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Relation between Ion Accumulation of Salt-Sensitive and Isolated Stable Salt-Tolerant Cell Lines of *Citrus aurantium*[1]

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ABSTRACT

Four selected NaCl-tolerant cell lines of Sour orange (*Citrus aurantium*) were compared with the nonselected cell line in their growth and internal ion content of Na^+, K^+, and Cl^- when exposed to increasing NaCl concentrations. No difference was found among the various NaCl-tolerant cell lines in Na^+ and Cl^- uptake, and all these cell lines took up similar or even larger amounts of Na^+ and Cl^- than the NaCl-sensitive cell line. Exposure of cells of NaCl-sensitive and NaCl-tolerant lines to equal external concentrations of NaCl, resulted in a greater loss of K^+ from the NaCl-sensitive cell line. This observation leads to the conclusion that growth and ability to retain high levels of internal K^+ are correlated. Exposure of the NaCl-tolerant cell lines to salts other than NaCl resulted in even greater tolerance to Na_2SO_4, but rather poor tolerance to K^+ introduced as either K_2SO_4 or KCl; the latter has a stronger inhibitory effect. The NaCl-sensitive cell line proved to be more sensitive to replacement of Na^+ by K^+. Analyses of internal Na^+, K^+, and Cl^- concentrations failed to identify any particular internal ion concentration which could serve as a reliable marker for salt tolerance.

In recent years it has been shown that variant cell lines, possessing a desired trait, can be selected from a population of cultured somatic cells of higher plants (4, 14, 19). Cells of important crops, having useful agricultural traits, such as tolerance to salt (6, 7, 9, 18), water stress (3), herbicides (4), or cold (8, 17) have been obtained. Such cell lines provide the starting material for possible regeneration of plants on the one hand, and a useful tool for elucidation of mechanisms of tolerance at the cellular level on the other hand.

Two mechanisms operate in the survival of salinity stress by various plants (12). Plants can either accumulate ions in response to high concentrations of salts in their environment, or protect themselves by exclusion of the salts and production of high concentrations of organic molecules to lower the osmotic potential of the cells. Cell cultures have mostly adopted the first mechanism, *e.g.* cells such as NaCl-selected alfalfa cells (6) accumulate NaCl when this salt is introduced into their growth medium.

Some aspects of salt tolerance in a NaCl-selected stable cell line of Shamouti orange (*Citrus sinensis* L. Osbeck) have been described previously (1, 2, 11). It was suggested that this cell line is indeed a variant line, and that its tolerance to salt most probably arises from partial avoidance. Recently, the isolation and stability of various NaCl-tolerant cell lines of another callus culture which originated from a different species of *Citrus*, namely Sour orange (*C. aurantium*), have been discussed (15). In the present study, the ionic content of the nonselected and NaCl-tolerant selected cells of Sour orange cell lines, exposed to media containing various salts, is reported. The analyses led to the conclusion that Sour orange NaCl-tolerant cells survive elevated levels of external salt through its accumulation.

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MATERIALS AND METHODS

Plant Material. Ovular callus of Sour orange (*Citrus aurantium*) and the NaCl-tolerant lines were obtained as described before (11, 15). Callus cells were subcultured monthly.

Medium. The basal medium was that of Murashige and Tucker (13) without any growth factors. Callus of NaCl-tolerant cells was routinely kept for over 1 year on medium containing 10 g L^{-1} NaCl.

Cell Growth. Cells were grown in solid medium (1% agar). Explants of about 50 mg were plated and determination of growth, expressed as gain in fresh weight, was carried out at maximal rate of growth, during or toward the end of the log phase (about 30 d). Growth data represent mean values of 10 repetitions, and each experiment was performed at least three times.

Ion Determination. Cells were collected and washed with 0.5 mM $CaSO_4$ for 5 min as described by Tal *et al.* (16), and then dried at 85°C for 24 h. Samples of 50 mg dry matter were wet ashed by H_2SO_4. Na^+ and K^+ were determined by Corning Evans Electroselenium Ltd flame photometer. Cl^- was determined according to Cotlove (5).

RESULTS AND DISCUSSION

The purpose of this study was to understand the mechanism by which several selected NaCl-tolerant cell lines of Sour orange survive at elevated salt levels, and if there are any differences among these cell lines. Moreover, we were interested to examine the degree of similarity between selected NaCl-tolerant cells of Sour orange and selected NaCl-tolerant cells of Shamouti orange, another *Citrus* species described before (2).

