Plant Physiol. (1983) 72, 685–690
0032-0889/83/72/0685/06/$00.50/0

Aspects of Salt Tolerance in a NaCl-Selected Stable Cell Line of *Citrus sinensis*[1]

Received for publication July 15, 1982 and in revised form February 25, 1983

GOZAL BEN-HAYYIM AND JOSHUA KOCHBA[2]
Institute of Horticulture, Agricultural Research Organization, The Volcani Center, Bet Dagan, Israel

ABSTRACT

A NaCl-tolerant cell line which was selected from ovular callus of 'Shamouti' orange (*Citrus sinensis* L. Osbeck) proved to be a true cell line variant. This conclusion is based on the following observations. (a) Cells which have been removed from the selection pressure for at least four passages retain the same NaCl tolerance as do cells which are kept constantly on 0.2 molar NaCl. (b) Na^+ and Cl^- uptake are considerably lower in salt-tolerant cells (R-10) than in salt-sensitive cells (L-5) at a given external NaCl concentration. (c) Growth of salt-tolerant cells is markedly suppressed upon replacement of NaCl by KCl, whereas the growth of salt-sensitive cells is only slightly affected. Accumulation of K^+ and Cl^- accompanies the inhibition of growth. Experiments carried out with sodium and potassium sulfate suggest that the toxic effect is due to the accumulated Cl^-. (d) Removal of Ca^{2+} from the growth medium severely inhibits the growth of salt-tolerant cells in the presence of NaCl, while it has a minor effect on growth of salt-sensitive cells in the presence of NaCl. (e) Electron micrographs show that the salt-tolerant cells have very big vacuoles when exposed to salt, while the size of the vacuoles of the salt-sensitive cells does not change.

Variant cell lines have been selected from a population of cultured somatic cells of higher plants following imposition of various stresses (2, 3, 25, 28, 30). Selection has commonly been practiced following exposure to salt, drought, cold, and herbicides. Of these agriculturally useful traits, resistance or tolerance to salt is becoming increasingly important among crops of agricultural value grown in arid zones. Selection of cell lines which exhibit tolerance to salt stress has been reported before (6, 7, 11, 21) but only recently Nabors *et al.* (22) were able to regenerate salt-tolerant plants from a salt-tolerant selected cell line of tobacco.

Citrus plants are among the crops most sensitive to salinity (8). Therefore, it is of great importance to obtain plants capable of growth with elevated salt levels in the irrigation water. To introduce better salt tolerance, selection for salt-tolerant genetic variants in tissue culture (spontaneous or by induced mutation) which also have regenerative capacity may provide useful starting material for conventional breeding.

A comprehensive review on the physiology of salt tolerance in plants (19) summarizes the range of known mechanisms of various plants to survive under salinity stress. Plants can either accumulate ions in response to high concentrations of salt in their environment, or protect themselves by salt exclusion and production of internal high concentrations of organic solutes, such as organic acids, amino acids, or sugars to lower the osmotic potential of the cell.

Ovular callus cultures of "Shamouti" orange (*Citrus sinensis* L. Osbeck) were developed. These cultures readily undergo embryogenesis in response to various treatments (14–17). The isolation of Shamouti orange cell lines with increased tolerance to NaCl has already been reported (1, 13). In the present paper, the ionic content of a nonselected and a NaCl-tolerant selected line exposed to media containing various salts is reported. An understanding of the cellular osmotic adjustment may be helpful in evaluation of the mechanism responsible for salt tolerance in the *Citrus* NaCl-tolerant variant cell line.

MATERIALS AND METHODS

Plant Material. Ovular callus of Shamouti orange (*Citrus sinensis* L. Osbeck) and the salt-tolerant lines were obtained as described before (1, 13). Callus cells were subcultured monthly.

Medium. The basal medium was that of Murashige and Tucker (20) without any growth factors. Callus of salt-tolerant cells (R-10) was routinely kept for over 1 year on medium containing 0.2 M NaCl.

Cell Growth. Cells were grown in a Petri dish as described before (1, 13). Explants of 100 to 120 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 (28–30 d). Dry weights were determined after incubation of the cells at 85°C for 24 h.

Ion Determination. Cells were collected and washed with 0.5 mM $CaSO_4$ for 5 min as described by Tal *et al.* (26), and then they were dried. Na^+ and K^+ were determined after H_2SO_4 wet ashing by an Eel flame photometer. Cl^- was determined according to Cotlove (5).

Organic Solutes. Cells were ground in cold solution of 0.8% NaCl and 0.2% $NaNO_3$ and centrifuged for 20 min at 12,000*g*. The proteins of the supernatant were precipitated by 80% hot ethanol and removed by a second centrifugation for 20 min at 12,000*g*. The second supernatant was dried, dissolved in water, and analyzed. Malate was determined according to Gutmann and Wahlefeld (10), free amino acids were determined by the ninhydrin method (23), and soluble sugars were determined by the anthrone method (24).

