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EFFECTS OF NaCl AND $CaCl_2$ ON CALLUS TISSUE OF *GOSSYPIUM HIRSUTUM* L. CV. ACALA SJ2

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The interactive effect of NaCl and $CaCl_2$ on the leaf and petiole-derived calli of Acala SJ2 *(Gossypium hirsutum. L.)*grown on Murashige and Skoog (1962) tissue culture medium was studied. Relative growth rate was used to assess the tolerance. NaCl reduced growth rate while calcium partially ameliorated the effects of NaCl. Water content of the callus decreased significantly with increasing salinity levels in the culture medium while reverse was true at high calcium level. NaCl resulted in higher Na^+ and lower K^+ and Mg^{+2}. The reverse was true for calcium up to 30 mol m^{-3}. C^{+2} concentration decreased with increasing salinity but increased at higher calcium levels. Cl^- contents increased with increasing salinity and calcium levels in the medium. NO^-_3, SO_4^{-2} and PO_4^{-3} were unaffected by salinity. In both kinds of callus, the highest calcium (up to 60 mol m^{-3}) and salinity (200 mol m^{-3}) levels increased proline accumulation. The correlation between relative growth rate and proline concentration was -0.98 (leaf callus) and -0.97 (petiole callus). The results suggested that salinity reduced the callus growth while calcium ameliorated its toxic effects. Proline acted as a compatible solute. The callus cultures from different explant sources of the same genotype behaved differently *in vitro.*

Key words : Salinity, Calcium nutrition, Callus tissue, *Gossypium hirsutum.*

Introduction

Salinity is one of the most important environmental factors limiting plant growth. Reduction in plant growth under salt stress is often attributed to physiological drought stress or water relations, ion toxicity and ion imbalance or the combination of these factors both *in vivo* and *in vitro* (Kurth *et al* 1986; Marschner 1995). Interspecific, intervarietal and even intravarietal differences exist in salinity tolerance of crop plants. The existence of genetic variability is a valuable source for screening and breeding for stress tolerance (Marschner 1995).

Cells and tissues play particular roles in the growth of plants. Metabolic functions of the cells at the molecular, biochemical and physiological levels may be disturbed by any factor that changes the environment. This distrubance in cell functions may reduce or enhance the growth of plants. Cotton (*Gossypium hirsutum* L.) is classified as a salt tolerant crop (Shimose and Sekiya 1991) but varietal and specific variation exists. Addition of NaCl to the culture medium has been reported in callus cultures of *Medicago sativa* (Shah *et al* 1990) and *Brassica* species (He and Cramer 1993). Shah *et al* (1990) have mentioned that the water contents of the calli of *Medicago sativa* are significantly reduced on NaCl-supplemented medium.

Calcium is known to ameliorate the detrimental effects of salinity in cotton (Kurth *et al* 1986). LaHaye and Epstein (1971) have reported that the tolerance of bean plants was increased by calcium and under salt stress, dry weights of roots, stem and leaves were increased. Calcium addition reduced leakage of K^+ from leaf discs of soybean (Leopold and Willing 1984).

Extensive studies have been carried out on the interaction between sodium and calcium. Many workers (Colmer *et al* 1994; Shah *et al* 1990) have described the essential role of calcium in selective cation transport, especially of potassium, in the cell, and functioning of Ca^{+2} in plant nutrition. CaCl2 at low concentrations has been reported to partially overcome the deleterious effects of NaCl (LaHaye and Epstein 1971; Cramer *et al* 1985; Gorham and Bridges 1995; Shah *et al* 1990). The protective effect of Ca^{+2} is due to the maintenance of membrane integrity and preventing the potassium leakage.

Tissue culture technology has many advantages in conducting physiological and biochemical studies of palnts. Brar and Khush (1994) have stated that it is much easier to screen 10^6-10^7 cells in small petri dishes than to screen a similar num-

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ber of whole plants in the field. Dracup (1991) has given five potential advantages of cell culture techniques over those used for plant breeding i.e. generation of large variability within genotypes duringh culture, evaluation and selection of large number of genotypes in the laboratory using relatively little space, reduction of time between generations, close control of the environment and cellular characteristics being masked in whole plants.

In the present work, the cellular response of leaf-and petiole-derived callus cultures of Acala SJ2 (*Gossypium hirsutum* L) to NaCl has been studied alone and in the presence of low and high concentrations of calcium.

Materials and Methods

Leaf and petiole-derived calli of Acala SJ2 (*Gossypium hirsutum* L.) were used. Culture media optimum for the growth of both type of calli (Akhtar 1996) was used. Each litre of medium contained 2,4-D and BAP (0.1 mg), thiamine-HCL (30 mg), myo-inositol (100 mg), glucose (45 g) (249 mol m^{-3}), $CaCl_2$ (15 mol m^{-3}), phytagel (2 g) and Murashige and Skoog (1962) basal salts excluding $CaCl_2$ at 5.8 pH. In experiments with petiole callus, thiamine and myo-inositol were added at the concentration of 25 and 200 mg l^{-1}, respectively.

All the callus cultures were incubated in the dark at 28°C. All cations, anions and proline were determinhed on per kg callus fresh weight basis. The measurement of relative growth rate, water contents, ionic and proline contents of callus are described as under.

Relative growth rate. Pre-weighed petri dishes containing 20 cm^3 of the culture media were inoculated with similar quantities of callus and inoculated petri dishes were reweighed to obtain the initial fresh weight of the callus. The petri dishes were incubated at 28°C in growth cabinet (Vindon Scientific Ltd., Diggle Oldham, England). After 28 days, calli were harvested and final fresh weight of the calli was obtained taking care to remove the entire callus from the petri dishes devoid of medium constituents. Growth of the callus cultures was expressed as relative growth rate $week^{-1}$ (RGR) (Shah *et al.*, 1990).

RGR = {In (final weight) - In (initial weight)} / 4 week

Water contents. 1 g of the fresh callus was oven dried 60°C for 48 hours in Gallenkamp size two oven. Dry weight of the callus was measured at the end of the drying period and water contents of callus cultures were calculated on a tissue dry weight basis, i.e. gram water g^{-1} dry weight of callus with the formula given by He and Cramer (1993) as under:

Water Contents = (Fresh weight - Dry weight)/ Dry weight

Cations and anions. Samples (0.5 g) of fresh callus were taken and placed into the glass tubes with 20 cm^3 of distilled water. The tubes were heated in a heating block for an hour at 100°C. After filtration the volume of the extract was made upto 25 cm^3 with distilled water in volumetric flasks. This filtrate was used as such to measure the concentrations of cations and anions by ion chromatography Twenty mm^3 of sap was diluted in an autoinjector vial with 1.4 cm^3 of cation eluent (20 mol m^{-3} methane sulphonic acid) and analyzed on a Dionex 2000 ion chromatograph fitted with a CS12 cation exchange column and a self-regenerating cation suppressor operated in auto-regeneration mode. The column was operated at 40°C. The system was automated by coupling to a Spark-Holland 'Marathon' autosampler fitted with a 5 mm^3 PEEK sample loop, and a Shimadzu CR5A plotting integrator linked to an Atari 1040 computer. Hot water extracts were centrifuged to remove particles and analyzed without further dilution.

For anion analysis the sap samples were diluted imme diately after extraction with 9 volumes of 20% propan-2-ol, mixed and re-centrifuged. Twenty mm^3 of the supernatant was then diluted in an autoinjector vial with 1.4 cm^3 of anion eluent (2.5 mol m^{-3} Na_2CO_3 + 2.4 mol m^{-3} $NaHCO_3$ in 2.5% propan-2-ol) and analyzed with the Dionex ion chromatograph fitted with an AS4A anion exchange column and an Anion Micro-Membrane Suppressor.

