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Full Length Research Paper

Influence of intracellular Na^+, K^+ and Cl^- on the salt tolerance in suspension cell cultures of *Medicago media*

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In the process of selection for salt tolerance, it is important to understand the physiological basis of ion management executed by the cells through the exclusion, accumulation or maintenance of ratios of specific ions. Intracellular accumulation of Na^+, K^+ and Cl^- ions in the cells *in vitro* was studied as a factor in salt tolerance in suspension cultures of *Medicago media* cv. Rambler. Cells selected for NaCl tolerance (R151-S) and the non-selected R151 (normal) cells were grown in MS medium containing 0, 100, 250, 400 and 550 mM NaCl, Na^+, Cl^- or K^+ for 5 weeks and examined for relative growth rate per week (RGR/week) and intracellular concentration of Na^+, Cl^- and K^+ ions. R151-S cells were found to be significantly more tolerant to the elevated Na^+ and Cl^- levels than R151. When the effects of Na^+ and Cl^- ions were compared, R151-S appeared to be more tolerant of higher concentrations of Cl^- as compared to the equimolar concentrations of Na^+. As the concentration of NaCl in the medium increased, the intracellular concentrations of Na^+ increased, though not at a proportional rate. Also, when the external Cl^- concentration increased, there was no significant change in the intracellular accumulation of Na^+. On the contrary, intracellular Na^+ decreased when the external K^+ was increased. In the cells adapted to high concentration of NaCl, the concentration of intracellular K^+ was lower than the non-adapted cells. The adequate intracellular K^+ concentration for optimum growth in *M. media* suspension cultures was found to be 3000 µM/g dry weight. Excess or deficiency of intracellular K^+ caused severe reduction in the growth rates in all cell lines.

Key words: Salt tolerance, *Medicago media*, suspension cell culture, intracellular ions, organic relative growth rate.

INTRODUCTION

Salinity is a major environmental stress of global concern that severely limits agricultural crop production world-wide. However, the increasing demand of the food intensifies the interest in the development of salinity-tolerant varieties. In spite of several studies conducted on the effects of salinity in plants, there has been little success in producing salt resistant crops (Ashraf and Ali, 2008). Farmers still prefer to use yield-selected rather than salt-selected lines in salty soils. Plant tissue culture techniques coupled with genetic transformation have the potential in obtaining salt tolerant cell lines in a wide range of species (Arzani, 2008). The cells and tissues can be maintained in culture for an indefinite period and can be trained to tolerate high salinity levels. There are several reports on salt tolerant cell lines in higher plants, but the trait disappeared in the regenerated plants (Tal, 1994). To make tissue culture as an efficient tool for obtaining stable salt tolerant plants, more information is needed on the physiological mechanisms of salt tolerance in cultured cells and the persistence of the character in regenerated plants (Rai et al., 2011).

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Abbreviations: **R151,** Control (normal) suspension cell culture of *Medicago media* cv. Rambler; **R151-S,** salt tolerant suspension cell culture of *Medicago media* cv. Rambler; **RGR,** relative growth rate.

Tolerance to salinity is influenced by a number of physiological, morphological and ontogenetic characters in plants, and is a complex multigenic trait. Molecular analysis has shown that acquired cellular salt-tolerance in alfalfa led to both constitutive and salt-inducible increases in gene expression in the salt-tolerant lines compared to the salt-sensitive parent lines (Winicov et al., 1989). Some transgenic plants are designed to over-express specific genes, known to be up-regulated by salt/drought stress (Pilon-Smits et al., 1995; Xu et al., 1996; Tian et al., 2006). The genes have been cloned from alfalfa and the function of the salt-induced transcripts (Alfin1); have been shown to possess salt dependent mRNA accumulation in salt-tolerant cultures and regenerated plants (Winicov and Bastola, 1999; Winicov et al., 2004).

Salinity tolerance in glycophytes is associated with the ability to exclude Na^+ from uptake (Tester and Davenport, 2003) and maintain high K^+/Na^+ ratios (Greenway and Munns, 1980; Chen et al., 2005). Ions such as Na^+ and Cl^-, can reach toxic levels in the plant and inhibit the growth due to ion build up in the cell wall (Flowers et al., 1991). Among the various ions Na^+ has received much attention because it interferes with K^+ uptake and Ca^{2+} and Mg^{2+} function (Serrano, 1996). In general, the uptake of Na^+ is balanced with the uptake of Cl^- and efflux of K^+ (Tyerman and Skerrett, 1999). Yamashita and Matsumoto (1996) indicated a passive Cl^- influx into root cells under high salinity. Plant cells are unlikely to tolerate high Cl^- concentrations in cytoplasm. It is likely that there are certain mechanisms to maintain low cytosolic Cl^- concentrations either by efflux across the plasma membrane or by efflux across the tonoplast to the vacuole (Yamashita and Matsumoto, 1996; Ashraf and Akram, 2009). Na^+ is found to affect K^+ uptake and distribution (He and Cramer, 1993). K^+ is important for osmotic adjustment and a decrease in K^+ absorption leads to reduced growth (Kafkafi, 1984).

In a bid to evaluate the salt tolerance, the growth parameters are determined in the suspension cell cultures of *Medicago media* in NaCl-selected (R151-S) and non-selected (R151) cell lines. The NaCl-selected cell line was also tested for the stability of the salt tolerant trait by growing in NaCl-free medium (R151-S1) for several subcultures and then transferring back to media with higher NaCl concentration. To investigate the toxic effects of individual Na^+ and Cl^- ions, the experiments are conducted using different concentrations of Na^+ (without Cl^-) and Cl^- (without Na^+). The novelty of the present work was to study the toxicity of individual salt ions (Na^+, Cl^- and K^+), and to understand the relationship between the external supply and intracellular ion accumulation.

MATERIALS AND METHODS

Suspension cultures were initiated from the young leaves of *M. media* cultivar Rambler from the greenhouse grown plants, designated as R151 (Al Rawahy, 2000). The cultures were maintained in MS medium (Murashige and Skoog, 1962) containing 1.0 mgL^{-1} kinetin and 5.0 mg/L^{-1} 2, 4-D. The cultures were incubated at 25 ± 2°C on a rotary shaker at 100 cycles per minute under illumination of 50 to 60 $\mu M/m^3/s$ for 16 h per day by cool white fluorescent lamps. The media was supplemented with various concentrations of NaCl. To select salt tolerant cell lines, a stepwise procedure was used by increasing the NaCl by 50 mM in every step of subculture. After 4 weeks in the culture, the surviving cells were transferred to a fresh medium with higher salinity. This way the cells capable of growing at 550 mM NaCl were obtained and designated as R151-S cell line. This selected cell line was maintained at least for 6 months in the media containing 550 mM NaCl. To test the persistence of the salt tolerance, these R151-S cells were cultured in MS media without any NaCl to obtain R151-S1 cell line. The R151-S1 cell cultures were maintained in NaCl-free medium for at least 6 months before they were transferred to NaCl containing media in further experiments. All experiments were performed with cells taken at the exponential growth phase and 10 replicate cultures were maintained for each treatment.