Electron Micrographs. Callus cells were fixed overnight in 4% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4. Then they were washed twice in buffer and incubated for 1 h in 1% OsO_4 in the same phosphate buffer. After dehydration they were embedded in Epon 812, sectioned, stained with 1.5% uranyl acetate in 30% ethanol followed by 0.1% lead citrate in H_2O, and examined with a JEOL 100-B electron microscope.

[1] This research was supported by Grant US-239-80 from the United States-Israel Binational Agricultural Research and Development Fund. This is Contribution No. 475-E, 1982 series, from the Agricultural Research Organization, The Volcani Center, Bet Dagan, Israel.

[2] Deceased May 15, 1981.

RESULTS

The salt tolerance of selected (R-10) and nonselected (L-5) *Citrus* cell lines and the stability of the selected line are presented in Figure 1. At all NaCl concentrations tested, R-10 cell line grew much better, and its increased tolerance toward NaCl was retained even after four consecutive transfers on medium without NaCl prior to the experiment (R-10a cells). This result, further supported by data to be presented later, indicates that the R-10 cell line is a true variant.

To study the mechanism by which R-10 cells tolerate NaCl in the medium, the concentration of Na^+, K^+, and Cl^- in L-5 and R-10 cells was determined. R-10 cells take up much less Na^+ and Cl^- compared with L-5 cells growing at the same external NaCl concentrations (Fig. 2). It should be noted that both cell lines contained similar concentrations of Na^+ and Cl^- when grown in the absence of NaCl. The per cent dry weight of both L-5 and R-10 cell lines was rather constant over the entire range of NaCl concentrations examined in this study. The values were 15 to 16% and 12 to 13% for L-5 and R-10 cell lines, respectively. It is also seen in Figure 2 that the concentration of K^+ in L-5 cells was not affected by the uptake of NaCl, while the concentration of K^+ in R-10 cells decreased as the concentration of NaCl increased.

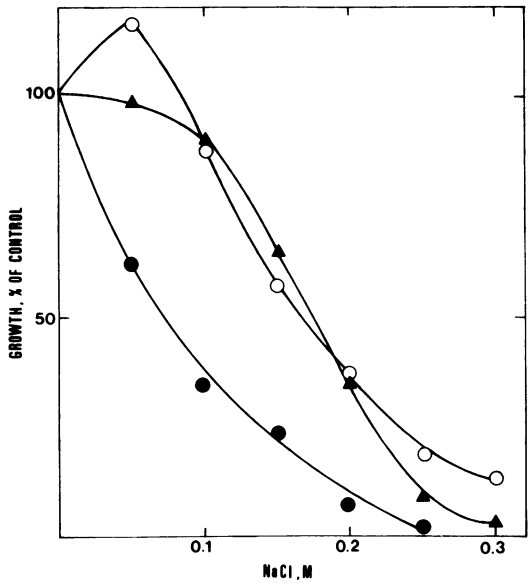

FIG. 1. Growth of nonselected (L-5) and NaCl-selected (R-10) cells as a function of NaCl concentrations. Growth was determined after 28 d and all other details are as described under "Materials and Methods." (●), L-5 cells; (○), R-10 cells; (▲), R-10 cells transferred four consecutive passages on medium without NaCl prior to the experiment (R-10a cells). 100% growth for L-5, R-10, and R-10a cells was 1.015, 1.275, and 1.515 g, respectively.

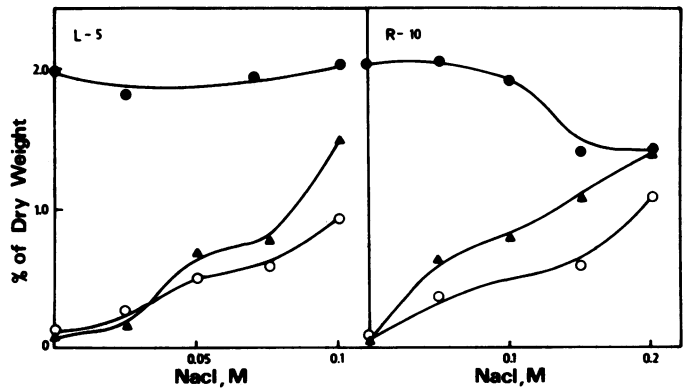

FIG. 2. Accumulation of Na^+, K^+, and Cl^- in L-5 and R-10 cells as a function of NaCl concentrations. Cells harvested in the experiment described in Figure 1 were analyzed as described in "Materials and Methods." (○), Na^+; (●), K^+; (▲), Cl^-.

The data presented in Figures 1 and 2 show that L-5 cells grown in the presence of 0.1 M NaCl gain about the same fresh weight and take up similar concentrations of Na^+ and Cl^- as do R-10 cells grown in the presence of 0.2 M NaCl. Thus, the performance of R-10 cells grown in the presence of 0.2 M NaCl was compared throughout this paper with L-5 cells grown in the presence of 0.1 M NaCl.