Proline. Proline was measured according to the method of Bates *et al* (1973).

Procedure. To select suitable salt concentration an experiment was set up with seven different concentrations (0, 50, 100, 150, 200, 250 and 300 mol m^{-3}) of NaCl at 3 mol m^{-3} $CaCl_2$ (Murashige and Skoog (1962) medium has this concentration of $CaCl_2$) using leaf and petiole-derived callus. All treatments had three replications. In second experiment, each of three concentrations of the NaCl (0, 100 and 200 mol m^{-3}) and $CaCl_2$ (0.03, 3.0 and 30 mol m^{-3}) were tested in all possible combinations using five replications. In the light of results obtained in second experiment e.g. the amelioration of adverse salinity effects by calcium up to 30 mol m^{-3}, it was decided to study its effects at higher concentrations (beyond 30 mol m^{-3}). The third experiment was designed with three concentrations of NaCl (0,100 and 200 mol m^{-3}) and five concentrations of $CaCl_2$ (3,15,30,45 and 60 mol m^{-3}) in all possible combinations using five replications with both leaf-and petiole derived calli.

Data analysis. The analysis of variance, means and standard error were computed by using minitab statistical package by computer. The treatment means were compared by Tukey's mehtbod (Anonymous 1993).

Results and Discussion

Relative growth rate (week^{-1}). Relative growth rate of leaf (a) and petiole (b) callus significantly (P=0.000) decreased with increasing NaCl salinity (Fig. 1). The colour and texture of the callus also changed from creamy white and friable to brown and hard with increased NaCl concentrations in the culture medium. However, some cells in the callus retained their white colour despite most of the callus cells turning brown. At 250 and 300 mol m^{-3} NaCl, the callus began to develop a darker brown colour, indicating necrosis and damage to the tissues. It is clear from the results that callus growth was reduced substantially and the brown colour and necrosis appeared at 250 and 300 mol m^{-3}. It was decided that 0, 100 and 200 mol m^{-3} NaCl will be used in the second and the third experiments.

Relative growth rate significantly reduced with increasing NaCl concentration in the medium (Fig. 2). Analysis of variance showed a significant NaCl effect on growth reduction (P=0.000). Calcium enhanced the relative growth rate under all conditions and this enhancement was more pronounced at 200 mol m^{-3} NaCl (Fig.2). Anlysis of variance also showed a highly significant effect of calcium (P=0.000). At low calcium (0.3 mol m^{-3}) a sharp and large reduction in growth occurred, with an increase in medium salinity. The interaction between NaCl and $CaCl_2$ was highly significant (P=0.000). As calcium chloride stimulated growth up to 30 mol m^{-3}, experiment no. 3 was designed to investigate the effects of higher concentrations (45 and 60 mol m^{-3}).

In the third experiment, a gradual decrease in growth of both kinds of calli occurred with increasing medium salinity (Fig.3). Calcium up to 30 mol m^{-3} tended to ameliorate the inhibitory effect of NaCl but at 45 and 60 mol m^{-3}. Highly significant effects of NaCl, calcium and their interaction were found (P=0.000).

Water contents (g g^{-1} dry wt of callus). The water contents (in first experiment) of the leaf (a) and petiole (b) callus subjected to NaCl stress decreased as function of higher salinity (Fig 4). Maximum reduction was found when callus was grown on 300 mol m^{-3} NaCl. The mean water contents at various levels of NaCl were significantly different from each

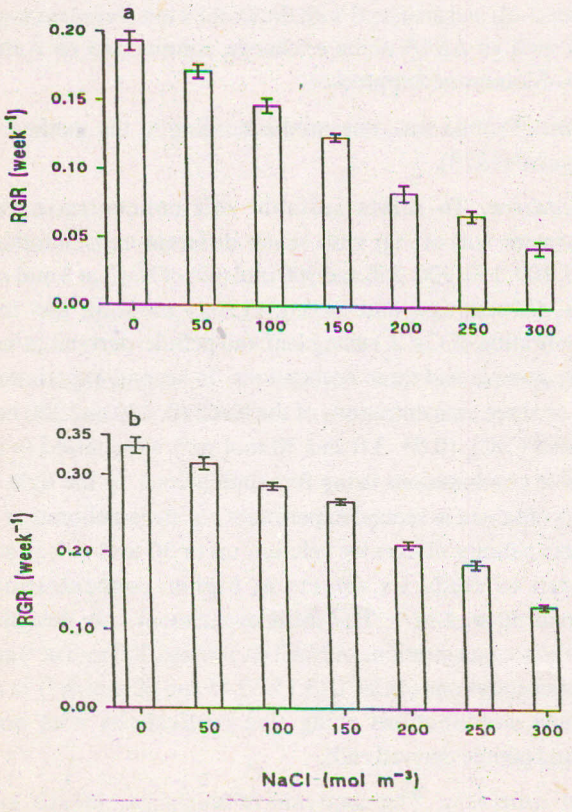

Fig 1 Effect of various concentrations of NaCl on relative growth rate of leaf (a) and petiole (b) callus cultures of Acala SJ2. The data points are means of 3 replications. The vertical bars represent SE (experiment no.1).

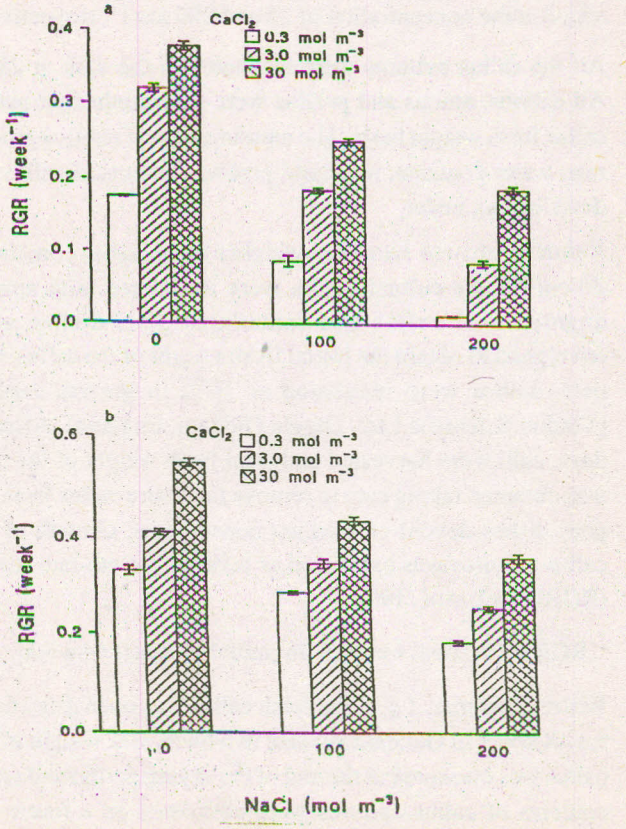

Fig 2 Effect of various concentrations of NaCl and $CaCl_2$ on relative growth rate of leaf (a) and petiole (b) callus cultures of Acala SJ2. The data points are means of 5 (a) and 3 (b) replications. The vertical bars represent SE (experiment no.2).