To establish effect of each of Na^+, Cl^- and K^+, 0.5 g of cells were transferred to medium supplemented with 0, 100, 250, 400 or 550 mM Na^+, Cl^- or K^+, respectively by adjusting the following macronutrients in the culture media: NO_3^-, NH_4^+, PO_4^{3-} and SO_4^{2-} (Table 1). The effect of NO_3^{-2}, NH_4^+, PO_4^{3-} and SO_4^{2-} nutrients on callus culture was tested before the experiment using MS media with highest concentration of above nutrients. There was no significant effect (*P>0.05*) in growth of cultures between MS medium and any of these treatments. Therefore, it can be assumed that any growth effect in these treatments is due to Na^+, Cl^- or K^+. The pH of media was adjusted to 5.7 ± 0.2 using NaOH in Na^+ and Cl^- treatments or KOH in K^+ treatment, the addition of small amount (< 3 mM) will not have significant effect. Osmotic potentials were measured (using a vapour pressure osmometer, 5100 C WESCOR) immediately after inoculation and there were no significant differences in osmotic potentials between cultures of different treatments

Also, the growth rates of R151, R151-S and R151-S1 cell cultures were obtained by measuring the final dry weight of cells at the end of the 5 weeks of growth period. Cells were harvested by filtering the sediment of cells through stainless steel fine-mesh (2 µ). About 0.5 g of cells were taken and washed twice with an iso-osmotic solution of D-sorbitol containing 3 mM $CaCl_2$ and dried in an oven at 60°C for 48 h and the final dry weight was recorded. Organic dry weight of cell samples were obtained by subtracting the weight of intracellular Na^+, Cl^- and K^+ burden from the final dry weights. Organic relative growth rate (organic RGR) per week of ten replicates was then calculated as:

$$\text{Organic RGR/ Week}_{sample} = ((\ln(\text{Organic Dry Weight}_{sample}) - \ln(\sum \text{Initial Organic Dry Weight})) / \text{weeks}.$$

The organic RGRs were used since the dry weights do not include the weights of Na^+, Cl^- and K^+ in the cells and genuine growth is not confused with weight increase due to salt uptake (Chaudhary et al., 1994). For the measurement of intracellular Na^+, Cl^- and K^+ ions, 0.1 g of dried sample of cells was mixed with 10 ml of double distilled water and kept in a boiling water bath for 1 h. The extract was made up to 30 ml and filtered through ash-free filter paper. Na^+ and K^+ ions were analyzed by using a flame photometer (Advanced Technical Services GmbH, England), calibrated using standard Na^+ and K^+ solutions (SIGMA). Chloride ion was measured by an EIL selective ion electrode (Chemlab Scientific Products Ltd, USA), calibrated with Cl^- standard solutions (Sigma). Concentrations of intracellular ions were expressed as μ mol g^{-1} dry weight.

Statistical analysis of the data was performed using the 'SPSS' 17.0. The data set was a good fit to a normal distribution Kolmogorov-Smirvon test. In order to assess significant differences between treatments, ANOVA was performed with post hoc test analyses based on the Duncan test. Throughout the study, values

Table 1. The concentration of Na^+, Cl^- or K^+ (mM) in modified MS medium.

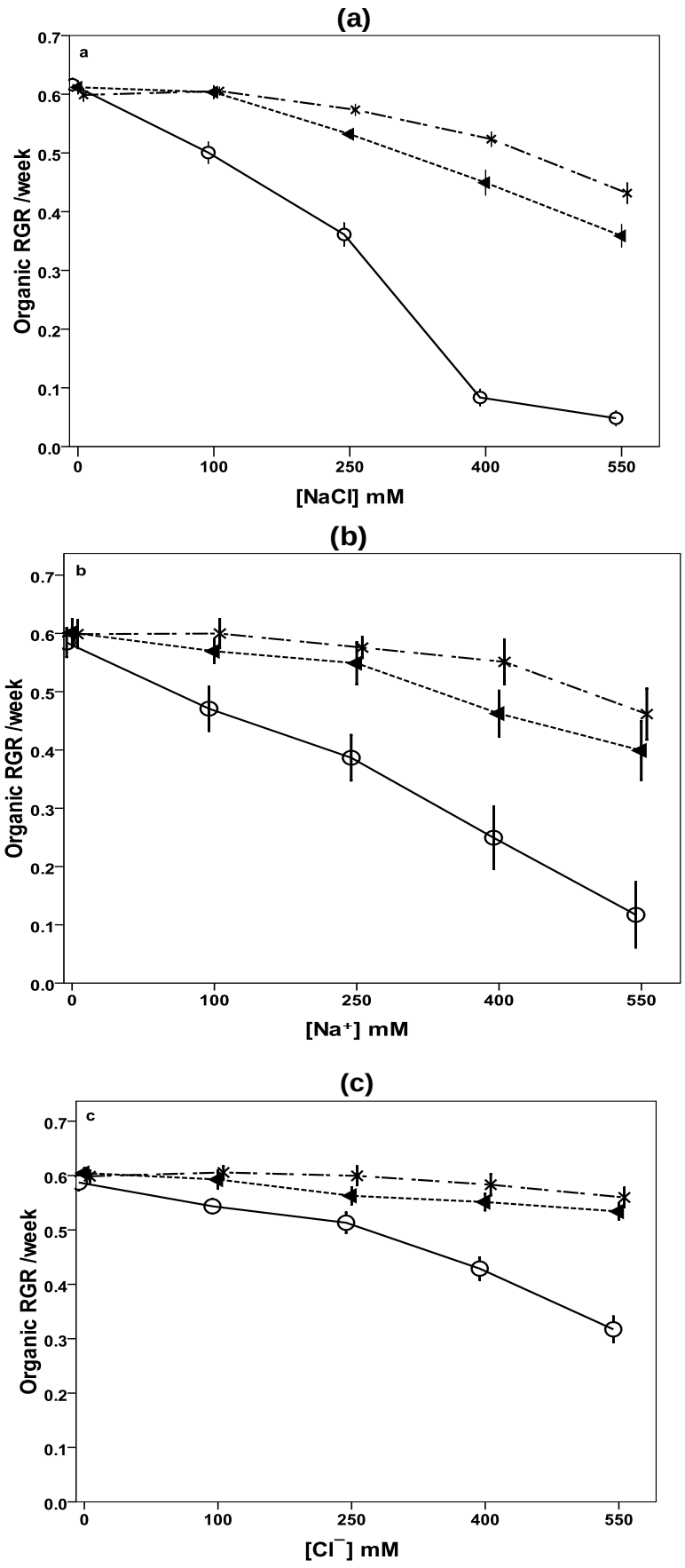
Treatment	Concentration (mM)								
	NO_3^{-2}	NH_4^+	PO_4^-	SO_4^+	Na^+	Cl^-	K^+	Ca^{2+}	Mg^{2+}
MS	395	206	13	15	0	30	200	30	14
***Na*$^+$**									
0	395	206	13	15	0	3	200	30	14
100	445	206	38	27	100	0	200	30	14
250	545	156	38	27	250	30	200	30	14
400	550	130	30	75	400	30	200	30	14
550	600	150	50	155	550	30	200	30	14
						30			
***Cl*$^-$**									
0	395	206	13	15	0	30	200	30	14
100	394	246	13	15	0	100	200	30	14
250	295	300	25	25	0	250	200	30	14
400	300	400	30	15	0	400	200	30	14
550	165	450	30	15	0	550	200	30	14
***K*$^+$**									
0	236	356	30	60	0	30	0	30	14
100	336	266	30	30	0	30	100	30	14
250	386	236	30	75	0	30	250	30	14
400	400	200	55	90	0	30	400	30	14
550	480	130	55	90	0	30	550	30	14