The relative growth of four NaCl-selected cell lines and the nonselected cell line is illustrated in Figure 1. At all NaCl concentrations tested, all four cell lines grew much better than the nonselected cell line, and only minor differences in relative growth were observed among the four selected cell lines. This is quite different from the situation described for the various NaCl-tolerant selected cell lines of Shamouti orange (15), where one can find more variable response in the ability of the selected cells to grow in the presence of NaCl concentrations higher than 0.1 M. It has already been reported that the NaCl-tolerant trait is a stable one for the four selected cell lines and that they perform similarly even after about 20 generations in the absence of

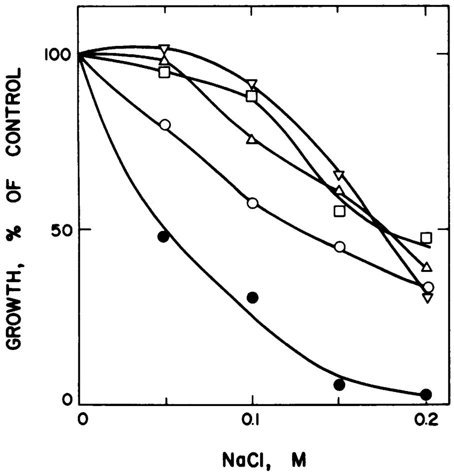

FIG. 1. Growth of NaCl-sensitive (SO-A) and NaCl-tolerant cell lines as a function of NaCl concentration. Growth was determined after 1 month; all other details are as described in "Materials and Methods." Mean values of five replications ± SD are given. (●), SO-A cells; (○), 01-7-2 cells; (△), 01-7-4 cells; (□), 01-7-3 cells; (▽), B-5-1 cells. 100% growth for SO-A, 01-7-2, 01-7-4, 01-7-3, and B-5-1 cells was 369 ± 31, 530 ± 32, 437 ± 15, 453 ± 25, and 398 ± 19 mg, respectively.

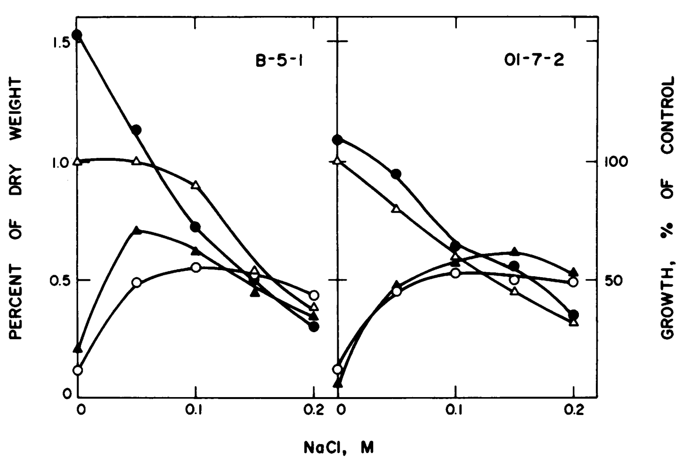

FIG. 2. Growth and internal Na^+, K^+, and Cl^- concentrations of two NaCl-tolerant cell lines as a function of NaCl concentration. Cells harvested in the experiment described in Figure 1 were analyzed as described in "Materials and Methods." (○), Na^+; (●), K^+; (▲), Cl^-; (△), growth. 100% growth for B-5-1 and 01-7-2 cells was 398 ± 19 and 530 ± 32 mg, respectively.

selection pressure (15).

To understand the enhanced ability of the NaCl-tolerant cell lines to grow in the presence of NaCl, the concentrations of Na^+, K^+, and Cl^- were determined in the NaCl-tolerant and NaCl-sensitive cell lines. The data presented in Figure 2 show the similarity between two NaCl-tolerant cell lines (01-7-2 and B-5-1) with respect to the change in their internal ion content of Na^+, K^+, and Cl^- as a function of increasing external NaCl concentrations. It should be noted that the performance of the other two NaCl-tolerant cell lines was essentially the same. Four conclusions can be drawn from the analysis of two NaCl-tolerant cell lines depicted in Figure 2. (a) Uptake of Na^+ reaches its maximum at an external NaCl concentration of about 0.05 M, and the level of this maximum is similar in the two cell lines. (b) Uptake of Cl^- also reaches its maximum at the same external NaCl concentration for one cell line (B-5-1), but keeps rising in the second one (01-7-2). Between the other two cell lines, one (01-7-3) resembles the performance of B-5-1 and the other (01-7-4) resembles that of 01-7-2. (c) K^+ is lost from the cells as a function of increasing external NaCl concentrations. Although there are minor differences in the content of K^+ in the cells growing in the absence of NaCl, the content becomes very similar in the presence of NaCl. Those cell lines having higher concentrations of internal K^+ lose it more rapidly. (d) Growth of cells in the presence of increasing NaCl concentrations is gradually inhibited from 0 to 0.2 M; thus, neither internal Na^+ nor Cl^- concentration can be directly correlated with salt tolerance. On the other hand, internal K^+ concentrations have a much better correlation with ability to grow on elevated NaCl levels.