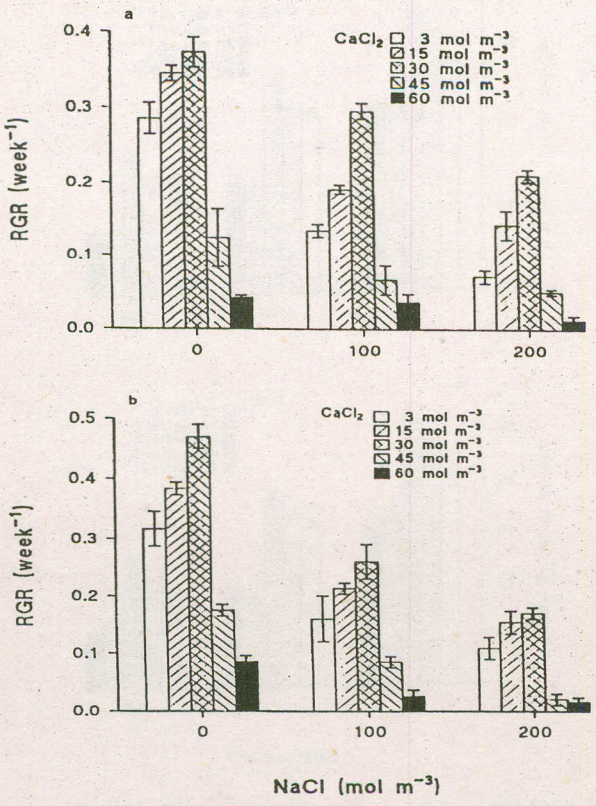

Fig 3 Effect of various concentrations of NaCl and $CaCl_2$ on relative growth rate of leaf (a) and petiole (b) callus cultures of Acala SJ2. The data points are means of 5 replications. The vertical bars represent SE (experiment no.3).

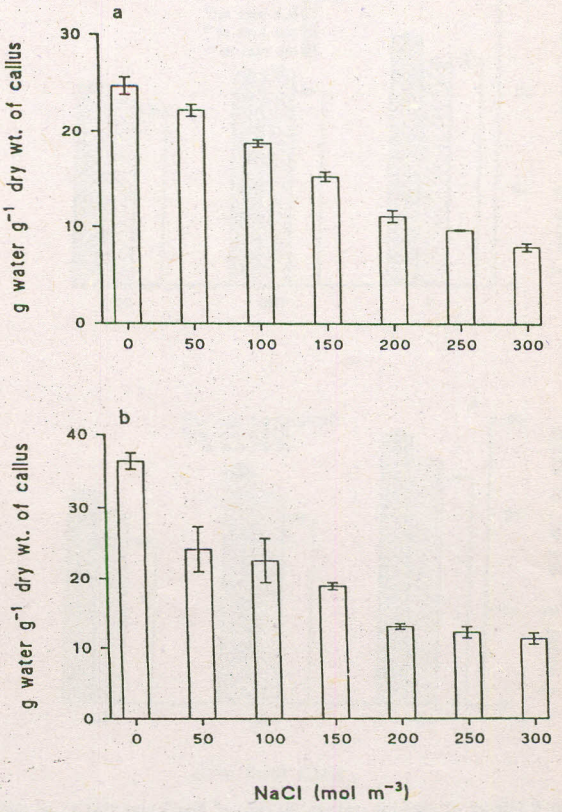

Fig 4 Effect of various concentrations of NaCl on water contents of leaf (a) and petiole (b) callus cultures of Acala SJ2. The data points are means of 3 replications. The vertical bars represent SE (experiment no.1).

other (P=0.000). The petiole callus had relatively higher water contents compared to leaf callus.

The water contents of the leaf (a) and petiole (b) callus cultures decreased on a medium supplemented with 100 and 200 mol m^{-3} NaCl (Fig 5). Statistical analysis showed a significant positive effect for $CaCl_2$ (P-0.000) but a highly significant negative effect of NaCl on water contents of callus (P=0.000); NaCl x $CaCl_2$ interation was significant (P=0.000).

Water contents of the leaf (Fig. 6a) and petiole (Fig. 6b) callus grown on various salinity levels decreased significantly (P=0.000). Although the water contents increased upto 30 mol m^{-3} calcium but decreased considerably at 45 and 60 mol m^{-3} (P=0.000). Highly significant interaction was found between NaCl and $CaCl_2$ (P=0.000).

Inorganic contents. Significant differences were found between Na^+ concentrations of the leaf and petiole callus at various NaCl levels in the culture medium (P=0.000). The calli grown on medium devoid of NaCl had minimum Na^+ (Table 1). The increasing salinity resulted in a gradual increase in Na^+ concentrations of the callus. Leaf callus had higher Na^+ at 200-300 mol m^{-3} external NaCl compared to petiole callus.

The potassium ion concentrations of the leaf and petiole callus were higher in control than with supplemented NaCl (Table 1). This decrease was more pronounced in leaf callus. The analysis of variance revealed significant differences between mean K^+ concentrations in the callus grown at various concentrations of NaCl (P=0.000).

Na^+/K^+ ratio significantly increased (Table 1) in both types of calli as a result of increasing NaCl concentration in the culutre medium. This ratio was higher in leaf calli compared to petiole calli at higher NaCl concentrations.

No significant differences were found between mean Mg^{+2} concentrations of the petiole callus at various levels of NaCl (P<0.920). There was a slight decrease in magnesium ion concentrations in the callus as a function of increased medium salinity (Table 1). Magnesium ions were not estimated in leaf callus.

The mean calcium ion (Ca^{+2}) concentrations in petiole callus (Table 1) were not statistically different at various

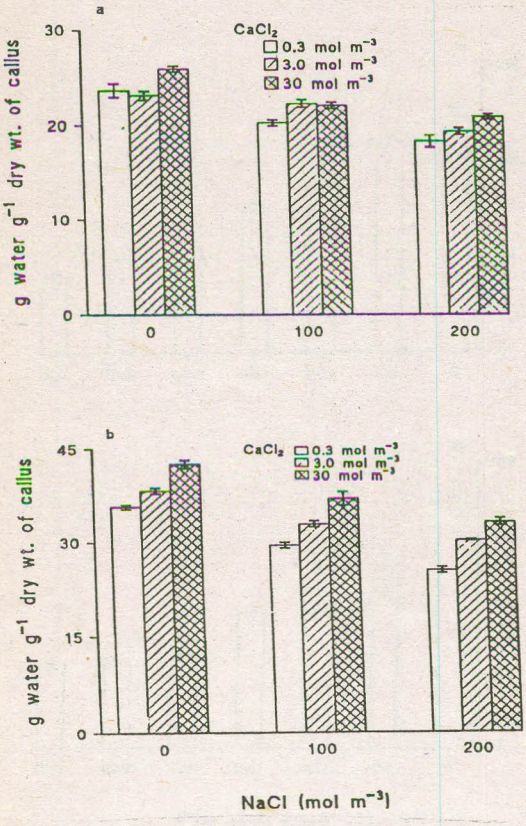

Fig 5 Effect of various concentrations of NaCl and $CaCl_2$ on water contents of leaf (a) and petiole (b) callus cultures of Acala SJ2. The data points are means of 5 (a) and 3 (b) replications. The vertical bars represent SE (experiment no.2).