were presented as mean ± SE, and the levels were considered significantly different at P<0.05.

RESULTS

Figure 1a to d gives the organic RGR per week of R151, R151-S and R151-S1 cell lines at different concentrations of NaCl, Na^+, Cl^- and K^+ treatments, respectively. With increasing external NaCl concentrations, the R151 showed significant decrease in growth and complete necrosis at higher concentrations of NaCl, as compared to R151-S and R151-S1 selected cell lines. As the Na^+ concentration increased, there was significant decrease in growth in all the three cell lines. However, in R151, there was sharp decrease with discoloration and necrosis at higher concentrations of Na^+ in the external medium. Similarly, less severe trend was noticed in the case of Cl^- treatments. In the case of K^+ treatment, there was an increase in the growth at 100 and 250 mM, followed by a decrease at 400 and 550 mM. The organic RGRs per week of all the three cell lines in the absence of K^+ (0 mM K^+) were similar to that at 550 mM K^+ treatment. Statistical analysis showed no significant difference in the growth of cell lines between NaCl and Na^+ treatments and between Na^+ and Cl^- treatments, while highly significant differences (P<0.01) was observed in the growth of cell lines between NaCl and Cl^- treatments, NaCl and K^+ treatments, Na^+ and K^+ treatments and Cl^- and K^+ treatments.

Figure 2a to d displays the intracellular Na^+ ion content expressed on a dry weight basis at different NaCl, Na^+, K^+ and Cl^- treatments, respectively. As the concentration of NaCl and Na^+ in the medium increased, so did the intracellular concentrations of Na^+. R151-S and R151-S1 cell lines showed significantly (*P<0.01*) lower intracellular accumulation of Na^+ ions than R151 cell line at all concentrations above 0 mM NaCl and Na^+. In case of Cl^- treatment, there were no significant changes in the intracellular accumulation of Na^+ as Cl^- concentration increased. Significantly, higher (*P<0.01*) intracellular Na^+ accumulated in R151-S and R151-S1 cell lines compared to R151 cell line. Similarly, in K^+ treatments, the intracellular Na^+ concentrations of R151-S and R151-S1 cell lines were significantly higher (*P<0.01*) compared with R151 cell line. As the external K^+ ion concentration increased the intracellular Na^+ ion concentration decreased significantly (*P<0.05*) in R151-S and R151-S1 cell lines, but not in R151 cell lines. Statistical analysis showed no significant difference in the concentrations of


(a)
a
Organic RGR /week
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0
100
250
400
550
[NaCl] mM
(b)
b
Organic RGR /week
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0
100
250
400
550
[Na+] mM
(c)
c
Organic RGR /week
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0
100
250
400
550
[Cl−] mM

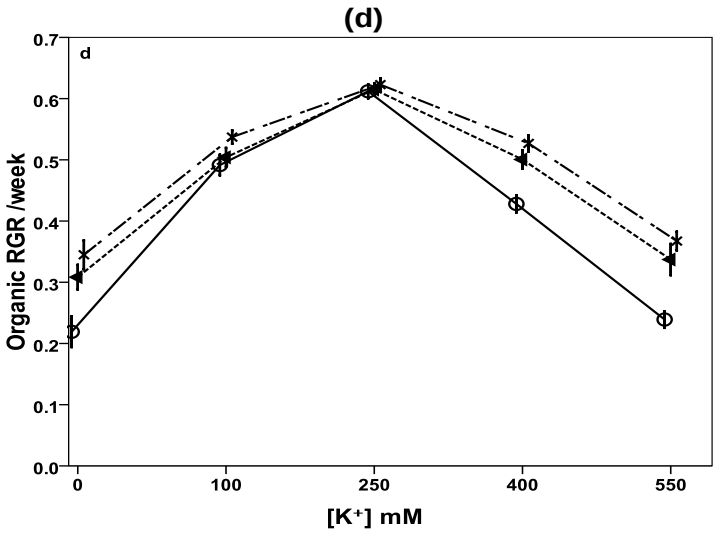
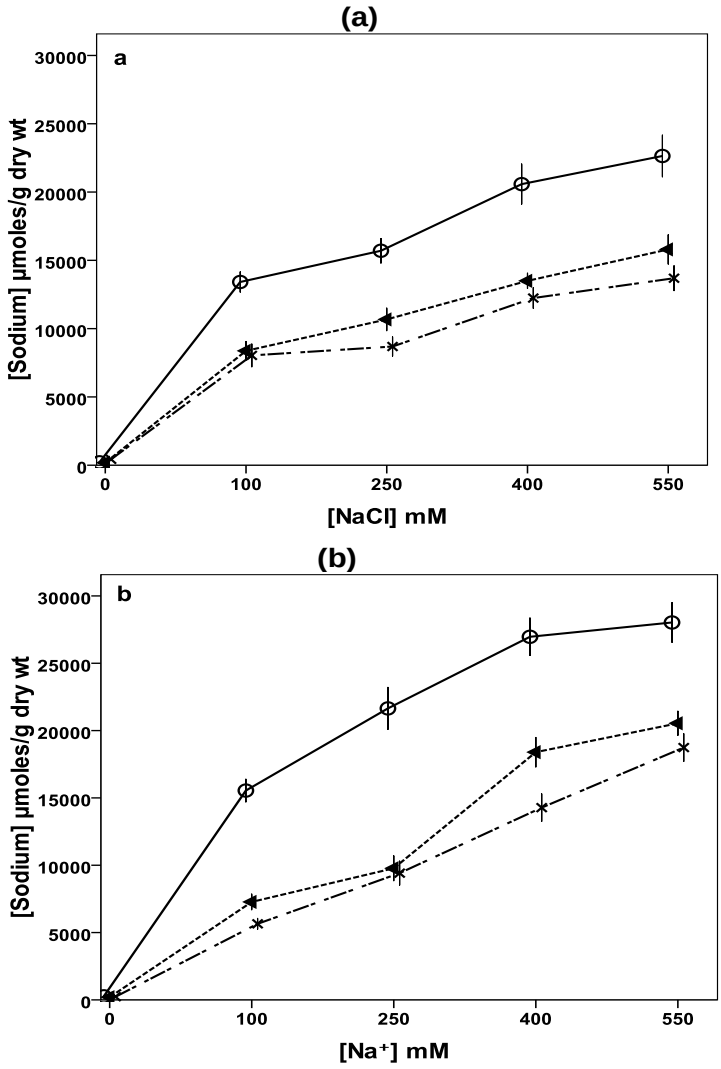


Figure 1. Organic RGR per week of *M. media* cell lines grown in the growth media containing 0, 100, 250, 400 and 550 mM of (a) NaCl, (b) Na^+, (c) Cl^- and (d) K^+ (d). Cell lines: (○) R151; (◀) R151-S; (x) R151-S1.