A comparison of growth and ion content between one NaCl-tolerant cell line (01-7-4) and the NaCl-sensitive cell line (SO-A) is shown in Figure 3. Both Na^+ and Cl^- were taken up by the NaCl-tolerant cell line to the same or an even greater extent than by the NaCl-sensitive cell line (Fig. 3A). However, there was a marked difference between the two cell lines in the extent of reduction of internal K^+ as a function of increasing external NaCl concentrations (Fig. 3B). At the same external NaCl concentrations, the NaCl-sensitive cell line lost much more K^+ than did the NaCl-tolerant cell line. The addition of growth curves of the two cell lines (Fig. 3B) suggests a rather poor correlation between growth and the degree of Na^+ or Cl^- uptake, but a rather good correlation between growth and loss of internal K^+. This behavior, namely, the pattern of the change of internal ion content in response to increasing external NaCl concentration of Sour orange NaCl-tolerant cells, resembles the pattern already described for several plant cell systems. Heyser and Nabors (10) and Watad *et al.* (18) described the ion uptake of nonadapted and salt-adapted tobacco cells growing in the presence of NaCl. Both studies showed a similar increase of the internal NaCl concentration in the wild type and selected cells as a function of increasing external NaCl concentrations. However, Heyser and Nabors (10) did not find any difference in the internal K^+ concentration between the two types of cells, while Watad *et al.* (18) showed a decrease of internal K^+ concentration as a function of increasing external NaCl concentration with the selected cells retaining slightly higher K^+ concentrations than the nonselected ones. This latter pattern was also found in the salt-selected alfalfa cells by Croughan *et al.* (6). However, in the case of calli derived from salt-sensitive and salt-tolerant tomato plants (16), the level of internal K^+ was practically constant over a wide range of NaCl salinity for the salt-sensitive cells and decreased significantly in the salt-tolerant cells. The level of internal Na^+ and Cl^- was much higher in the calli derived from wild salt-tolerant plants than in the calli derived from the salt-sensitive cultivated species.

All these studies, including the present one dealing with the performance of NaCl-tolerant Sour orange cells, describe plant

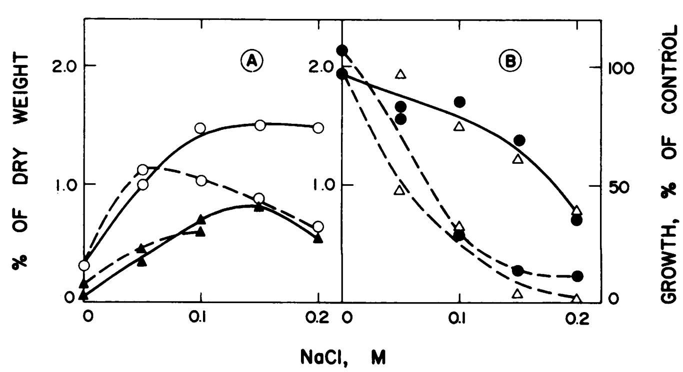

FIG. 3. Growth and internal Na^+, K^+, and Cl^- concentrations of the NaCl-sensitive and one NaCl-tolerant cell lines as a function of NaCl concentration. (○), Na^+; (●), K^+; (▲), Cl^-; (△), growth. (---), SO-A; (——), 01-7-4. 100% growth for SO-A and 01-7-4 was 369 ± 31 and 437 ± 15 mg, respectively.

cell systems where accumulation of Na^+ and Cl^- takes place. In some plant systems, such as Sour orange, salt tolerance can be attributed to a reduced loss of internal K^+. However, other factors should be involved in the evaluation of the mechanism of salt tolerance, especially for those plant systems where K^+ levels remained unchanged. Moreover, as will be discussed later, high internal K^+ concentration, even in the case of Sour orange cells, is not enough to protect these cells when salts, other than NaCl, are present.