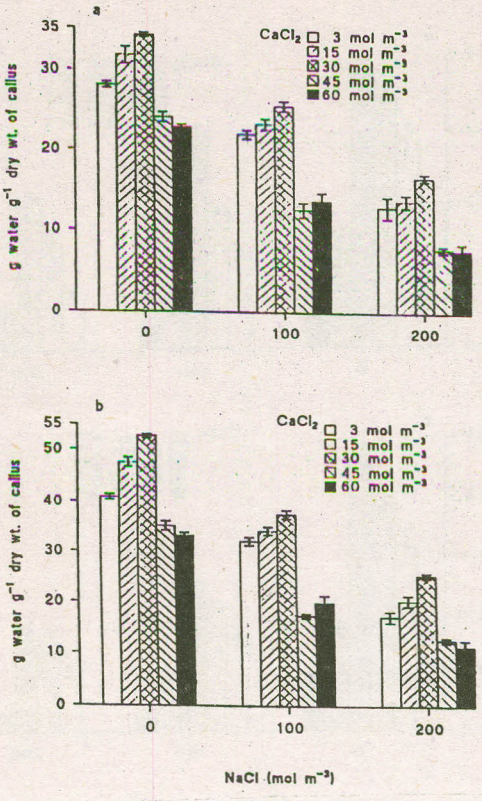

Fig 6 Effect of various concentrations of NaCl and $CaCl_2$ on water contents of leaf (a) and petiole (b) callus cultures of Acala SJ2. The data points are means of 3 replications. The vertical bars represent SE (experiment no.3).

concentrations of NaCl (P<0.840), although at 100-300 mol m^{-3} NaCl these were reduced to some extent compared to control. Calcium ions were not estimated in leaf callus.

The increasing concentrations of NaCl in the culture medium resulted in an increase in Cl^- concentrations of the leaf and petiole callus (Table 2). Differences between the means at various NaCl levels were highly significant (P=0.000). Control had a minimum Cl^- concentration while at 300 mol m^{-3} NaCl, the Cl^- concentrations of callus were the highest.

Nitrate ion concentrations of the leaf and petiole callus were not significantly affected by increasing salinity levels (Table 2). The supplemented NaCl resulted in a slight (not significant) decrease in PO_4^{-3} concentrations of the leaf callus from 50-300 mol m^{-3} (Table 2). The control had maximum phosphate ion concentration while minimum was found on 300 mol m^{-3} NaCl. No phosphate ions were found in the petiole callus.

Sulphate ion (SO_4^{-2}) concentrations in the leaf callus (Table 2) remained unaffected by various concentrations of NaCl in the culture medium. Analysis of variance showed no significant differences between mean sulphate ion concentrations of the callus when grown on supplemented NaCl. Increasing Na Cl in the culture medium resulted in an increase in sulphate ion (SO_4^{-2}) concentration in petiole callus. The means were significantly different from each other in petiole (P=0.000) while non-significant in leaf callus (P<0.230).

Sodium ion concentrations in the leaf (a) and petiole (b) callus increased significantly (P=0.000) as a result of increased NaCl in the culture medium (Fig 7) Calcium treatments decreased the sodium accumulation at higher concentrations of NaCl. The interation between NaCl and $CaCl_2$ was significant (P=0.000).

A highly significant decrease in K^+ (Fig 8) was observed at higher NaCl concentrations (P=0.000). Calcium in the culture mediuim increased the K^+ concentration in callus at higher NaCl. There was no significant interaction between NaCl and $CaCl_2$.

Calcium at higher concentrations significantly decreased Na^+/K^+ ratio which increased in both kinds of callus with increasing salinity in the culture medium. NaCl and calcium effects and their interaction were highly significant.

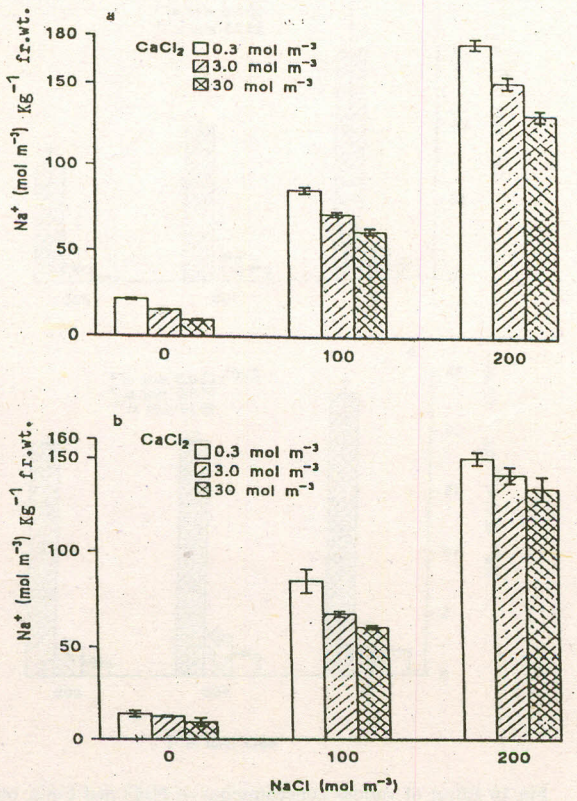

Fig 7 Effect of various concentrations of NaCl and $CaCl_2$ on Na' contents of leaf (a) and petiole (b) callus cultures of Acala SJ2. The data points are means of 5 (a) and 3 (b) replications. The vertical bars represent SE (experiment no.2).

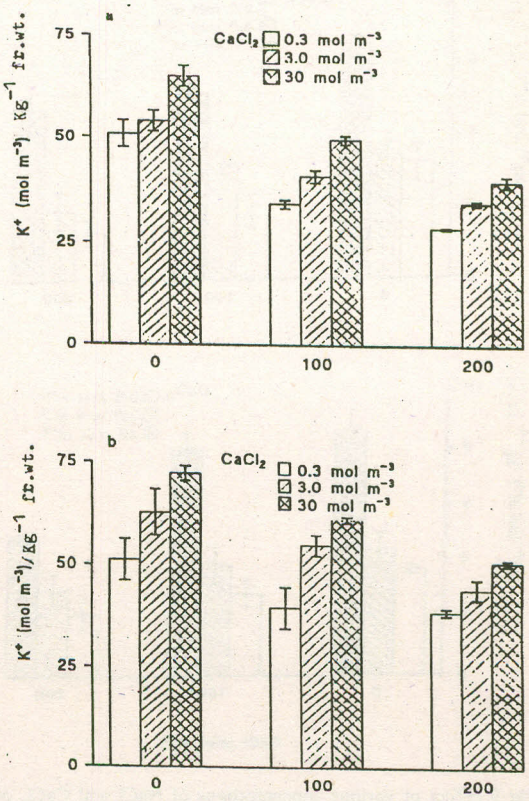

Fig 8 Effect of various concentrations of NaCl and $CaCl_2$ on K' contents of leaf (a) and petiole (b) callus cultures of Acala SJ2. The data points are means of 5 (a) and 3 (b) replications. The vertical bars represent SE (experiment no.2).

NaCl salinity in the culture medium resulted in a significant decrease in magnesium ion concentrations in the leaf and petiole callus (Fig 9). Higher calcium concentrations (3 and 30 mol m^{-3}) significantly increased Mg^{+2} ion concentrations (35 and 166% compared to control) of the callus. The calcium effect was significant (P< 0.030 and 0.000) while that of NaCl was highly significant (P=0.000). The interaction between NaCl and $CaCl_2$ was non-significant (P<0.92 and 0.10). Mg^{+2} was higher in petiole callus compared to that in leaf callus.

The calcium ion concentrations of the leaf and petiole callus were reduced compared to control at 100 and 200 mol m^{-3} NaCl (Fig 10). The addition of higher concentrations of $CaCl_2$ in the medium resulted in a significant increase in calcium ion concentrations of the callus. The interaction between NaCl and $CaCl_2$ was non-significant in leaf (P<0.32) but significant in petiole callus (P<0.02).

Anion concentrations were not measured in leaf callus cultures. In petiole callus both NaCl and calcium at their higher concentrations resulted in a significant increase in chloride (Cl^-) ion concentrations (Fig. 11a). The interaction between the two factors was significant (P=0.000). Salinity decreased the NO^-_3 concentrations (Fig 11b) of the petiole callus while calcium at higher concentrations resulted in a small increase in nitrate ion concentrations. NaCl and calcium interaction was non-significant (P<0.330). No sulphate and phosphate ions were detected in petiole calli.