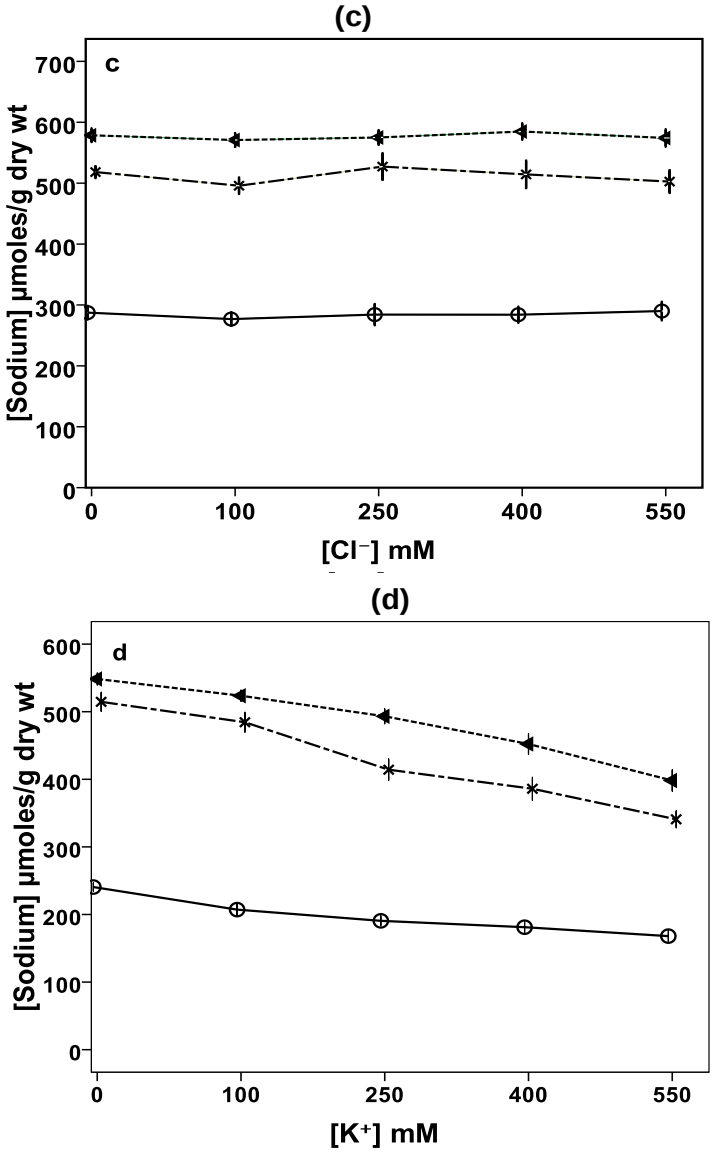
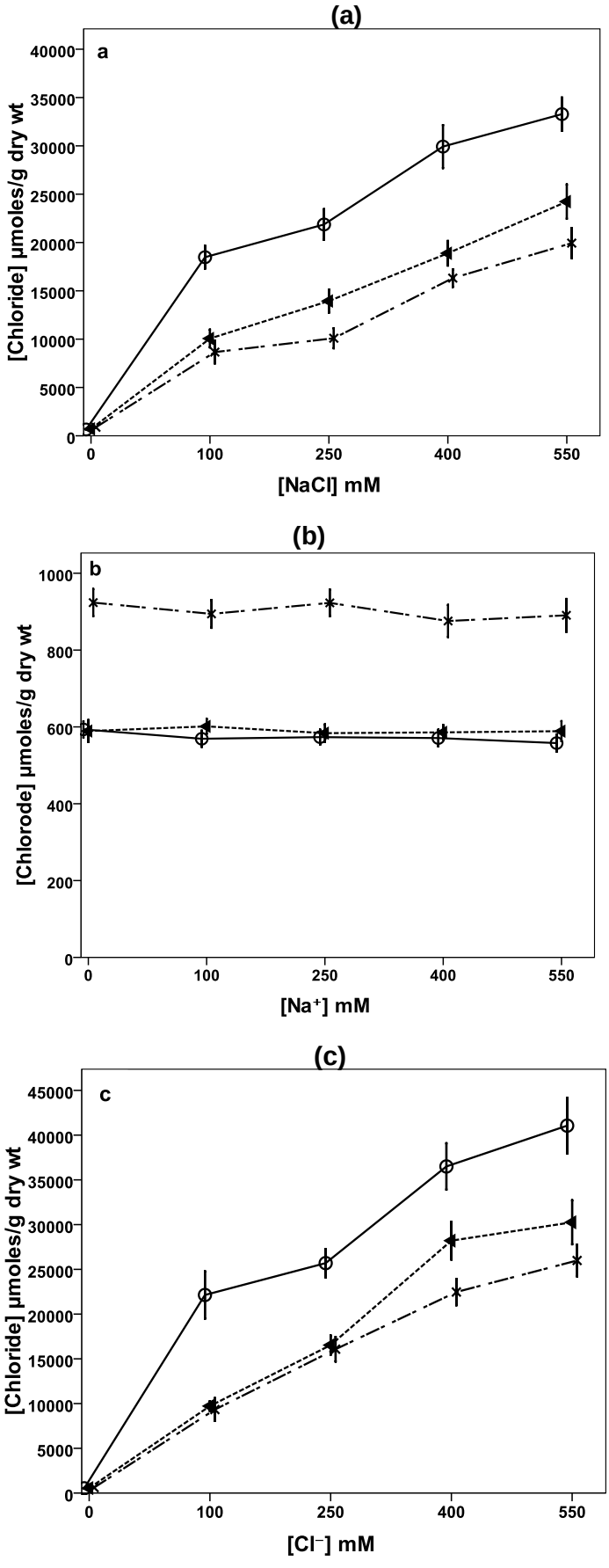


Figure 2. Sodium ion concentration in cells of *M. media* cell lines grown in the growth media containing 0, 100, 250, 400 and 550 mM of (a) NaCl, (b), Na^+ (c) Cl^- and (d) K^+ . Cell lines: (○) R151; (◄) R151-S; (x) R151-S1.