It should be noted that the percentage of dry weight of both NaCl-sensitive cell line and the four NaCl-tolerant cell lines was rather constant over the entire range of NaCl concentrations examined in this study. The values were 7 to 8% for the NaCl-sensitive cell line (SO-A) and 6 to 7% or 7 to 8% for the various NaCl-tolerant cell lines.

In an attempt to understand the role of Na^+, K^+, and Cl^- in the nature of the tolerance of the NaCl-tolerant cell lines, various salts were introduced into the growth medium. The growth of two NaCl-tolerant cell lines was examined when Na^+ was replaced by K^+ and Cl^- by SO_4^{2-}. Replacement of NaCl by Na_2SO_4 resulted in even better tolerance to the latter (Fig. 4). This is true for the other two NaCl-tolerant cell lines as well as for the NaCl-sensitive cell line (data not shown). Replacement of NaCl by KCl had an inhibitory effect which was more pronounced in one NaCl-tolerant cell line (01-7-2) than the other (01-7-4). The other two NaCl-tolerant cell lines resemble 01-7-2, being more severely inhibited by KCl. Replacement of Na_2SO_4 by K_2SO_4 has also an inhibitory effect on all four NaCl-tolerant cell lines, although to a lesser extent than KCl.

The performance of NaCl-tolerant cell lines as a function of replacement of Na^+ by K^+ was compared with that of the NaCl-sensitive cell line and the results are summarized in Figures 5, 6, and 7. Figure 5 shows the growth of the NaCl-sensitive cell line (SO-A) and one NaCl-tolerant cell line (01-7-2) in the presence of Na_2SO_4 and a combination of Na_2SO_4 and K_2SO_4. At all concentrations tested, SO-A was more sensitive than the NaCl-tolerant cell line to partial replacement of Na^+ by K^+. Inhibition of growth of the latter occurred only at a high salt concentration

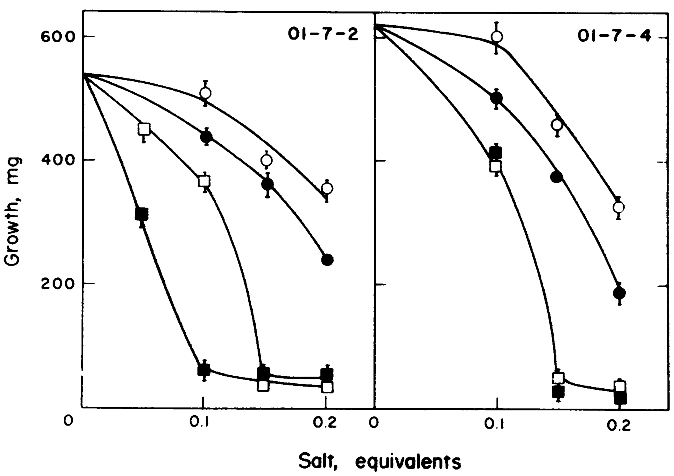

FIG. 4. Effect of various salts on growth of two NaCl-tolerant cell lines. Cells were harvested after 1 month. (●), NaCl; (○), Na_2SO_4; (■), KCl; (□), K_2SO_4.

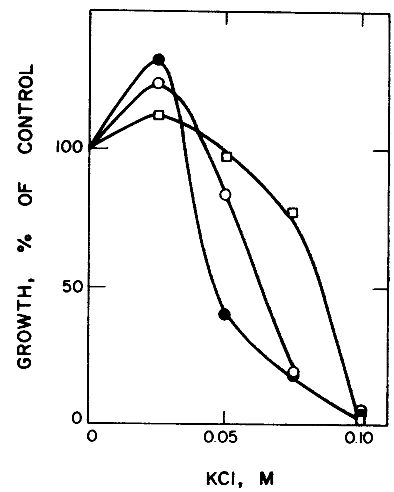

FIG. 6. Growth of the NaCl-sensitive (SO-A) and two NaCl-tolerant cell lines as a function of KCl concentration. 0 KCl means 0.1 M NaCl and any concentration of added KCl was deducted from the NaCl to maintain the osmolarity and the concentration of Cl^- constant. (●), SO-A; (○), 01-7-2; (□), 01-7-4. 100% growth for SO-A, 01-7-2, and 01-7-4 was 166 ± 14, 244 ± 16, and 320 ± 31 mg, respectively.