In the thired experiment, sodium ion concentrations of the leaf and petiole callus increased with increasing salinity (Tables 3 and 4). The statistical analysis showed highly significant effects of NaCl and $CaCl_2$ (P=0.000). $CaCl_2$ upto 45 mol m^{-3} resulted in a decrease in Na^+ in leaf callus but Na^+ concentrations increased at the highest calcium levels (60 mol m^{-3}). In petiole callus 45 and 60 mol m^{-3} calcium resulted in an increase in Na^+ ion concentrations. There was significant interaction between NaCl and calcium (P<0.015 and <0.018).

A highly significant (P+0.000) decrease in K^+ contents of leaf and petiole calli was observed at higher NaCl concentrations (Table 3 and 4). The potassium ion concentrations of the callus increased up to 30 mol m^{-3} calcium but decreased at higher concentrations. There was significant interaction

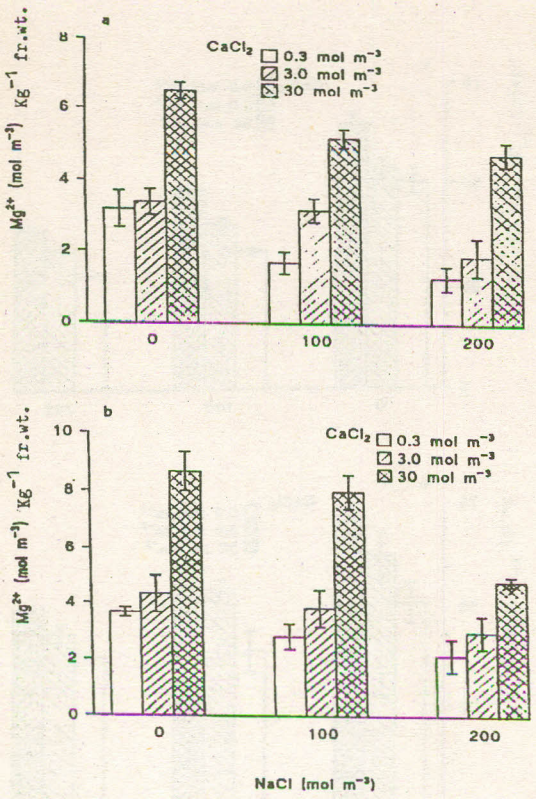

Fig 9 Effect of various concentrations of NaCl and $CaCl_2$ on Mg^{+2} contents of leaf (a) and petiole (b) callus cultures of Acala SJ2. The data points are means of 5 (a) and 3 (b) replications. The vertical bars represent SE (experiment no.2).

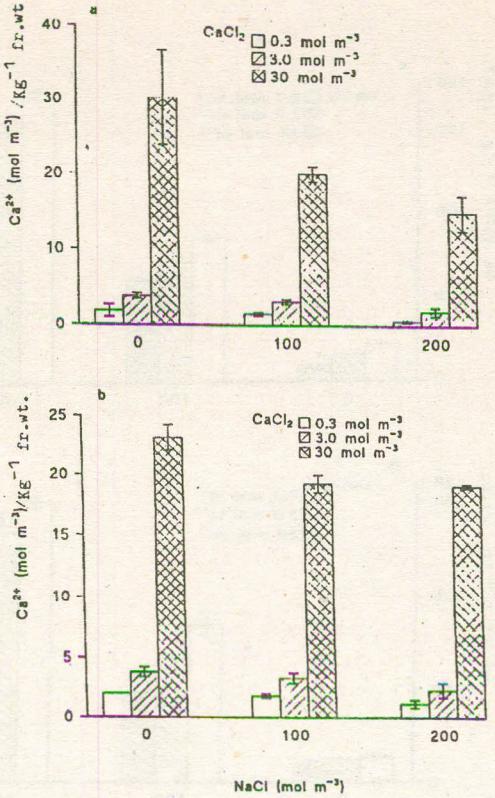

Fig 10 Effect of various concentrations of NaCl and $CaCl_2$ on Ca^{+2} contents of leaf (a) and petiole (b) callus cultures of Acala SJ2. The data points are means of 5 (a) and 3 (b) replications. The vertical bars represent SE (experiment no.2).

between NaCl and $CaCl_2$ in leaf and petiole callus (P=0.000).

Increasing calcium levels up to 30 mol m^{-3} in the medium resulted in reduced Na^+/K^+ ratio in both types of calli (Table 3 and 4), increasing NaCl concentrations in the medium tended to increase the Na^+/K^+ ratio. The statistical analysis of the data obtained revealed highly significant effects of both NaCl, $CaCl_2$ and their interaction (P= 0.000) in both kinds of callus.

Salinity resulted in a decrease in magnesium ion concentrations of the leaf callus (Table 3) and petiole callus (Table 4). Calcium from 3-30 mol m^{-3} increased the magnesium concentrations while higher calcium concentrations (45-60 mol m^{-3}) reduced Mg^{+2} ion concentrations. NaCl and calcium effects were highly significant (P=0.000). Mg^{+2} concentration were maximum at 30 and 60 mol m^{-3} calcium with 0 mol m^{-3} NaCl. The interaction between NaCl and $CaCl_2$ was significant in leaf callus (P=0.000) while no interaction was found in petiole callus (P<0.089).

Calcium ion concentrations of the leaf callus were significantly (P+0.000) reduced compared to control at 100 and 200 mol m^{-3} NaCl (Table 3 & 4). Addition of higher concentrations of $CaCl_2$ in the medium resulted in a significant (P=0.000) increase in calcium ion concentrations of the leaf and petiole callus. Non-significant interaction between NaCl and calcium regarding their effects on Ca^{+2} concentration in leaf (P<0.057) and petiole (P<0.542) calli was computed. Anions were not measured in this experiment.

Proline accumulation. There was a significant increase in proline concentrations of the leaf and petiole callus (Fig 12) at increasing NaCl in the culture medium compared to control (first experiment). Maximum mean proline concentrations were found at 300 mol m^{-3} NaCl. The treatment means were different from each other statistically (P=0.000). More proline accumulated in petiole calli (0.37 mol m^{-3}) compared to leaf calli (0.33 mol m^{-3}).

Proline concentrations in leaf and petiole callus relative to cultural NaCl and $CaCl_2$ are shown in Fig 13 (second experiment) and in Fig 14 (third experiment). Proline concentrations in the callus significantly increased with increasing NaCl concentration in the growth medium (P=0.000). The highest NaCl and high calcium concentration resulted in the accumulation of significantly more proline than at low calcium. Analysis of variance showed a significant effect of calcium (P=0.000). There was

significant interaction between NaCl and $CaCl_2$ (P=0.000) suggesting that thecalcium effect was different at different NaCl levels:

Growth is a result of cell division and elongation and both these processes are affected by increasing salinity that reduces the size, volume and production of cells (Kurth *et al* 1986). In cotton roots, the reduction in cell size and cell production have been reported at 150 mol m^{-3} NaCl at low calcium (0.4 mol m^{-3}) by Kurth *et al* (1986) while no reduction was found at the same salinity but at 10 mol m^{-3}) calcium. In the present studies, higher calcium (30 mol m^{-3}) at high NaCl (100 and 200 mol m^{-3}) may have increased the cell size and production (not measured) and ultimately the growth of callus cultures.