Electron micrographs of L-5 and R-10 cells grown in the presence and absence of NaCl were compared. As seen in Figure 3, L-5 cells, whether grown in the presence or absence of NaCl, look very much the same, and a small vacuole, if any, can be observed. However, R-10 cells grown in the presence of NaCl have a very big vacuole, which occupies about 90% of the cell. When these cells are grown in the absence of NaCl for four consecutive transfers (R-10a cells, see Fig. 1), the big vacuoles disappear.

In an attempt to understand the inhibitory effect of NaCl and the mechanism(s) by which R-10 cells reduce their accumulation of ions, cells were subjected to various salt solutions causing a comparable lowering of water potential in the medium. It has been shown (1) that R-10 cells are equally tolerant to various sodium salts, such as SO_4^{2-}, NO_3^-, and Br^-. However, replacing NaCl by KCl turned the NaCl-tolerant cell line into a much more sensitive one, while it had only a slight effect on the nonselected cells (13). As the last experiment was carried out with other salt-sensitive and salt-tolerant cell lines, it was repeated with L-5 and R-10 cell lines and extended over a wider range of K^+ concentrations. The data presented in Table I and Figure 4 show the growth capacity of the salt-sensitive (L-5) and salt-tolerant (R-10) cell lines at various salt concentrations. It is rather clear that R-10 cells were more sensitive than L-5 cells to the addition of 0.05 and 0.1 M KCl into the medium (Fig. 4). The differential response suggests again that the two cell lines represent two variants with respect to their mechanisms of salt tolerance. The data presented in Figure 5 show that there is an increase in the amount of K^+ taken up by the cells of both lines which parallels the increased K^+ concentrations in the medium. At an external concentration of 0.05 M KCl, both types of cells double the amount of accumulated K^+. It is also seen in Figure 5 that the amount of Cl^- taken up was correlated positively with the K^+ concentration in the medium, although the Cl^- concentration in the medium was kept constant. The decrease in the accumulation of K^+ and Cl^- by R-10 cells at KCl concentrations above 0.05 M was most probably due to cell mortality. This assumption is based on the fact that transferring these cells back to a medium containing 0.2 M NaCl did not relieve the growth inhibition. The decrease in the accumulation of Na^+ in these cells occurred only after the accumulation of K^+ reached its maximum. Thus, replacement of NaCl by KCl in the medium increased total cation and anion uptake.

Table I shows that both cell lines responded similarly to the addition of 0.1 M K^+ ions when added as K_2SO_4 salt (0.05 M K_2SO_4 for L-5 and 0.05 M K_2SO_4 + 0.05 M Na_2SO_4 for R-10 cells). The relative good growth of R-10 cells on this concentration of K_2SO_4 (0.05 M), in contrast to no growth at 0.1 M KCl, was used to determine the extent of accumulation of K^+ into the cells. The experiment was carried out at varying conditions, namely, when Cl^- was absent (only SO_4^{2-}) and when SO_4^{2-} was gradually replaced by Cl^- at a constant external concentration of K^+. Such an analysis should answer the question of whether the inhibitory effect of KCl on R-10 cells is due to excess accumulation of K^+ or Cl^-. The data presented in Figure 6 show that K^+ accumulation is high and the growth of R-10 cells is not impaired in the absence

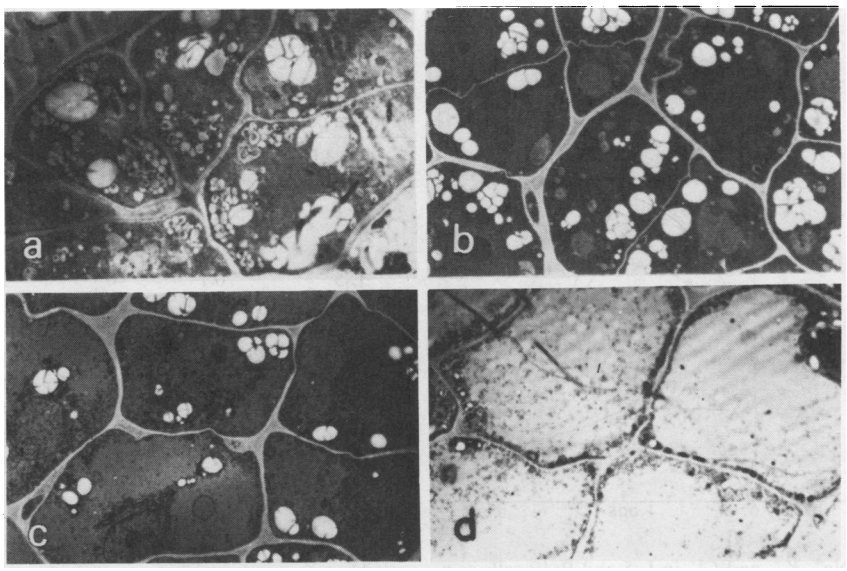

FIG. 3. Electron micrographs of L-5 and R-10 cells grown in the presence and absence of salt. a, L-5 cell in the absence of NaCl. b, L-5 cell grown in the presence of 0.1 M NaCl. c, R-10 cells in the absence of NaCl. d, R-10 cells grown in the presence of 0.2 M NaCl. Sections were prepared as described in "Materials and Methods"; × 2400.

