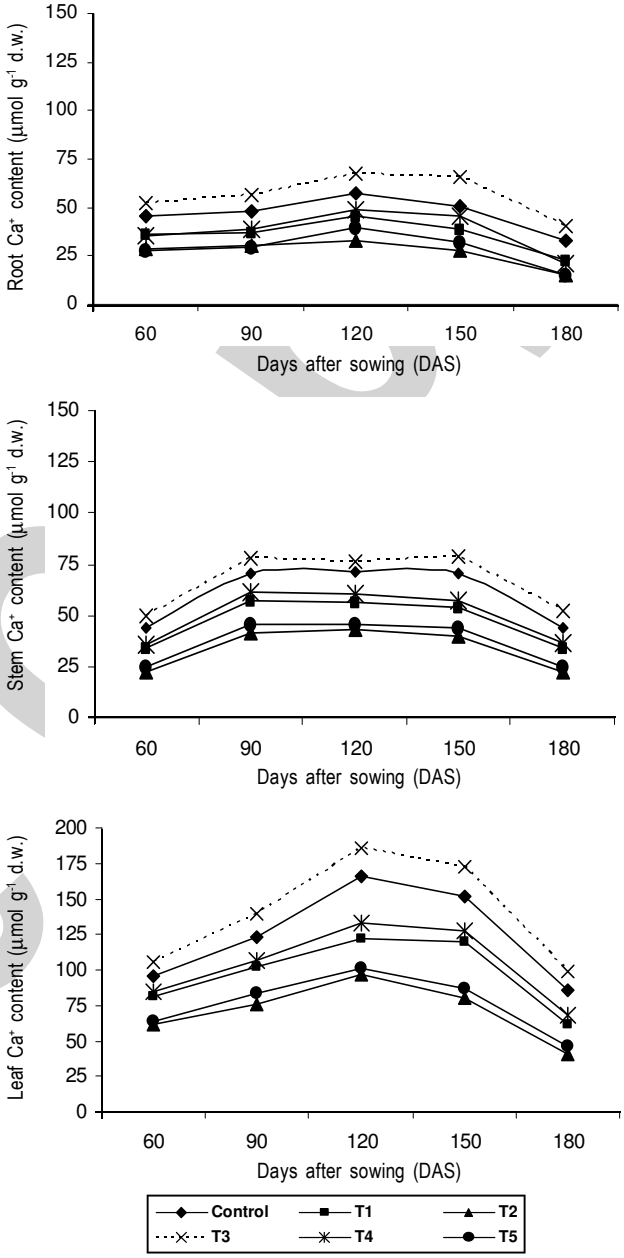

Fig. 2: Changes in calcium content (µmol g^{-1} d.w.) in the root (A), stem (B) and leaves (C) as induced by NaCl (T_1, T_2), $CaCl_2$ (T_3) and NaCl + $CaCl_2$ (T_4, T_5) treatments at different time intervals

cytosol (Mahajan and Tuteja, 2005). It promoted plant growth and dry weight and minimized the adverse effect of NaCl in the present study, as it did also in the case of *Cassia angustifiolia* (Arshi *et al.,* 2006).

Leaf dry weight (Table 2) as well as leaf area (Table 3) increased with plant age both in the control and the NaCl-treated plants. With the increasing concentration of NaCl, both these parameters were markedly inhibited as compared with control. Reduction in the leaf dry weight and the leaf area was nearly 62 and 56% under the influence of 160 mM NaCl. The salinity-induced reduction in the leaf area might be due to inhibition of cell division and cell expansion under salt stress (Serraj and Sinclair, 2002). A decrease in leaf size under stress conditions allows the conservation of energy, thereby launching the appropriate defence response and also reducing the risk of heritable damage (Chaparzadeh *et al.*, 2004).