The relative growth rate of both type of callus (leaf and petiole) was reduced as a result of medium salinity (Figs 1,2,3). The extent of this inhibition increased with increasing salinity and was relieved by calcium chloride. Nevertheless, the growth of callus was not completely halted even under the highest NaCl concentration (300 mol m^{-3}). The results are in accordance with the findings of Kerimov *et al* (1993) who reported that the initiation and growth of cotton callus were inhibited at higher salinity. The callus grown at 250 and 300 mol m^{-3} NaCl developed brown colour and necrosis of tissue. Similar browning and necrosis of callus tissue has been reported in wheat (Mukhtar and Hasnain 1994). Mukhtar and Hasnain (1994) and Gossett *et al* (1994) have reported reduction in callus growth at higher salinity in wheat and

Table 1

Effect of various concentrations of NaCl on cation concentrations of leaf and petiole callus of Acala SJ2.

	NaCl (mol m^{-3})						
Cation	0	50	100	150	200	250	300
	Leaf Callus Cultures						
Na^+	23.6±1.4	42.1±0.9	91.4±0.5	115.4±3.0	171.1±3.0	194.8±2.4	235.2±2.5
K^+	50.8±4.0	41.7±0.8	37.9±1.2	27.7±1.4	21.7±0.7	18.8±0.5	11.7±0.6
$Na^+ K^+$	0.5±0.04	1.0±0.04	2.4±0.1	4.2±0.2	7.9±0.4	10.4±0.1	20.2±1.0
	Petiole Callus Cultures						
Na^+	25.5±4.9	63.8±3.6	97.7±0.7	122.0±1.1	151.2±2.2	169.3±10.0	208.5±6.8
K^+	51.3±0.2	36.8±1.9	34.0±1.8	32.7±1.4	30.2±0.7	29.0±4.0	28.0±3.8
$Na^+ K^+$	0.5±0.1	1.8±0.2	2.9±0.1	3.8±0.1	5.0±0.1	6.1±1.1	7.7±1.1
Mg^{+2}	4.8±2.3	4.0±1.3	3.7±0.2	3.5±0.3	3.3±0.2	3.2±0.2	3.0±1.0
Ca^{+2}	4.3±2.1	4.2±0.2	3.5±0.8	3.3±1.0	3.0±0.3	3.0±0.0	2.7±0.2

The data are means of three replication ± standard error of means (experiment No.1).

Table 2

Effect of various concentrations of NaCl on anion concentrations of leaf and petiole callus of Acala SJ2.

	NaCl (mol m^{-3})						
Anion	0	50	100	150	200	250	300
	Leaf Callus Cultures						
Chloride (Cl^-)	11.4±0.5	73.4±5.1	99.2±1.6	142.7±2.7	195.9±5.0	224.1±14.0	291.0±7.8
Nitrate (NO^-_3)	53.6±4.0	54.2±1.4	47.8±2.3	48.5±2.3	46.6±4.1	46.2±4.3	44.9±1.9
Phosphate (PO_4^{-3})	3.1±0.4	2.9±0.3	2.6±0.5	2.6±0.1	2.2±0.2	2.1±0.2	2.3±0.1
Sulphate (SO_4^{-2})	2.6±0.1	2.5±0.2	2.5±0.2	2.2±0.3	2.0±0.3	2.0±0.1	2.0±0.1
	Petiole Callus Cultures						
Chloride (Cl)	6.2±0.4	66.0±1.3	92.8±19.5	142.2±5.0	182.7±0.7	215.7±10.9	259.7±6.5
Nitrate (NO_3)	25.2±2.2	30.0±3.3	26.5±5.2	26.3±1.8	26.0±3.0	27.3±3.6	25.0±1.3
Sulphate (SO4-2)	0.0±0.0	0.9±0.9	0.4±0.4	1.7±0.9	4.9±1.0	7.0±1.3	8.0±0.5

The data are means of three replication ± standard error of means (experiment No.1).

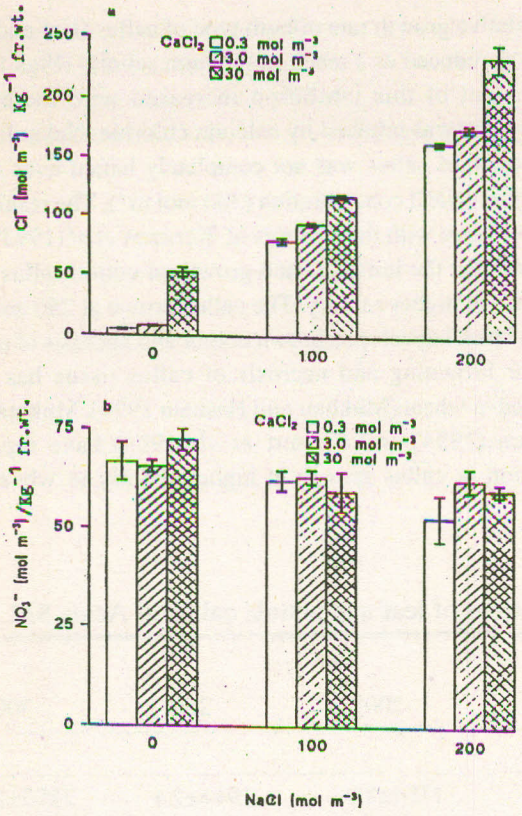

Fig 11 Effect of various concentrations of NaCl and $CaCl_2$ on Cl (a) and NO_3 (b) contents of petiole callus cultures of Acala SJ2. The data points are means of 3 replications. The vertical bars represent SE (experiment no.2).

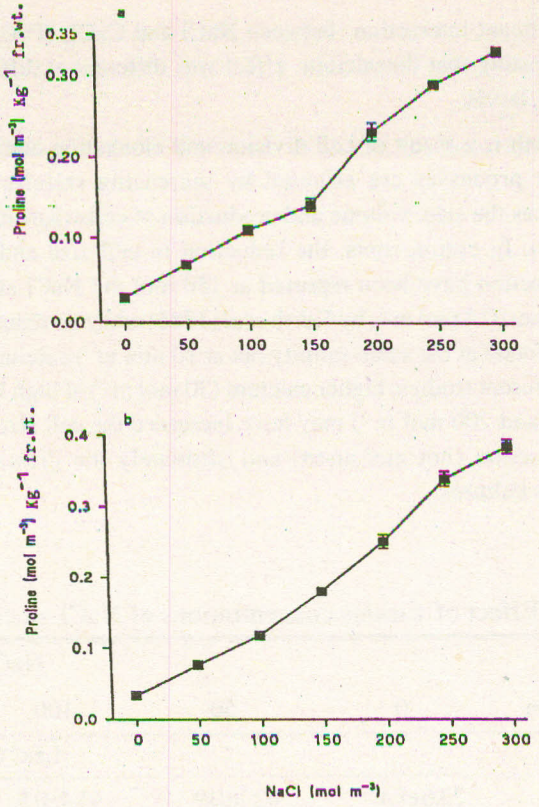

Fig 12 Effect of various concentrations of NaCl on proline contents of leaf (a) and petiole (b) callus cultures of Acala SJ2. The data points are means of 3 replications. The vertical bars represent SE (experiment no.1).