Table I. *Effect of Various Na^+ and K^+ Salts on Shamouti Salt-Sensitive (L-5) and Salt-Tolerant (R-10) Cell Lines*

Growth was determined after 28 d; 100% of L-5 and R-10 cells was 0.49 and 0.51 g, respectively.

Cell Line	Salt	Growth
	M	*% control*
L-5	NaCl, 0.1	100
L-5	NaCl, 0.05 + KCl, 0.05	95
L-5	KCl, 0.1	38
L-5	Na_2SO_4, 0.05	94
L-5	K_2SO_4, 0.05	57
L-5	Na_2SO_4, 0.025 + K_2SO_4, 0.025	80
R-10	NaCl, 0.2	100
R-10	NaCl, 0.15 + KCl, 0.05	60
R-10	NaCl, 0.1 + KCl, 0.1	0
R-10	Na_2SO_4, 0.1	80
R-10	K_2SO_4, 0.1	0
R-10	Na_2SO_4, 0.05 + K_2SO_4, 0.05	64

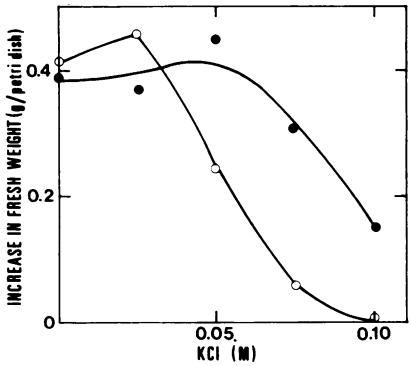

FIG. 4. Growth of L-5 and R-10 cells as a function of KCl concentrations. 0 KCl means 0.1 M and 0.2 M NaCl for L-5 and R-10 cells, respectively. Any concentration of added KCl was deducted from the NaCl to maintain the osmolarity and the concentration of Cl^- constant. Growth was determined after 30 d. (●), L-5 cells; (○), R-10 cells.

of Cl^- (medium containing 0.05 M Na_2SO_4 and 0.05 M K_2SO_4). When KCl was introduced into the medium, the uptake of K^+ remained quite constant (note that the external concentration of K^+ was constant) and Cl^- was accumulated. Chloride accumulation was related linearly to its concentration in the medium and was lower at a given KCl concentration than the observed value when the rest of the salt present was NaCl (compare Figs. 4 and 6). Other anions, such as NO_3^- and Br^-, behaved in a similar manner to Cl^-, namely, R-10 cells were tolerant to their sodium salts but highly sensitive to their potassium salts. No other anion behaving similarly to SO_4^{2-} was found. Anions known to be impermeable, such as phosphate, succinate, and citrate, suppressed growth of both cell lines even if introduced as sodium salts.

The data presented in Figures 1 and 2 suggest that R-10 cells are more salt-tolerant because they are somehow capable of restricting the accumulation of NaCl. It was shown in several systems (18, 29) that Na^+ accumulation is related to the concentration of Ca^{2+} present in the solution. High Na^+/Ca^{2+} ratios result in greater Na^+ accumulation, while increasing Ca^{2+} concentrations in the medium reduced Na^+ uptake. Therefore, in order to determine whether Ca^{2+} ions play any role in the mechanism of NaCl tolerance of R-10 cells, these cells as well as control L-5 cells were grown in the presence (control medium) and absence of Ca^{2+}, in the presence and absence of NaCl. The data presented in Table II show that only R-10 cells grown in the presence of NaCl, for either many generations prior to the experiment or on immediate exposure to it (R-10a cells), were severely affected by the absence of Ca^{2+}. L-5 cells exposed to NaCl in the absence of Ca^{2+} grew rather well. Omission of Mg^{2+} from the growth medium had only a minimal effect on R-10 cells grown in the presence of 0.2 M NaCl, as well as on L-5 cells grown in the presence or absence of NaCl and R-10 cells in the absence of NaCl (data not shown). The different response of R-10 and L-5 cells to the absence of Ca^{2+} indicates once more the genetic variability between those cell lines. The data presented in Figure 7 show the dependence of R-10 cells grown in the presence of 0.2 M NaCl, and of L-5 control cells, on the concentration of Ca^{2+} required for maximal gain in fresh weight. It is evident that Ca^{2+} ions are definitely required for growth of R-10 cells and saturation is achieved at rather low concentrations (1.5–2.0 mM).