The combination of salts (NaCl + $CaCl_2$) reduced the leaf dry weight and leaf area but the reduction was less than one with the NaCl treatment alone. $CaCl_2$ treatment improved both these characters with reference to the control. Thus, calcium appeared to

Table - 1: Changes in root and stem lengths (cm plant^{-1}) of *C. intybus* L. as affected by NaCl (T_1, T_2), $CaCl_2$ (T_3) and NaCl + $CaCl_2$ (T_4, T_5) treatments at different time intervals

Treatments	Days after sowing (DAS)				
	60	90	120	150	180
			Root length		
Control	7.30^{b} ± 0.45	9.47^{b} ± 1.11	19.62^{b} ± 0.85	26.39^{b} ± 0.66	29.69^{b} ± 0.88
T_1	6.12^{c} ± 0.66	8.32^{c} ± 0.23	17.27^{c} ± 0.22	23.58^{c} ± 0.55	25.71^{cd} ± 0.15
T_2	5.09^{e} ± 0.23	6.12^{d} ± 0.55	14.68^{e} ± 0.42	20.14^{d} ± 0.12	22.83^{e} ± 0.11
T_3	8.86^{a} ± 0.23	11.38^{a} ± 0.66	23.78^{a} ± 0.55	30.64^{a} ± 0.32	34.01^{a} ± 0.17
T_4	6.54^{c} ± 0.44	8.76^{c} ± 0.33	18.22^{b} ± 0.10	23.86^{c} ± 0.11	26.44^{c} ± 0.62
T_5	5.62^{d} ± 0.44	6.60^{d} ± 0.15	15.48^{d} ± 0.33	21.56^{d} ± 0.12	23.72^{de} ± 0.15
			Stem length		
Control	103.72^{b} ± 2.15	132.59^{b} ± 0.72	160.77^{b} ± 2.77	166.59^{b} ± 1.46	167.32^{b} ± 1.46
T_1	94.88^{b} ± 1.02	120.36^{c} ± 1.12	140.67^{c} ± 1.05	149.56^{c} ± 2.79	149.55^{d} ± 1.62
T_2	78.25^{c} ± 1.72	93.68^{e} ± 0.98	120.58^{d} ± 1.17	119.56^{e} ± 2.23	119.65^{f} ± 1.85
T_3	128.61^{a} ± 1.11	157.44^{a} ± 1.09	187.54^{a} ± 0.95	189.62^{a} ± 2.54	191.12^{a} ± 1.56
T_4	97.77^{ab} ± 1.66	125.32^{c} ± 2.56	146.32^{c} ± 2.09	153.87^{c} ± 1.27	157.52^{c} ± 1.55
T_5	83.36^{c} ± 1.58	100.88^{d} ± 2.86	127.65^{d} ± 1.78	126.35^{d} ± 1.58	131.66^{e} ± 1.09

The values (Mean ± SE) are based on fifteen root and shoot samples and the mean was separated by the Duncan's multiple range test (DMRT), p≥0.05

Table - 2: Changes in root, stem and leaf dry weight (g plant^{-1}) of *C. intybus* L. as affected by NaCl (T_1, T_2), $CaCl_2$ (T_3) and NaCl + $CaCl_2$ (T_4, T_5) treatments at different time intervals