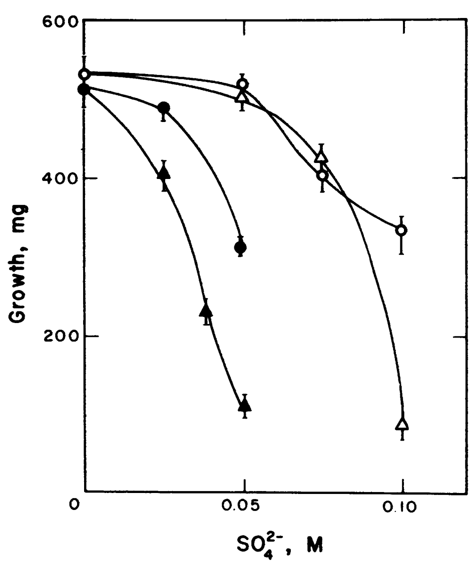

FIG. 5. Growth of the NaCl-sensitive (SO-A) and NaCl-tolerant (01-7-2) cell lines on Na_2SO_4 and Na_2SO_4 + K_2SO_4. Cells were harvested after 1 month. (●, ○), Na_2SO_4; (▲, △), Na_2SO_4 + K_2SO_4 in equal molarity. (●, ▲), SO-A; (○, △), 01-7-2.

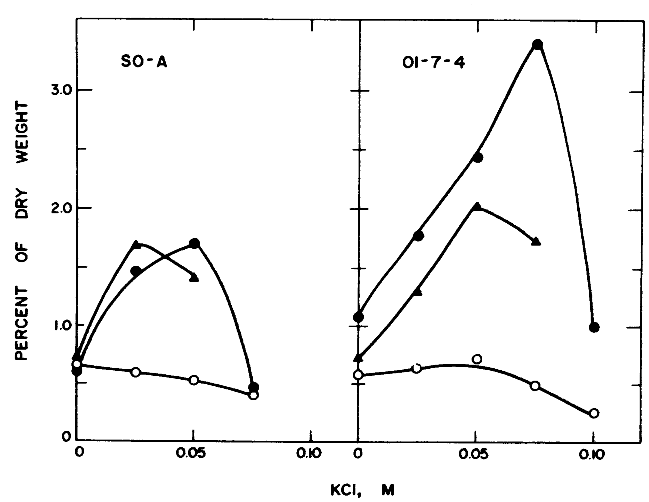

FIG. 7. Accumulation of Na^+, K^+, and Cl^- in the NaCl-sensitive (SO-A) and NaCl-tolerant (01-7-4) cell lines as a function of KCl concentration. Cells harvested in the experiment described in Figure 6 were analyzed as described in "Materials and Methods." (○), Na^+; (●), K^+; (▲), Cl^-.

Table I. *Effect of Various Salts on Growth and Ion Content of Sour Orange Salt-Sensitive (SO-A) and Two Salt-Tolerant Cell Lines (01-7-2, 01-7-4)*

Growth was determined after 1 month. Cells were washed and analyzed as described in "Materials and Methods." Growth is shown as mean values of five replications ± SD.

Cell Line	Salt	Growth	Na^+	K^+	Cl^-
	M	*mg*	*% of dry wt*		
SO-A	—	408 ± 25	0.12	0.90	0.15
	NaCl, 0.05	247 ± 22	0.37	0.56	0.50
	Na_2SO_4, 0.025	357 ± 38	0.52	1.16	0.11
	NaCl, 0.1	152 ± 10	0.48	0.51	0.75
	Na_2SO_4, 0.05	309 ± 20	0.59	0.63	0.12
	Na_2SO_4, 0.025 + K_2SO_4, 0.025	108 ± 11	0.46	1.38	0.13
01-7-2	—	671 ± 35	0.14	1.58	0.17
	NaCl, 0.1	481 ± 28	0.56	1.40	0.96
	Na_2SO_4, 0.05	540 ± 17	0.80	1.51	0.15
	NaCl, 0.2	238 ± 16	0.60	0.47	0.59
	Na_2SO_4, 0.1	337 ± 50	0.74	0.78	0.17
	Na_2SO_4, 0.05 + K_2SO_4, 0.05	50 ±	—	—	—
01-7-4	—	626 ± 33	0.20	1.84	0.28
	NaCl, 0.1	446 ± 20	0.72	1.74	1.12
	Na_2SO_4, 0.05	515 ± 24	1.06	1.82	0.30
	NaCl, 0.2	240 ± 18	0.66	0.67	0.82
	Na_2SO_4, 0.1	335 ± 24	0.86	0.90	0.26
	Na_2SO_4, 0.05 + K_2SO_4, 0.05	130 ± 10	0.54	1.07	0.28