Preliminary experiments in the search for possible candidates for osmoregulation in R-10 cells gave negative results. No excess of malic acid, soluble sugars, or free amino acids was accumulated in R-10 cells grown in 0.2 M NaCl over L-5 cells grown in the

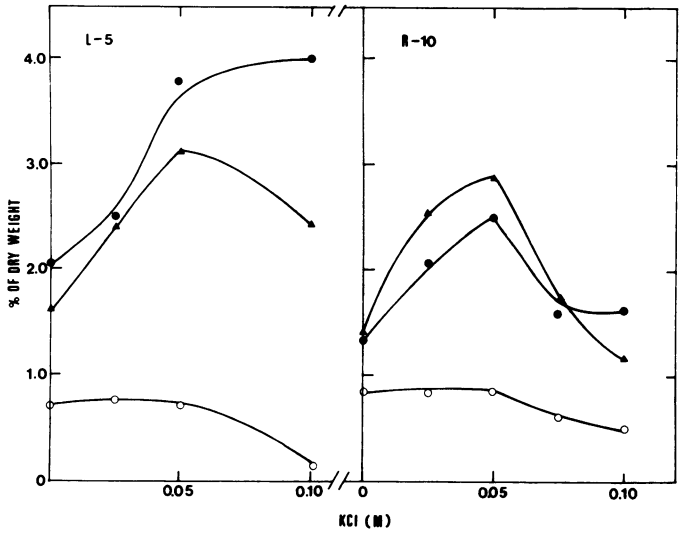

FIG. 5. Accumulation of Na^+, K^+, and Cl^- in L-5 and R-10 cells as a function of KCl concentrations. Cells harvested in the experiment described in Figure 4 were analyzed as described in "Materials and Methods." (○), Na^+; (●), K^+; (▲), Cl^-.

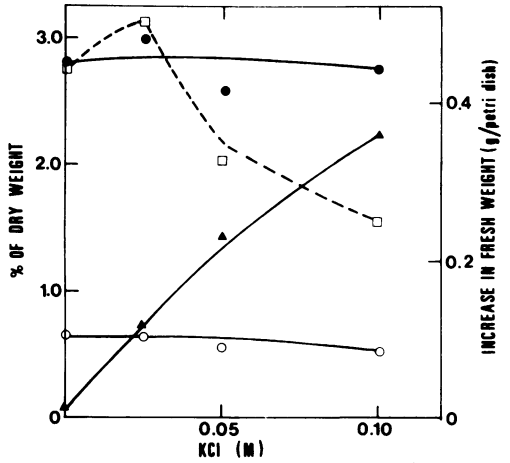

FIG. 6. Growth and accumulation of Na^+, K^+, and Cl^- in R-10 cells as a function of external Cl^- concentrations. Growth was determined after 30 d. 0 KCl means 0.05 M Na_2SO_4 plus 0.05 M K_2SO_4. Any concentration of added KCl was deducted from the K_2SO_4 to maintain the concentration of K^+ constant. Growth, □---□; Na^+, ○——○; K^+, ●——●; Cl^-, ▲——▲.

absence of NaCl. Research into other possible organic solutes is now underway.

DISCUSSION

The purpose of this study was first to establish that a NaCl-tolerant selected cell line of Shamouti orange is indeed a variant cell line, and second, to understand the mechanism by which these cells survive at elevated salt levels.

From the data presented in this paper, there are good indications that the R-10 cell line which was selected on a medium containing 0.2 M NaCl is a true genetic variant. First, the acquired tolerance to NaCl which was achieved through a prolonged procedure of passages on medium containing NaCl is a stable trait, namely, it is not lost when the cells are transferred through numerous generations in the absence of selection pressure (Fig. 1). Second, the extent of Na^+ and Cl^- uptake at a given external NaCl concentration is lower in the selected cells than that observed for

Table II. *Effect of Ca^{2+} and Mg^{2+} on Growth of Selected and Nonselected Cell Lines in the Presence and Absence of NaCl*

Growth was determined after 28 d. It should be noted that the basal medium contains 3 mM $CaCl_2$ and 3 mM $MgCl_2$.

Cell Line	NaCl	$CaCl_2$	$MgCl_2$	Growth (±SE)
	M	mM	mM	g
L-5	0	0	3	0.880 ± 0.095
	0	3	3	1.080 ± 0.110
L-5	0.1	0	3	0.495 ± 0.090
	0.1	3	3	0.610 ± 0.050
R-10	0	0	3	1.453 ± 0.140
	0	3	3	1.563 ± 0.280
R-10	0.2	0	3	0.120 ± 0.010
	0.2	3	3	0.640 ± 0.035
R-10a[a]	0.2	0	3	0.120 ± 0.010
	0.2	3	3	0.792 ± 0.080
R-10	0.2	3	0	0.465 ± 0.060
	0.2	3	3	0.530 ± 0.050

[a] See Figure 1.