Table 3

Effect of various concentrations of NaCl and $CaCl_2$ on cation concentrations and Na^+/K^+ ratio of leaf callus of Acala SJ2

NaCl	$CaCl_2$	Na^+	K^+	Na^+/K^+	Mg^{+2}	Ca^{+2}
0	3	19.8±103	44.8±2.9	0.4±0.2	4.8±0.3	6.2±0.9
	15	17.3±0.9	48.3±1.2	0.3±0.01	7.3±0.5	13.3±1.7
	30	11.7±0.9	74.8±3.1	0.2±0.1	8.8±0.3	21.3±2.1
	45	10.8±2.6	43.7±1.2	0.3±0.1	4.7±0.2	34.8±1.7
	60	17.5±9.3	48.0±2.0	0.4±0.2	5.3±0.3	51.7±0.7
100	3	112.3±226	39.3±2.1	2.8±0.5	3.4±0.1	3.3±0.2
	15	84.8±2.0	41.2±0.9	2.1±0.01	4.5±0.3	12.7±0.8
	30	68.7±2.9	57.2±1.0	1.2±0.04	6.7±0.2	19.2±1.0
	45	82.8±5.3	43.2±0.7	1.9±0.1	4.3±0.2	29.3±0.9
	60	85.7±1.9	43.8±1.2	2.0±0.1	5.2±0.2	47.3±0.4
200	3	162.3±2.2	36.3±3.5	4.6±0.5	1.0±0.0	1.3±0.6
	15	154.8±1.9	41.0±1.8	3.8±0.1	3.2±0.6	7.0±1.2
	30	145.3±4.7	50.7±0.9	2.9±0.1	3.7±0.2	18.3±0.2
	45	147.8±7.9	39.8±0.9	3.7±0.2	3.3±0.2	26.3±2.2
	60	193.5±4.9	42.8±1.0	4.5±0.1	4.0±0.3	40.5±1.4

The data are means of three replication ± standard error of means (experiment No.3)

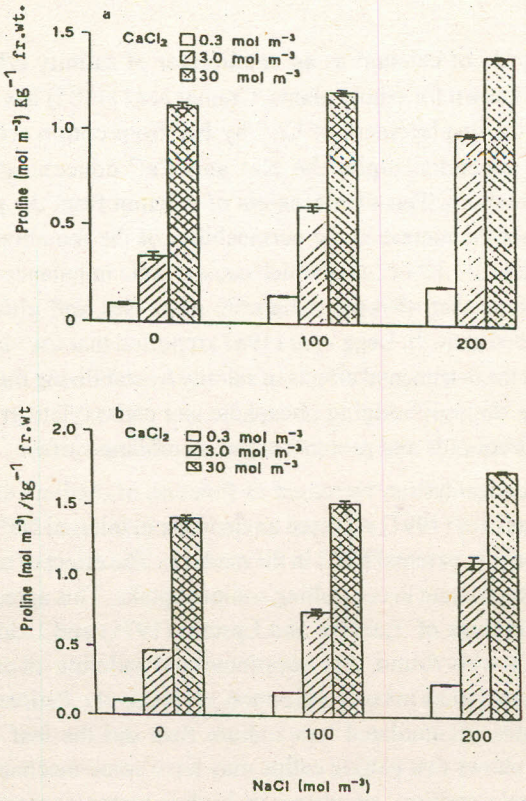

Fig 13 Effect of various concentrations of NaCl and $CaCl_2$ on proline contents of leaf (a) and petiole (b) callus cultures of Acala SJ2. The data points are means of 5 replications. The vertical bars represent SE (experiment no.2).

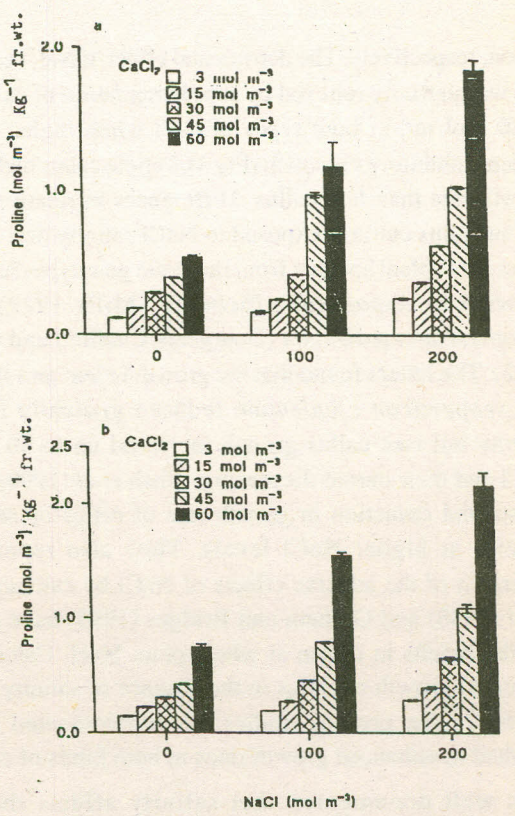

Fig 14 Effect of various concentrations of NaCl and $CaCl_2$ on proline contents of leaf (a) and petiole (b) callus cultures of Acala SJ2. The data points are means of 3 replications. The vertical bars represent SE (experiment no.3).

Table 4

Effect of various concentrations of NaCl and $CaCl_2$ on cation concentrations and Na/K ratio of petiole callus of Acala SJ2

NaCl	$CaCl_2$	Na^+	K^+	Na^+/K^+	Mg^{+2}	Ca^{+2}
0	3	9.2±0.1	49.7±2.2	0.2±0.01	5.7±0.2	3.2±0.7
	15	8.8±0.7	49.7±2.9	0.2±0.01	5.8±0.6	14.3±0.3
	30	6.9±0.3	61.5±0.8	0.1±0.00	7.3±0.7	24.2±0.3
	45	11.2±3.4	54.3±1.2	0.2±0.06	5.8±1.0	30.7±0.6
	60	17.4±0.6	44.7±1.2	0.4±0.00	7.3±0.4	43.8±1.3
100	3	86.5±6.3	48.2±2.3	1.8±0.8	4.2±1.0	2.7±0.2
	15	71.8±4.5	49.5±0.6	1.5±0.1	4.8±0.6	12.2±0.9
	30	60.6±0.9	52.8±1.6	1.1±0.04	5.8±0.4	21.0±0.5
	45	71.2±1.6	43.6±0.8	1.6±0.1	5.7±0.3	30.5±1.2
	60	79.8±2.7	30.1±1.1	2.7±0.1	5.3±0.2	42.8±2.9
200	3	158.6±2.0	47.2±1.5	3.7±0.1	1.7±0.7	1.5±0.3
	15	142±3.1	48.3±0.7	2.9±0.04	4.5±0.5	8.0±1.5
	30	133.8±1.8	49.6±0.5	2.7±0.06	5.5±0.6	17.7±2.2
	45	146.2±6.1	35.7±0.6	4.1±0.1	5.7±0.2	28.3±0.2
	60	159.0±2.0	22.7±1.4	7.1±0.5	4.2±0.2	41.5±2.0

The data are means of three replication ± standard error of means (experiment No.3)

cotton, respectively. The detrimental effect of NaCl on growth rate was partially relieved by supplementation of calcium up to 30 mol m^{-3} in both types of calli while higher calcium became inhibitory to growth (Fig 3). Petiole callus had a higher growth rate than leaf callus. Differences in relative growth rate in callus cultures exposed to NaCl salinity derived from different explant sources from the same genotypes have been reported in *Asparagus officinalis* (Mills 1989) and in *Lycopersicon esculentum* (Bourgeais-Chaillou and Guerrier 1992). The latters found that the growth in leaf and stem calli of *Lycopersicon esculentum* reduced gradually by NaCl salinity but root callus growth increased up to 50 mol m^{-3} NaCl and then started decreasing. Shah *et al* (1990) found a substantial reduction in growth rate of callus of *Medicago* species at higher NaCl levels. They also reported the reversion of the adverse effects of NaCl by calcium. Kurth *et al* (1986) and Gorham and Bridges (1995) have reported similar results in cotton at whole plant level. Calcium also increases growth of callus in the absence of salinity (Akhtar 1996). In the present studies the supplemented calcium resulted in enhanced growth rates in both kinds of callus.