Na^+ in cell lines between NaCl and N^+ treatments and between Cl^- and K^+ treatments, while highly significant differences ($P<0.01$) were observed in the Na^+ accumulation in cell lines between NaCl and Cl^- treatments, NaCl and K^+ treatments, Na^+ and Cl^- treatments and Na^+ and K^+ treatments.

Figure 3a to d shows the intracellular Cl^- ion content of cell lines in different external concentrations of NaCl, Na^+, K^+ and Cl^- treatments, respectively. There were significant (*P<0.01*) increases in intracellular Cl^- as the external concentrations of NaCl and Cl^- increased, but no changes in Na^+ and K^+ treatments. In case of NaCl and Cl^- treatments, significantly higher (*P<0.01*) concentrations of intracellular Cl^- were found in R151 cell line compared with other two cell lines, while in Na^+ and K^+ treatments significantly higher levels of Cl^- were found in R151-S cell line. Statistical analysis showed no significant differences in the concentrations of Cl^- in cell lines between NaCl and Cl^- treatments and between Na^+ and K^+ treatments, while highly significant differences ($P<0.01$) were found in the Cl^- accumulation in cell lines between NaCl and Na^+ treatments, NaCl and K^+ treatments, Na^+ and Cl^- treatments and Cl^- and K^+ treatments.

Intracellular concentrations of K^+ in cell lines can be seen in Figure 4a to d. Highly significant decreases in K^+


(a)
a
[Chloride] µmoles/g dry wt
40000
35000
30000
25000
20000
15000
10000
5000
0
0
100
250
400
550
[NaCl] mM
(b)
b
[Chlorode] µmoles/g dry wt
1000
800
600
400
200
0
0
100
250
400
550
[Na+] mM
(c)
c
[Chloride] µmoles/g dry wt
45000
40000
35000
30000
25000
20000
15000
10000
5000
0
0
100
250
400
550
[Cl−] mM

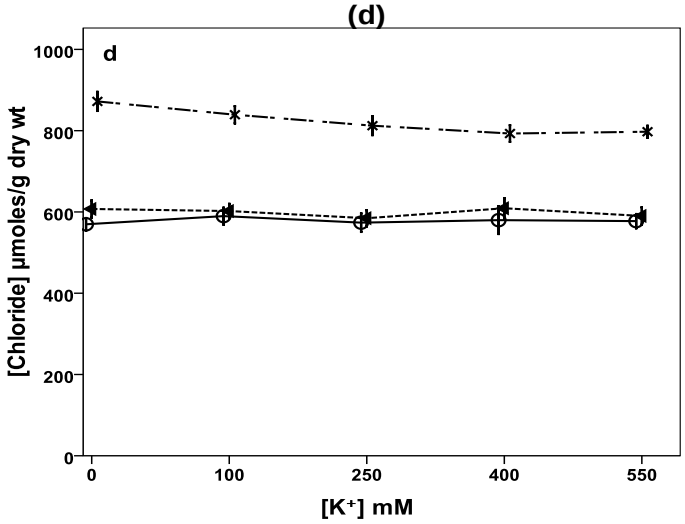
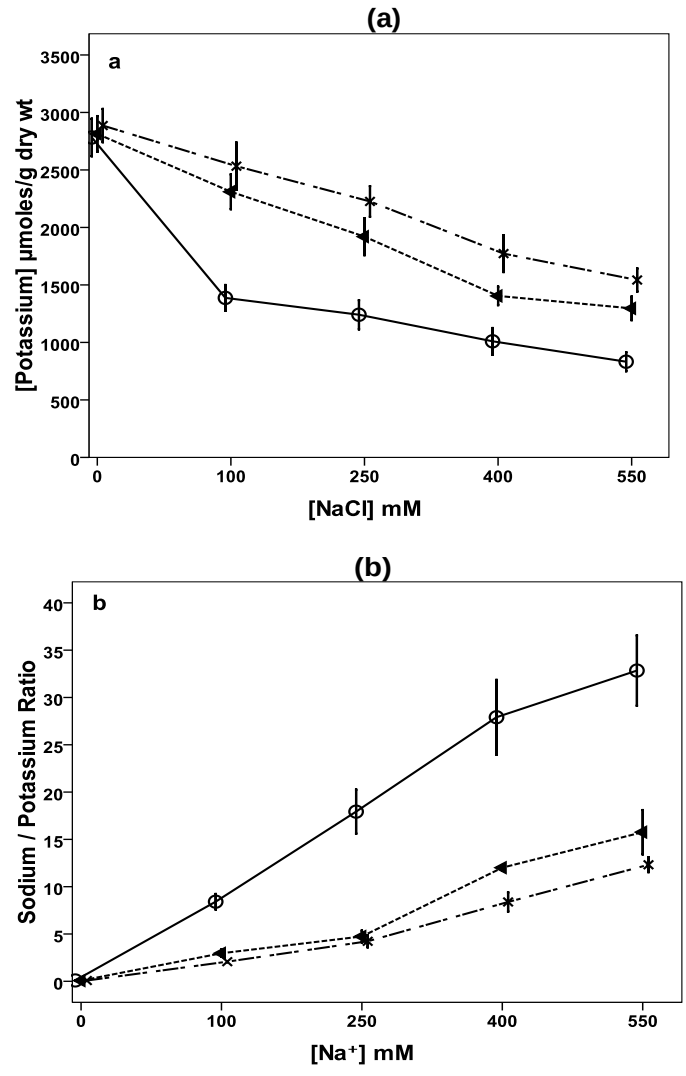


Figure 3. Chloride ion concentration in cells of *M. media* cell lines grown in the growth media containing 0, 100, 250, 400 and 550 mM of (a) NaCl, (b), Na^+ (c) Cl^- and (d) K^+ . Cell lines: (○) R151; (◄) R151-S; (x) R151-S1.