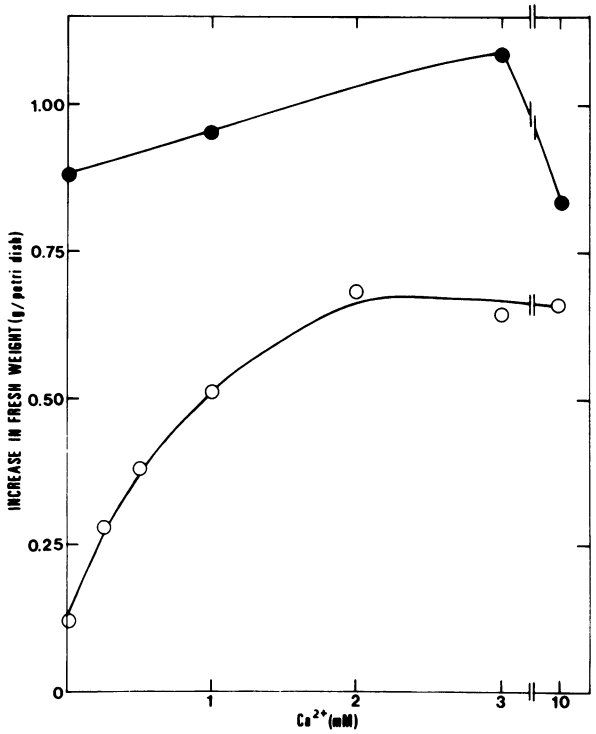

FIG. 7. Growth of R-10 cells in the presence of salt, and of L-5 cells in the absence of salt, as a function of Ca^{2+}. Growth was determined after 28 d. (●), L-5 cells; (○), R-10 cells in 0.2 M NaCl.

the nonselected cells (Fig. 2). Third, R-10 cells are very sensitive to replacement of NaCl by KCl, while the nonselected cells (L-5) are relatively indifferent to this replacement (Fig. 4). This finding suggests that R-10 and L-5 cells may differ in either the mechanism by which Na^+, K^+, and Cl^- are accumulated, or in compartmentation of these ions within the cells. Additional evidence for the assumption that L-5 and R-10 cell lines differ in their mechanism(s) of ion accumulation is provided by the marked effect which the lack of Ca^{2+} in the medium has on the sensitivity of R-10 cells toward NaCl. This characteristic is not shared by L-5 cells grown in the presence of NaCl (Table II). The morphological differences found between L-5 and R-10 cells also suggest that these cells belong to distinct variant lines. Confirmation of the R-10 cell line as a true variant is important because Shamouti orange

callus cells (L-5) are capable of embryogenesis and, subsequently, plant regeneration (14–17). Only a stable genetic variant can have the potential eventually to regenerate plants possessing the desired trait selected for at the callus level. It is an absolute requirement but not necessarily a sufficient one. It was reported that a stable cold-resistant carrot cell line did not regenerate cold-resistant plants (27). However, NaCl-resistant tobacco plants were regenerated from NaCl-resistant callus (22). As preliminary results indicate that Shamouti orange embryos regenerated from R-10 cells survive in a medium containing NaCl much better than do embryos regenerated from nonselected cells (13), it is hoped that this will also be characteristic of plants following regeneration.

There are several systems described in the literature in which ion uptake, especially Na^+, K^+, and Cl^-, was analyzed for nonadapted and NaCl-adapted cultures. Heyser and Nabors (12) describe the ion uptake of tobacco cells selected in the presence of 0.13 M NaCl, and Croughan *et al.* (6) describe the ion uptake of alfalfa cell cultures selected in 10 g L^{-1} NaCl. Tal *et al.* (26) describe the ion uptake of cell cultures derived from either salt-sensitive or salt-tolerant tomato plants. In all these studies, as well as in *Citrus* cell cultures, Na^+ and Cl^- uptake increases as a function of external concentrations of NaCl. However, a comparison between the extent of uptake of these ions in nonselected and selected line of each crop shows varied response. Whereas in the case of alfalfa there is essentially no difference between the extent of uptake of Na^+ and Cl^- by NaCl-selected and nonselected cell lines, the response of tomato callus derived from NaCl-resistant plants is rather different from that of the callus derived from NaCl-sensitive plants (26). At a given external concentration of NaCl, the callus derived from salt-resistant plants accumulates larger amounts of both Na^+ and Cl^- than does the callus derived from salt-sensitive plants. A comparison between NaCl-sensitive and NaCl-tolerant *Citrus* cell lines reveals an opposite pattern of Na^+ and Cl^- uptake. At a given external concentration of NaCl, the salt-tolerant (R-10) cells accumulate less Na^+ and less Cl^- than do the salt-sensitive (L-5) cells (Fig. 2). This result suggests that while the salt-selected alfalfa cell line survives salinity by a general shift toward a halophytic mode of salt tolerance, the salt-selected *Citrus* cell line most probably survives the elevated levels of salinity by partial avoidance of NaCl. Tal *et al.* (26) show a direct relationship between ion accumulation under saline conditions in tomato plants and the callus derived from them. Therefore, it is reasonable to hypothesize that such a relationship may also hold in the opposite direction, namely, in going from a salt-tolerant callus line to the plants regenerated from it.