Treatments	Days after sowing (DAS)				
	60	90	120	150	180
			Root dry weight		
Control	0.184^{b} ± 0.05	0.603^{b} ± 0.22	2.01^{b} ± 0.09	4.23^{b} ± 0.11	6.99^{b} ± 0.10
T_1	0.142^{d} ± 0.09	0.351^{d} ± 0.04	1.19^{b} ± 0.05	2.65^{d} ± 0.09	4.58^{d} ± 0.05
T_2	0.072^{f} ± 0.06	0.251^{e} ± 0.06	0.650^{e} ± 0.06	1.55^{f} ± 0.10	1.88^{f} ± 0.09
T_3	0.225^{a} ± 0.04	0.748^{a} ± 0.06	2.41^{a} ± 0.02	4.99^{a} ± 0.09	8.12^{a} ± 0.10
T_4	0.159^{c} ± 0.12	0.462^{c} ± 0.07	1.41^{c} ± 0.04	3.06^{c} ± 0.14	5.12^{c} ± 0.14
T_5	0.090^{e} ± 0.09	0.276^{e} ± 0.08	0.790^{d} ± 0.07	1.89^{e} ± 0.11	2.44^{e} ± 0.09
			Stem dry weight		
Control	20.74^{b} ± 1.05	31.80^{b} ± 1.02	44.54^{b} ± 0.64	49.62^{b} ± 1.09	51.25^{b} ± 1.32
T_1	15.96^{d} ± 0.59	25.14^{d} ± 0.74	32.69^{d} ± 1.10	38.82^{c} ± 1.07	40.18^{c} ± 1.05
T_2	11.25^{e} ± 1.01	18.43^{e} ± 0.22	26.69^{e} ± 1.09	30.56^{d} ± 1.66	28.49^{e} ± 1.02
T_3	23.65^{a} ± 0.02	36.66^{a} ± 1.22	52.36^{a} ± 1.08	57.86^{a} ± 1.08	59.62^{a} ± 0.24
T_4	17.26^{c} ± 1.00	27.42^{c} ± 2.46	35.66^{c} ± 1.28	40.57^{c} ± 1.04	42.18^{c} ± 0.66
T_5	12.33^{e} ± 1.48	19.22^{e} ± 3.22	28.12^{e} ± 1.07	32.63^{d} ± 3.08	32.55^{d} ± 2.65
			Leaf dry weight		
Control	1.63^{b} ± 0.05	3.57^{b} ± 0.13	7.76^{b} ± 0.15	6.12^{b} ± 0.19	2.01^{b} ± 0.12
T_1	1.36^{c} ± 0.04	2.84^{c} ± 0.11	5.88^{d} ± 0.18	4.29^{d} ± 0.08	1.32^{d} ± 0.05
T_2	0.98^{d} ± 0.09	2.02^{e} ± 0.07	3.91^{f} ± 0.41	2.71^{f} ± 0.46	0.76^{f} ± 0.05
T_3	1.81^{a} ± 0.02	4.12^{a} ± 0.05	8.99^{a} ± 0.10	7.24^{a} ± 0.10	2.45^{a} ± 0.10
T_4	1.44^{c} ± 0.08	3.09^{c} ± 0.07	6.31^{c} ± 0.17	4.77^{c} ± 0.18	1.53^{c} ± 0.16
T_5	1.09^{d} ± 0.04	2.35^{d} ± 0.12	4.42^{e} ± 0.48	3.09^{e} ± 0.27	0.91^{e} ± 0.10

The values (Mean ± SE) are based on fifteen root, shoot and leaf samples and the mean was separated by the Duncan's multiple range test (DMRT), p≥0.05

Table - 3: Changes in leaf area (cm^2 $plant^{-1}$) of *C. intybus* L. as affected by NaCl (T_1, T_2), $CaCl_2$ (T_3) and NaCl + $CaCl_2$ (T_4, T_5) treatments at different time intervals

Treatments	Days after sowing (DAS)				
	60	90	120	150	180
Control	$40.17^{b} \pm 5.03$	$110.54^{b} \pm 4.63$	$595.10^{b} \pm 21.22$	$1132.2^{b} \pm 11.58$	$745.17^{b} \pm 16.38$
T_1	$31.58^{c} \pm 2.22$	$84.54^{d} \pm 2.11$	$464.88^{d} \pm 15.18$	$827.21^{d} \pm 40.66$	$503.66^{d} \pm 11.28$
T_2	$23.66^{e} \pm 1.41$	$60.10^{f} \pm 2.33$	$327.56^{f} \pm 23.26$	$567.43^{f} \pm 41.87$	$325.10^{f} \pm 25.86$
T_3	$47.75^{a} \pm 0.98$	$131.10^{a} \pm 2.58$	$736.26^{a} \pm 15.11$	$1385.2^{a} \pm 15.87$	$925.45^{a} \pm 15.86$
T_4	$33.65^{c} \pm 2.91$	$93.45^{c} \pm 5.66$	$488.25^{c} \pm 15.10$	$942.41^{c} \pm 18.79$	$589.63^{c} \pm 12.87$
T_5	$26.87^{d} \pm 2.57$	$71.220^{e} \pm 4.37$	$377.96^{e} \pm 16.81$	$686.63^{e} \pm 10.22$	$376.58^{e} \pm 10.66$

The values (Mean ± SE) are based on leaves of fifteen plants and the mean was separated by the Duncan's multiple range test (DMRT), $p \geq 0.05$

Table - 4: Changes in proline content (µg g^{-1} f.w.) of *C. intybus* L. as affected by NaCl, (T_1, T_2), $CaCl_2$ (T_3) and NaCl + $CaCl_2$ (T_4, T_5) treatments at different time intervals