It is well documented that salinity affects the water contents of plants (Gorham *et al* 1985). Water contents of the callus cultures decreased significantly on a NaCl supplemented medium. The present findings support the results of Shah *et al* (1990) who reported decreased water contents at higher NaCl concentration in *Medicago* species. However, the increase in water contents as a result of calcium treatment does not agree with their results. They found no effect of calcium on water contents of callus. Mukhtar and Hasnain (1994) attributed the salinity-induced callus growth reduction to the reduced cell colume due to loss of water from the cells under salinity stress. They added that the reduced cell volume may also be advantageous since solute accumulation necessary for osmotic adjustment of a small cell would be energetically less costly relative to that of a large cell.

The results obtained by Gorham and Bridges (1995) from Acala SJ2 at the whole plant level at various calcium and NaCl concentrations and in the present study at the cellular level, suggested that salt tolerance may operate both at cellular and whole plant level. In both cases the growth was reduced as a result of salinity and supplemented calcium ameliorated this growth reduction. Similar results have been reported by Gossett *et al* (1993) who found NaCl to reduce the growth of cotton cultivars (Acala 1517-88 and Deltapine 50) in whole plants and in corresponding callus cultures. They concluded that the salt tolerance mechanism operates at cellular level.

The role of calcium as an ameliorator of salinity effects is well known for whole plants. Cramer *et al* (1985) has shown that the displacement of Ca^{+2} by Na^{+} from cotton root cells was dependent upon the Na^{+} and Ca^{+2} concentration of the medium. This displacement of calcium from the plasma membrane increased the permeability of the membrane and leakage of K^{+} occured which caused ionic imbalance within the cells that affected metabolic processes and ultimately reduced growth. Legg *et al* (1982) reported that Ca^{+2} ameliorated the detrimental effects of salinity by stabilizing the membrane through bridging phosphate and carboxylate group of phospholipids and proteins at the membrane surface.

Na^{+} concentration increased as function of medium salinity. Abbas *et al* (1991) reported an increase in internal Na^{+} due to increase in external NaCl in the medium. The effect of calcium was significant in controlling sodium uptake. This agrees with the findings of LaHaye and Epstein (1971) and Leidi *et al* 1991) who found that supplemented calcium decreased sodium uptake in bean and cotton, respectively. Petiole callus cultures accumulated less sodium than did the leaf callus. This shows that petiole callus may have some mechanism to exclude sodium, resulting in higher water contents and enabled it to grow better under high salt stress than leaf callus.

Potassium ion (K^{+}) concentrations of both the types of callus decreased with increasing medium salinity. These results are consistent with those of Abbas *et al* (1991) and Shah *et al* (1990). Interestingly, K^{+} concentrations were significantly higher at high calcium concentrations upto only 30 mol m^{-3} than at lower concentrations (0.3, 3 and 15 mol m^{-3}). Both high sodium and low potassium concentrations in both kinds of callus contributed to higher Na^{+}/K^{+} ratios at low calcium treatments. Lower Na^{+}/K^{+} ratio at higher calcium treatments could be interpreted according to the suggestions of Cramer *et al* (1985) that the Na^{+} displaces calcium from the plasma membrane of the root cells, which enhances the efflux of K^{+}, raising the Na^{+}/K^{+} ratio. High Na^{+}/K^{+} ratio is toxic to metabolic processes of the plant cells. Similar results have been reported by Shah *et al* (1990).

Increasing medium salinity decreased calcium (Ca^{+2}) ion concentrations in both the types of callus. This decrease was more pronounced at lower external calcium concentrations of the medium and higher NaCl levels. In both calli the Ca^{+2} concentrations was higher at high calcium treatments, reflecting availability of calcium ions. These results support the findings of Shah *et al* (1990) who reported that calcium ion concentrations of the *Medicago* species callus were affected by salinity and calcium ion concentrations were higher of high calcium level. Inhibited uptake of calcium by salinity has

been reported in cotton (Shimose and Skeiya 1991). He and Cramer (1993) found that Ca^{+a} concentration decreased with increasing salinity.

Magnesium (Mg^{+2}) ion concentrations in the callus decreased with increasing salinity (first experiment). Supplemented calcium increased Mg^{+2} (second experiment) but magnesium ion concentration decreased beyond 30 mol m^{-3} $CaCl_2$ (third experiment). Similar results have been reported in cotton (Gorham and Bridges 1995; Shimose and Sekiya 1991) and in *Phaseolus vulgaris* (Abbas *et al* 1991).

Among anions, Cl^- ion concentration of the callus increased as a result of increased salinity and calcium chloride. These results are consistent with the results obtained at the whole plant level (Gorham and Bridges 1995). Higher medium salinity and calcium levels did not affect nitrate, phosphate and sulphate ion concentrations of callus. Gorham and Bridges (1995) while working with hydroponic culture of Acala SJ2 reported that other anions were not affected by calcium while chloride ions increased.

Of all the ions studied, the K^+ ion concentration of the callus cultures seems to play an important role in the ionic balance of the calli. Leigh and Jones (1984) suggested that for normal growth of the cell the optimum concentration of potassium ions in the cytoplasm ranged from 100 to 200 mol m^{-3}. As all metabolic processes in the cell occur at proper ionic concentrations, decrease or increase of ion concentration below a critical point or above a maximum level affects metabolic processes and ultimately growth is influenced. K^+ ions act as enzyme cofactors and an increase in the Na^+/K^+ ratio decreaseds protein translation efficiency. K^+ is the major cytosolic cation and the major counter ion to proton pumps and pH regulators (Yeo 1981).

The results obtained with the callus cultures show a large increase in proline concentration under NaCl stress as compared to control. Proline accumulation in response to salinity levels has been reported at cellular level in *Lycopersicon esculentum* (Bourgeais-Chaillou and Guerrier 1992) and at whole plant level in *Phaseolus vulgaris* (Abbas *et al* 1991).

Paleg *et al* (1985) reported that proline protects protein-containing systems against the unfavourable consequences of dehydration and other stresses. Exogenous proline up to 400 mol m^{-3} does not depress enzyme activities, hence it is considered a compatible solute (Pollard and Jones 1979). Lone *et al* showed that exogenous proline enhanced K^+/Na^+ discrimination in transport from root to shoot in cultured barley embryos. Chu *et al* (1976), using iso-osmotic solutions of polyethylene glycol, NaCl, KCl, Na_2SO_4, $CaCl_2$ and $MgCl_2$, found that excised barley leaves or the whole plants accumulated elevated levels of proline and the highest level was found with $MgCl_2$ and $CaCl_2$. High levels of proline at high external calcium treatments at the highest medium salinity (200 mol m^{-3}) showed interactions of NaCl and calcium, while Chu *et al* (1976) were looking at the response of proline with individual types of salinity as the stress. Calcium induced proline accumulation may be through an osmotic effect.

Conclusions. On the basis of results obtained in the present study it can be concluded that NaCl stress reduces the growth rate and water contents of callus cultures. Supplemented calcium enhances the growth rate and weter contents of callus up to 30 mol m^{-3} under salt stress and is inhibitory to growth at 45 and 60 mol m^{-3}. Na^+ concentration of the cells increases and K^+ concentration decreases at higher salinity levels, resulting in higher Na^+/K^+ ratio. NaCl stress decreases the calcium ion concentrations of callus. Higher external Ca^{+2} concentration increases the potassium, calcium and magnesium ion concentrations of callus. Proline concentrations in callus cultures increase with increasing medium salinity and calcium enhances proline concentrations. Calcium partially reversed the detrimental effects of NaCl through:

- regulating K^+ concentrations, whether as enhanced uptake or reduced leakage,
- increasing proline accumulation at high salinity,
- decreasing Na^+ accumulation and
- decreasing Na^+/K^+ ratio.

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