The extent of accumulated K^+ as a function of external concentrations of NaCl is also different among the various cell cultivars. While the K^+ concentration of the nonselected alfalfa cells was lower than that found in the NaCl-selected cells upon addition of NaCl into the growth medium, the reverse pattern was obtained with both selected and nonselected *Citrus* cell lines, as well as calli derived from salt-sensitive and salt-tolerant tomato plants. In the latter systems, salt-tolerant calli had lower K^+ concentration upon addition of NaCl than did the salt-sensitive ones. It seems possible to explain this result in the *Citrus* cell culture if one assumes that K^+ is accumulated mostly in the cytoplasm. While there is no difference in the relative contribution of cytoplasm to the cell volume of L-5 cells in the presence or absence of salt, such a difference is observed in R-10 cells. Upon addition of NaCl to R-10 cells, the size of vacuoles increases so that the cytoplasmic volume decreases. Thus, the observed decrease of accumulated K^+ may arise from a shrinkage of cytoplasmic volume rather than from a decrease in its internal concentration. In that case, there is no difference, in respect to internal concentration of K^+, between L-5 and R-10 cells and tobacco cells grown in the absence or presence of salt (12).

The accumulation of Na^+ and K^+ by *Citrus* cell lines is not dependent on the nature of the anion, but is only a function of the external concentrations of these ions. However, the accumulation of Cl^-, which is also a function of external concentrations of Cl^-, is markedly enhanced by the presence of K^+ (Fig. 5). Since increased accumulation of K^+ by itself does not affect the growth of either L-5 or R-10 cells (Table I and Fig. 6), it may be concluded that Cl^- is the toxic element for *Citrus* callus cells grown in the presence of NaCl. However, this conclusion is oversimplified. Calculations of internal Cl^- content show that L-5 cells grown at 0.1 M NaCl (50% inhibition of growth; see Fig. 1) have 1.5% Cl^- by dry weight (Fig. 2), while the same cells grown at 0.05 M NaCl + 0.05 M KCl have about 3% Cl^- by dry weight (Fig. 5), and no further inhibition of growth is observed (Fig. 4). When the same calculations are made for R-10 cells at concentrations of 0.2 M NaCl or 0.15 M NaCl + 0.05 M KCl, one finds a correlation between higher levels of Cl^- accumulation and further inhibition of growth (Figs. 4 and 5). At present, we do not have any explanation for the difference between the performance of L-5 and R-10 cells in the presence of KCl. Thus, the above conclusion, concerning the toxicity of accumulated Cl^-, seems valid at least in respect to R-10 cells. Similar results were described by Cooper and Gorton (4) and Furr and Ream (9), where a correlation was found between salt tolerance of various *Citrus* trees and their accumulation of Cl^-. This similarity implies that *Citrus* callus may serve as a reliable model and obviously a much easier system to study the mechanism of salt tolerance in *Citrus* plants.

R-10 cells seems to survive in elevated levels of NaCl by avoiding its accumulation. The enhanced sensitivity of these cells to the lack of Ca^{2+} may suggest that extrusion of Na^+ from the cells depends on uptake of Ca^{2+}. It was shown by Lahaye and Epstein (18) that in *Phaseolus vulgaris* the accumulation of Na^+ decreased upon addition of Ca^{2+}. Such a decrease was also found in soybean (29). These data may explain the preliminary results described in this study, but more thorough analysis of ion content at various Ca^{2+} concentrations is required. The main reason for presenting these results at such an early stage is that they lend support to the evidence for R-10 cells being a variant cell line rather than evaluation of any mechanism concerning salt tolerance.

In conclusion, the *Citrus* NaCl-tolerant cell line selected from ovular callus of Shamouti orange is a stable and true variant isolated during a prolonged procedure of selection. This cell line tolerates NaCl by its partial avoidance and the accumulated Cl^- seems to be the toxic ion, as suggested previously for the effect of NaCl on *Citrus* trees. Thus, callus may serve as a reliable and easy model system for studying the mechanism of salt tolerance in *Citrus*.

Acknowledgment—I wish to thank Dr. R. Ganmor, of the Institute of Soils and Water, ARO, the Volcani Center, for her invaluable expertise in the determinations of the ions.

LITERATURE CITED

1. Ben-Hayyim G, J Kochba 1982 Growth characteristics and stability of tolerance of citrus callus cells subjected to NaCl stress. Plant Sci Lett 27: 87–94
2. Chaleff RS, PS Carlson 1974 Somatic cell genetics of higher plants. Annu Rev Genet 8: 267–278
3. Chaleff RS, MF Parsons 1978 Direct selection *in vitro* for herbicide-resistant mutants of *Nicotiana tabacum*. Proc Natl Acad Sci USA 75: 5104–5107
4. Cooper WC, BS Gorton 1952 Toxicity and accumulation of chloride salts in citrus on various rootstocks. Proc Am Soc Hort Sci 59: 143–146
5. Cotlove E 1965 Determination of Cl^- in biological materials. *In* D Glick, ed, Methods of Biochemical Analysis, vol 12. Interscience Publishers, New York, pp 227–392
6. Croughan TP, SJ Stavarek, DW Rains 1979 Selection of a NaCl tolerant line of cultured alfalfa cells. Crop Sci 18: 959–963
7. Dix PJ, HE Street 1975 Sodium chloride-resistant cultured cell lines from *Nicotiana sylvestris* and *Capsicum annum*. Plant Sci Lett 5: 231–237
8. Furr JR, JB Carpenter, AA Hewitt 1963 Breeding new varieties of *Citrus* fruits and rootstocks for the southwest. J Rio Grande Val Hort Sci 17: 90–107
9. Furr JR, CL Ream 1969 Breeding *Citrus* rootstocks for salt tolerance. *In*