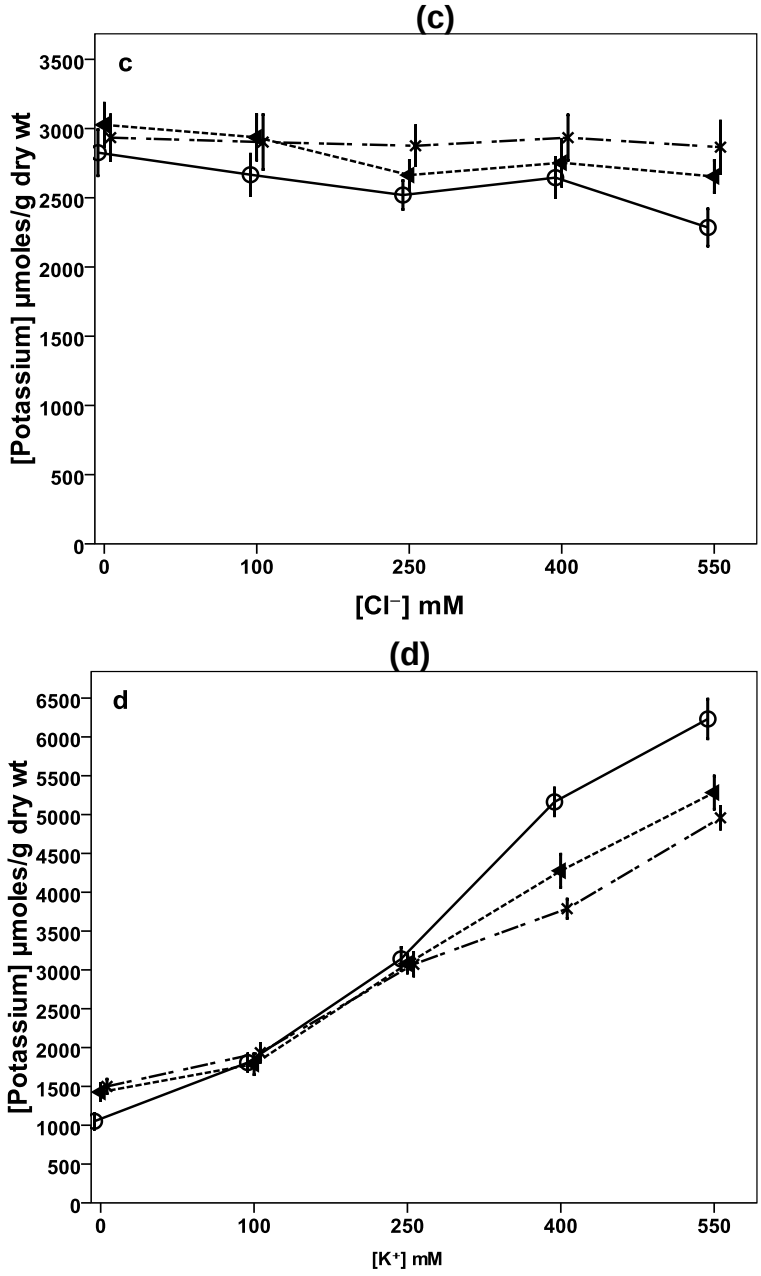
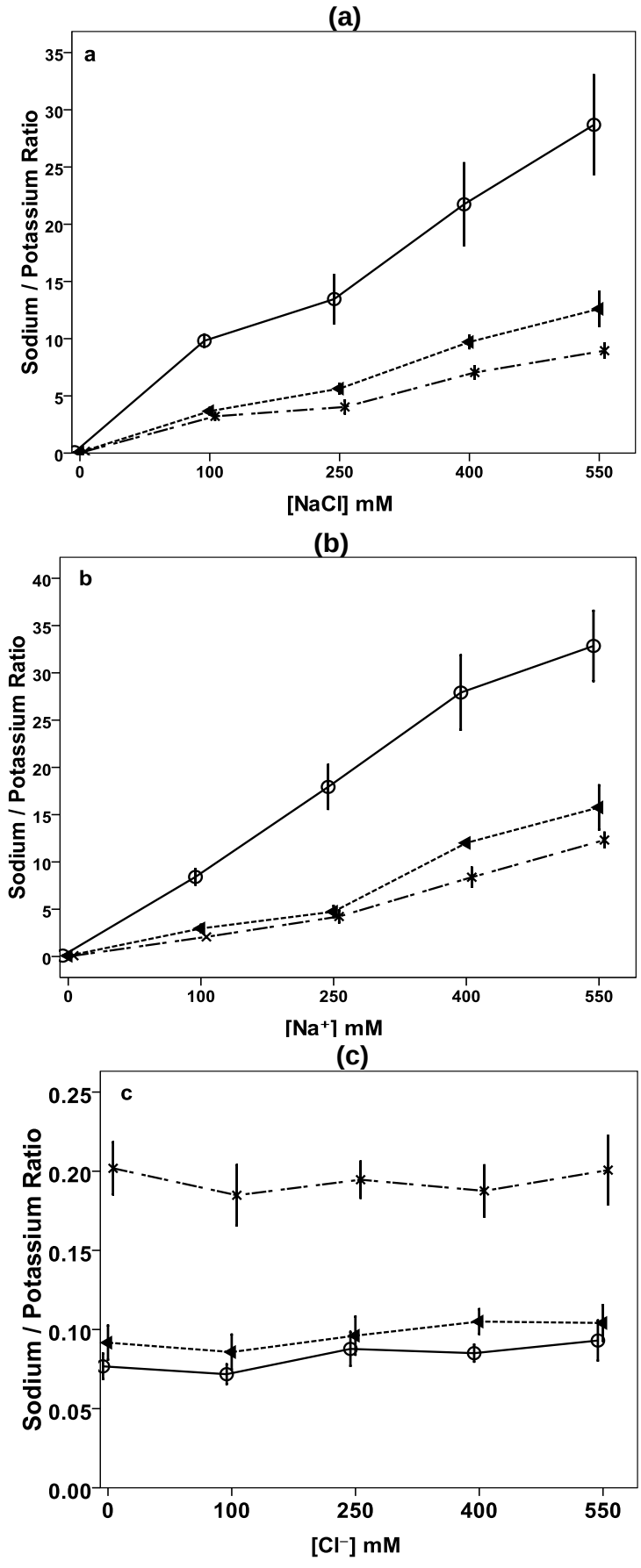


Figure 4. Potassium ion concentration in cells of *M. media* cell lines grown in the growth media containing 0, 100, 250, 400 and 550 mM of (a) NaCl, (b), Na^+ (c) Cl^- and (d) K^+ . Cell lines: (○) R151; (◄) R151-S; (x) R151-S1.

concentrations in all three cell lines were found as a function of external NaCl and Na^+ treatments, while highly significant (*P<0.01*) increase as the function of external K^+ treatments in all three cell lines. No significant changes in the K^+ levels in all cell lines occur as external Cl^- increase. There was no significant difference in the concentrations of K^+ in cell lines between NaCl and Na^+ treatments, while highly significant differences (P<0.01) were found in the K^+ concentrations in cell lines between NaCl and Cl^- treatments, NaCl and K^+ treatments, Na^+ and Cl^- treatments and Na^+ and K^+ treatments.