(above 0.075 M SO_4^{-2}), or when K^+ was the only cation present in the medium (Fig. 4). Figure 6 shows the results of gradual replacement of Na^+ by K^+ in the presence of Cl^-. Two NaCl-tolerant cell lines were compared with the NaCl-sensitive cell line for their growth capacity under constant Cl^- concentration and various combinations of Na^+ and K^+. At a very low concentration of K^+, stimulation of growth was observed for all cell lines. At KCl concentrations higher than 0.025 M, inhibition of growth was observed for all cell lines, the nonselected being the most sensitive one. Analyses of the ion content of the various cells growing under these conditions, *i.e.* variable combinations of NaCl and KCl, are depicted in Figure 7. Inhibition of growth was accompanied by a small decrease in the uptake of Na^+ and a very high increase in both K^+ and Cl^- uptake. The decrease of K^+ and Cl^- uptake in external concentration of KCl above 0.05 M in the case of the NaCl-sensitive cell line and above 0.075 M in the case of the NaCl-tolerant cell lines is the result of a significant inhibition of growth and of the death of a large fraction of the cells.

In an attempt to correlate growth capacity with ion content of the cells, more data concerning the internal concentration of Na^+, K^+, and Cl^- of the NaCl-sensitive and two NaCl-tolerant cell lines growing in the presence of various salts are summarized in Table I. Examination of the data revealed two interesting properties. First, the enhanced growth of all cell lines on medium containing Na_2SO_4, compared with NaCl, was accompanied by a higher internal concentration of Na^+. Thus, internal Na^+ concentration cannot be directly correlated with growth. Second, the level of internal Cl^- concentration is also an unsatisfactory marker for salt tolerance. NaCl-tolerant cell lines accumulate similar amounts of Cl^- as does the salt-sensitive line (Fig. 3), and replacement of Cl^- by SO_4^{2-}, especially in the presence of K_2SO_4 (Table I), was no less inhibitory. Third, an important role for internal K^+ concentrations in the mechanism of salt tolerance for Sour orange cells was suggested (Fig. 3). However, replacement of Na^+ salts by K^+ salts markedly increased the internal levels of K^+ with a concomitant significant decrease of growth (Table I; Figs. 5–7). Thus, there seems to be not one particular ion whose internal concentration can serve as a reliable marker for a degree of salt tolerance in these cells. The apparent paradox in the role of K^+ in the presence of NaCl and other salts containing K^+ may be resolved when techniques, determining the location of K^+ in the cell, will be available. Otherwise, other factors, such as molecules important for osmoregulation, will be analyzed. So far, we have not detected any known type of molecule in the Sour orange system.

The Sour orange NaCl-tolerant cell lines differ from a Shamouti orange NaCl-tolerant cell line in two major properties. One is the nature of the ion uptake and the second is the relative sensitivity of the NaCl-sensitive and the NaCl-tolerant cells to exposure to salts containing K^+. While the NaCl-tolerant Shamouti orange cell line survives the raised levels of external NaCl by at least partial exclusion of Na^+ and Cl^- (2), the Sour orange NaCl-tolerant cell lines belong to a class of salt accumulators. The NaCl-tolerant Shamouti orange cells were found to be much more sensitive to replacement of NaCl by KCl (2). However, in the case of Sour orange, the NaCl-sensitive cells were more sensitive (Fig. 6). Moreover, while replacement of Na_2SO_4 by K_2SO_4 had almost no effect on the NaCl-sensitive Shamouti orange cells, such a replacement had a marked effect on the Sour orange NaCl-sensitive as well as NaCl-tolerant cells (Fig. 5).

In conclusion, four Sour orange NaCl-tolerant cell lines selected from ovular callus have a similar degree of salt tolerance as well as very similar behavior in changing their internal ion content in response to various concentrations of NaCl and other salts. All the experiments described in this study were designed to find whether a correlation between tolerance to NaCl and internal concentration of major ions exists. Research into other possible parameters involved in this tolerance, such as high concentrations of organic solutes is now underway. The mechanism operating in the Sour orange system seems to differ from the one operating in another species of *Citrus*, namely, *Citrus sinensis*.

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