Proceedings 1st Int Citrus Symp, vol 1. University of California, Riverside, pp 373–380
10. GUTMANN I, AW WAHLEFELD 1974 L-Malate determination with malate dehydrogenase and NAD. *In* HV Bergmeyer ed, Methods of Enzymatic Analysis. Academic Press, New York, pp 1585–1588
11. HASEGAWA PM, RA BRESSAN, A HANDA 1980 Growth characteristics of NaCl-selected and non-selected cells of *Nicotiana tabacum* L. Plant Cell Physiol 21: 1347–1355
12. HEYSER JW, MW NABORS 1981 Osmotic adjustment of cultured tobacco cells (*Nicotiana tabacum* var. Samsum) grown on sodium chloride. Plant Physiol 67: 720–727
13. KOCHBA J, G BEN-HAYYIM, P SPIEGEL-ROY, H NEUMANN, S SAAD 1982 Selection of stable salt-tolerant callus cell lines and embryos in *C. sinensis* and *C. aurantium*. Z Pflanzenphysiol 106: 111–118
14. KOCHBA J, P SPIEGEL-ROY 1977 The effects of auxin, cytokinins and inhibitors on embryogenesis in habituated ovular callus of the "Shamouti" orange (*Citrus sinensis*). Z Pflanzenphysiol 81: 283–288
15. KOCHBA J, P SPIEGEL-ROY, H NEUMANN, S SAAD 1978 Stimulation of embryogenesis in *Citrus* ovular callus by ABA, ethephon, CCC and alar and its suppression by GA_3. Z Pflanzenphysiol 89: 427–432
16. KOCHBA J, P SPIEGEL-ROY, H NEUMANN, S SAAD 1982 Carbohydrate control of somatic embryogenesis in subcultured callus of *Citrus* cultivars. Z Pflanzenphysiol 105: 359–368
17. KOCHBA J, P SPIEGEL-ROY, H SAFRAN 1972 Adventive plants from ovules and nucelli in *Citrus*. Planta 106: 237–245
18. LAHAYE PA, E EPSTEIN 1971 Calcium and salt toleration by bean plants. Physiol Plant 25: 213–218
19. MAAS EV, RH NIEMAN 1978 Physiology of plant tolerance to salinity. *In* GA Yung, ed, Crop Tolerance to Suboptimal Land Conditions. American Society of Agronomy Special Publication. Crop Science Society of America, Madison, WI, pp 277–299
20. MURASHIGE T, DPH TUCKER 1969 Growth factor requirements of *Citrus* tissue culture. *In* H Chapman, ed, Proceedings 1st Int Citrus Symp, vol 3. University of California, Riverside, pp 1155–1161
21. NABORS MW, A DANIELS, L NADOLNY, C BROWN 1975 Sodium chloride tolerant lines of tobacco cells. Plant Sci Lett 4: 155–159
22. NABORS MW, SE GIBBS, CS BERNSTEIN, ME MEIS 1980 NaCl tolerant tobacco plants from cultured cells. Z Pflanzenphysiol 97: 13–17
23. ROSEN H 1957 A modified ninhydrin colorimetric analysis for amino acids. Arch Biochem Biophys 67: 10–15
24. SCOTT JR TA, E MELVIN 1953 Determination of dextran with anthrone. Anal Chem 25: 1656–1666
25. SCOWCROFT WR 1977 Somatic cell genetics and plant improvement. Adv Agron 29: 39–82
26. TAL M, H HEIKIN, K DEHAN 1978 Salt tolerance in the wild relatives of the cultivated tomato: responses of callus tissue of *Lycopersicon esculentum*, *L. peruvianum* and *Solanum pennellii* to high salinity. Z Pflanzenphysiol 86: 231–240
27. TEMPLETON-SOMERS KM, WR SHARP, RM PFISTER 1981 Selection of cold resistant cell lines of carrots. Z Pflanzenphysiol 103: 139–148
28. WIDHOLM JM 1977 Selection and characterization of biochemical mutants. *In* W Barz, E Reinhard, MH Zenk, eds, Plant Tissue Culture and Its Biotechnological Application. Springer-Verlag, Berlin, pp 112–122
29. WIENEKE J, A LAUCHLI 1980 Effects of salt stress on distribution of Na^+ and some other cations in two soybean varieties differing in salt tolerance. Z Pflanzenernaehr Bodenkd 143: 55–67
30. ZENK MH 1977 Haploids in physiological and biochemical research. *In* KJ Kasha, ed, Haploids in Higher Plants. University of Guelph, pp 339–353