The intracellular Na^+/K^+ ratios of cell lines at different NaCl, Na^+, Cl^- and K^+ treatments are presented in Figure 5a to 5d. The Na^+/K^+ ratios are significantly higher (*P<0.01* in R151; *P<0.05* in R151-S and R151-S1) under both NaCl and Na^+ treatments, while an opposite trend was noticed in the ratios of Na^+/K^+ when cells were treated with K^+. No changes occurred in all cell lines as the level of Cl^- in the external medium increased. There were no significant differences in Na^+/K^+ ratios among


(a)
a
Sodium / Potassium Ratio
35
30
25
20
15
10
5
0
0
100
250
400
550
[NaCl] mM
(b)
b
Sodium / Potassium Ratio
40
35
30
25
20
15
10
5
0
0
100
250
400
550
[Na+] mM
(c)
c
Sodium / Potassium Ratio
0.25
0.20
0.15
0.10
0.05
0.00
0
100
250
400
550
[Cl−] mM

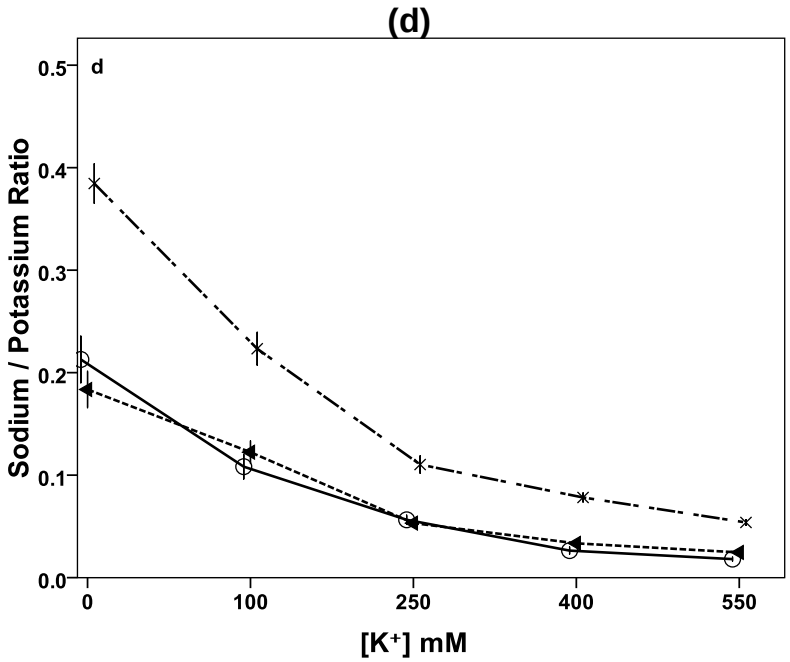


Figure 5. Na+/K+ ratio in cells of *M. media* cell lines grown in the growth media containing 0, 100, 250, 400 and 550 of (a) NaCl, (b), Na^+ (c) Cl^- and (d) K^+ . Cell lines: (○) R151; (◄) R151-S; (x) R151-S1.

cell lines between NaCl and Na^+ treatments, while highly significant differences (*P<0.01*) were observed among cell lines between NaCl and Cl^- treatments, NaCl and K^+ treatments, Na^+ and Cl^- treatments, Na^+ and K^+ treatments and Cl^- and K^+ treatments. Close examination of data on growth rates and intracellular K^+ revealed that the adequate intracellular K^+ concentration for optimum growth in these cell lines is about 3000 μmoles/g dry weight. Interestingly, similar intracellular K^+ levels were found when these NaCl-selected and non-selected cell lines were grown under non-stress conditions (0 mM NaCl (Figure 4a), O mM Na^+ (Fig 4b) and 0 mM Cl^- (Fig 4c)). Another interesting point to consider is that the level of Na^+/K^+ ratio of all three cell lines at 250 mM K^+ ion treatment was about 0.08, which is about the same when these cell cultures were grown under non-stress conditions (0 mM NaCl (Figure 5a), O mM Na^+ (Figure 5b) and 0 mM Cl^- (Figure 5c)).

DISCUSSION

Significant reduction in organic RGR per week in R151 non-selected cell line in comparison with R151-S and R151-S1 selected cell lines of *M. media* when treated with 100, 250, 400 and 550 mM NaCl, indicate the superiority of the selected cell lines in combating with the NaCl stress. All cell lines grew well in the absence of NaCl (0 mM), which is a control treatment in MS media without any stress. In spite of growing the R151-S1 selected cell line for several passages in the absence of NaCl, the trait was not lost, thus indicating the persistence of NaCl tolerance in the absence of NaCl selection pressure. Absence of necrosis and discoloration under NaCl, K^+ and Cl^- treatments proves the ability of these cells to survive under different stresses. The R151-S and R151-S1 selected cell lines were also more tolerant to Na^+ stress and showed higher growth, while the growth of R151 non-selected cell line significantly decreased with the increase in external Na^+ ion concentrations culminating in necrosis at 250 mM Na^+ treatment.

Reduction in growth was reported earlier in NaCl- non-adapted *Citrus aurantium* cell lines (Ben-Hayyim et al., 1985) and in non-selected cell line of *Vigna radiata*, (Gulati and Jaiwal, 1992). The inhibition of cell growth can be attributed to the higher uptake of Na^+ ions by cells. In the present study, NaCl selected cell lines were more tolerant and accumulated significantly lower intracellular Na^+ than non-selected cell line, suggesting that the selected cell lines have developed a mechanism of partial exclusion of Na^+ ions. Similar view was expressed by Houshmand et al. (2005) in wheat and Gandonou et al. (2005) in sugarcane experiments.

The ionic treatments showed that intracellular Na^+ and Cl^- contents of the selected and non-selected cell lines increased as a function of the external NaCl concentrations of the growth medium. At 0 mM NaCl (control medium), the intracellular Na^+ and Cl^- ion concentrations remained low in all three cell lines. However, there were

marked differences between cell lines in the levels of intracellular Na^+ and Cl^- that increased as a function of increased external NaCl concentration. There was greater influx of both Na^+ and Cl^- ions in R151 non-selected cell line than R151-S and R151-S1 selected cell lines. Similar trends were found in *Medicago sativa* suspension cultures (Shah et al., 1990; Chaudhary et al., 1994), callus cultures of blue berry (Muralitharan et al., 1992). This trend suggest that the NaCl-selected cell lines have developed Na^+ and Cl^- ion exclusion or avoidance mechanisms to combat NaCl stress. However, in sour orange NaCl-tolerant cell lines accumulate higher amount of Na^+ and Cl^- ions than NaCl sensitive cell lines (Storey and Walker, 1999).

An Interesting point to note was that selected cell lines R151-S and R151-S1 were found to contain relatively higher intracellular Na^+ compared with R151 in both Cl^- and K^+ treatments where no external Na^+ was supplied. Note that R151-S was maintained in high NaCl medium before these experiments, whereas R151-S1 cells were maintained in NaCl free medium for more than 6 months before the experiment and then treated with only Cl^- or K^+ but still contain about 50% more Na^+ compared with R151 cells. It is possible that the additional intracellular Na^+ may be due to their ability to accumulate Na^+ within salt adapted cells or may have compartmentalized into vacuoles. This view gathers support from the studies of Blumwald et al. (2000), Fukuda et al. (2004), Ashraf and Akram (2009) and Summart et al. (2010) in different plant species. Na^+ is pumped in to the vacuole before its concentrations are built up in cytoplasm which is catalyzed by Na^+/H^+ antiporter (Tyerman and Skerrett, 1999). Using Na^+/H^+ antiporters of the vacuolar membranes, the plant cells compartmentalize Na^+ into vacuoles and separate it from the cytosolic enzymes (Blumwald et al., 2000). This process helps to balance against the low extracellular osmotic potential created by the salt stress.