Treatments	Days after sowing (DAS)				
	60	90	120	150	180
Control	$19.32^{e} \pm 10.11$	$27.53^{d} \pm 20.56$	$35.39^{d} \pm 24.45$	$69.43^{e} \pm 8.96$	$81.12^{e} \pm 9.45$
T_1	$29.54^{c} \pm 3.15$	$44.87^{c} \pm 5.69$	$69.87^{c} \pm 18.03$	$121.84^{c} \pm 39.88$	$147.23^{d} \pm 11.45$
T_2	$39.87^{b} \pm 2.22$	$68.77^{b} \pm 8.58$	$110.85^{b} \pm 22.58$	$221.96^{b} \pm 31.47$	$345.14^{b} \pm 11.32$
T_3	$25.44^{d} \pm 0.06$	$45.89^{c} \pm 0.02$	$65.77^{c} \pm 0.06$	$115.43^{d} \pm 0.08$	$140.24^{d} \pm 0.05$
T_4	$38.64^{b} \pm 10.44$	$68.83^{b} \pm 33.11$	$106.17^{b} \pm 55.11$	$222.18^{b} \pm 33.55$	$308.26^{c} \pm 55.12$
T_5	$48.30^{a} \pm 6.39$	$82.59^{a} \pm 15.33$	$134.48^{a} \pm 55.41$	$312.44^{a} \pm 40.22$	$454.27^{a} \pm 37.55$

The values (Mean ± SE) are based on five individual readings and the mean was separated by the Duncan's multiple range test (DMRT), $p \geq 0.05$

mitigate the adverse effect of NaCl. Salt stress inhibited cell division, cell enlargement and consequently leaf expansion. Calcium may favour cell elongation and cell expansion, ultimately increasing the leaf area (Hernandez and Almansa, 2002).

Proline content was enhanced with the NaCl, $CaCl_2$ and combined treatments but the maximum (six-fold) enhancement over the control was observed with combined salts (NaCl + $CaCl_2$) treatment as against the individual treatments of NaCl and $CaCl_2$ that caused a four-times higher and two-times higher proline accumulation respectively (Table 4). Proline is considered as a compatible solute involved in osmotic adjustment (Azooz *et al.,* 2004). It may act as a non-toxic osmotic solute preferentially stabilizing the structure of macromolecules and organelles (Kavi Kishore *et al.,* 2005; Sumithra *et al.,* 2006).

Addition of $CaCl_2$ + NaCl increased the proline content mainly due to breakdown of proline-rich protein and fresh synthesis of proline and amino acids (Jun *et al.,* 2000). Increased proline in the stressed plants may be an adaptation to compensate the energy for growth and survival and thereby helps the plant to tolerate stress, as observed in spinach leaves (Ozturk and Demir, 2003), *Catharanthus roseus* (Jaleel *et al.,* 2007b) and *Cassia angustifolia* (Arshi *et al.,* 2005).

In our study, compared with the control, the Na^+ contents increased significantly in the NaCl-treated plants with each concentration; the maximum accumulation taking place in the leaves, followed by the stem and the roots. $CaCl_2$ application reduced the Na^+ content. The combined salt application increased the Na^+ content in different plant parts but the increase was less than one caused by the NaCl application alone (Fig. 1). Accumulation of inorganic ions, predominantly of Na^+ has a role in the process of osmotic adjustment (Gzik, 1996; Arshi *et al.*, 2005). High Na^+ levels in the external medium greatly reduce the physiochemical activity of the dissolved calcium (Cramer *et al.*, 1986) and may thus displace Ca^{2+} from the plasma membrane of the root cells (Cramer *et al.*, 1985).

Calcium (Ca^{2+}) contents also decreased under the influence of NaCl and combined treatments of NaCl + $CaCl_2$, but the reduction was less with combined treatments than with NaCl alone. Roots were more severely affected than stem and leaves. On the contrary, $CaCl_2$ treatments increased the calcium content of these plant parts significantly (Fig. 2). Salinity also disturbs the level of Ca^{2+} and alters K^+/Na^+ selectivity (Melgar *et al.,* 2006).

Our findings highlight the positive role of $CaCl_2$ in salt-strees mitigation in *C.intybus* L. plant.

Acknowledgments

The authors are grateful to their laboratory colleagues for technical help during the experimental phase of the study. The first author thanks the CSIR, New Delhi, for granting her Research Associateship.

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