Several vacuolar Na+/H+ antiporter genes have been isolated from a number of plant species, such as AtNHX1 and AtNHX2 in Arabidopsis, TaNHX1 and TaNHX2 in wheat, OsNHX1 in rice, GhNHX1 in cotton, and HbNHX1 in *Hordeum* (Apse et al., 1999; Fukuda et al., 2004; Wang et al., 2004; Yokoi et al., 2002; Wu et al., 2004; Lü et al., 2005). In Arabidopsis, the plants with the over-expressing AtNHX1 had much higher salt tolerance than wildtype plants. The discovery of the Salt-Overly-Sensitive (SOS) pathways gives a better understanding of how Na^+ is sensed in a cellular system (Zhu, 2002; Türkan and Demiral, 2009). The plasma membrane Na^+/H^+ antiporter SOS1 make one of the possible Na^+ sensors which is also involved in Na^+ efflux. The electrochemical gradient of protons generated by two vacuolar H^+ translocating enzymes H^+-ATPase and H^+-PPase is the driving force for this process. The ability of plant cells to maintain low sodium in the cytosol is associated with the ability of the plants to grow under high salt regimes (Ashraf and Harris, 2004). High concentration of Na^+ disturbs the intracellular ion homeostasis, which may lead to membrane dysfunction, attenuation of metabolic activity and thereby causes growth inhibition, leading to cell death (Rus et al., 2004; Ashraf and Akram, 2009).

Moreover, in case of Cl^- treatments, the reduction in growth in the cultures was found to be relatively lesser in comparison with equimolar treatments of NaCl and Na^+, suggesting Na^+ has a greater toxic effect than Cl^- in *M. media* cells. This was clearly noticed in R151 non-selected cell line where the growth was significantly reduced at higher treatments of NaCl and Na^+, at the same time the intracellular Na^+ content was significantly higher as compared to the cultures that accumulated higher intracellular Cl^-. Chloride ion is the prevalent anion accompanying Na^+ and K^+ (Gulati and Jaiwal, 1992; Greenway and Munns, 1980; Jeschke and Wolf, 1988). The accumulation of intracellular K^+ was significantly higher in all three cell lines in Cl^- treatments as compared to Na^+ treatments. Perhaps Cl^- has a key physiological role in osmoregulation, whereas uptake and accumulation of Na^+ may conflict with K^+ as a nutrient, thus causing salt sensitivity. Higher K^+ contents have been considered to be associated with salt tolerance (Yang et al., 1990). The relation between growth and intracellular Na^+ content is complex, especially when the intercellular K^+ is considered. As the intracellular Na^+ increased, the intracellular K^+ followed an opposite trend. Such a decrease of intracellular K^+ content in the presence of Na^+ could indicate damage and may have been one of the factors for the reduction in growth. Similar view was expressed in cotton (Ashraf and Ahmad, 2000). Under salt conditions, metabolic toxicity of Na^+ is largely due to its ability to compete with K^+ for binding sites essential for cellular function (Bhandal and Malik, 1988). High K^+/Na^+ ratio is attributed to salt resistance (Qasim and Ashraf, 2006). Although, cells show high selectivity of K^+ over Na^+ ion, excessive amount of K^+ is detrimental.

In the present study, the intracellular concentrations of Na^+ were slightly reduced (although not significant in R151 and R151-S cells) as the external concentrations of K^+ increased in all three cell lines. The results indicate that lower intracellular Na^+ and adequate K^+ contents within the cells are crucial for the resumption of growth. The reduction in the growth with the reduction in intracellular K^+ ion content was clearly demonstrated in all cell lines when cultures were grown in different treatments of K^+. When external K^+ was not available, the intracellular K^+ content was significantly lower and the growth rates were also significantly lower, while intracellular Na^+ and Cl^- contents were normal. These cell lines were able to resume their optimum growth when external K^+ was supplied (250 mM). As the external K^+ increased to 550 mM, there was twofold increase the intracellular K^+ and interestingly, the growth rates decreased concomitantly in all three cell lines. Therefore, the maintenance of

adequate concentrations of K^+ ion is indeed essential for the normal growth of cell cultures of *M. media*. Storey and Walker (1999) reported that in cultured *Citrus sinensis* cell line, the adapted cells were capable in accumulating adequate amount of K^+. The increase in intracellular Cl^- content (without increase in Na^+ content) was found to have no significant effect in reduction in K^+ content in our cell cultures. Channels that are activated when the transmembrane potential is hyperpolarized are highly selective for K^+, while other channels that are activated when the membrane potential is depolarized are less selective, thus depolarization could be one mean by which sodium enters cells (Maser et al., 2002). Sodium can also enter via KUP/HAK/KT potassium transporters, cyclic-nucleotide-gated channels, glutamate-activated channels, LCT transporters, and HKT transporters, although, the relative role of each of these varies in different species (Tester and Davenport, 2003). In rice *OsHKT1* is down-regulated after osmotic shock with 150 mM NaCl in plants growing in a low K^+ (Golldack et al., 2002).

At higher concentrations of NaCl treatments, R151 cell line contained significantly higher ratio of Na^+ to K^+. It appears that the inhibition of K^+ uptake is reduced in NaCl stressed cells in the selected cell lines indicating that these cells had a higher K^+/Na^+ selectivity at the plasma membrane. Therefore, increased K^+/Na^+ selectivity of the K^+ uptake system might represent a significant adaptation to NaCl-stress. It is possible that the depolarisation opens the outward rectified channel allowing Na^+ influx and K^+ efflux under saline conditions and increasing conductivity. The results indicate that there is a decreasing Na^+ influx and increased K^+ efflux under NaCl stress leads to salt adaptation in *M. media* suspension cultures. R151 non-selected cell line significantly accumulated more Na^+ and released more K^+ ion than R151-S and R151-S1 selected cell lines. A similar conclusion was also made on tolerant cell lines of potato (Sabbah and Tal, 1990) and *Citrus sinensis* (Storey and Walker, 1999) that these cell lines efficiently excluded Na^+ and prevented the decrease of K^+ content. It is likely that K^+/Na^+ discrimination mechanism in plant cells is genetically controlled (Tyerman and Skerrett, 1999). However, the response of plant cells to K^+ ion increments is not uniform, especially in the presence of other ions. Many sensitive glycophytes fail to maintain adequate K^+ in the presence of high salt concentrations leading to damage (Poustini and Siosemardeh, 2004).

Conclusion

Increased levels of NaCl increased the intracellular concentrations of Na^+, while Cl^- did not show any significant change, whereas, K^+ increased with decreased Na^+. Salt tolerant trait was not lost in spite of growing the tolerant cell line in NaCl free media for several generations. Decrease in intracellular K^+ as a consequence of increased Na^+ uptake caused cellular damage and resulted in the reduction in growth and proliferation of suspension cells in culture.

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