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**Development of Plants from
Citrus Salt-Tolerant Embryos Regenerated
from Stable Salt-Tolerant Calli**

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Development of Plants from Citrus Salt Tolerant Embryos Regenerated from
Stable Salt Tolerant Calli

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ABSTRACT

Plants regenerated from salt-tolerant callus are essential in testing the feasibility of transferring this acquired trait. We regenerated plants from embryos of salt-tolerant Citrus callus that were previously treated with and without NaCl. Torpedo-shape embryos, required for further development of plants, could be obtained both in the presence and absence of NaCl. The presence of NaCl induced greening at an earlier stage. Further development of plants was carried out only in the absence of NaCl. Abnormal shoots, lacking internode, were formed from embryos developed in either treatments, although more normal leaves were observed in plants developed in the absence of NaCl. Normal looking roots were developed from embryos formed in the presence and absence of NaCl. Diploid number of chromosomes (18) was found in root tips of 6 plants.

We investigated physiological and biochemical aspects representing major functions for possible differences between salt-sensitive and salt-tolerant cells. Sugar uptake is similar in both types of cells, but starch is mainly accumulated in salt-sensitive cells growing in the presence of NaCl. Malate dehydrogenase activity is enhanced only in salt-tolerant cells growing in the presence of NaCl. The change in V_{max} is not accompanied by changes in K_m for either malate or NAD^+ . Intracellular malate content was significantly higher in the salt tolerant cells in the presence of salt, as compared with the salt sensitive cells. We established the role of internal potassium in maintaining growth of cells and the effect of external concentrations ratio of Ca^{2+}/Na^+ in determining internal K^+ content.

OBJECTIVES OF THE ORIGINAL RESEARCH PROPOSAL

The objectives of the original research proposal were to regenerate plants from salt-tolerant callus lines, test them for salt tolerance and try to correlate salt tolerance with major physiological processes occurring in the cell.

We regenerated plants from salt-tolerant callus of Shamouti orange (R-10), but the abnormal shoots did not allow us to subject them to conditions required for testing salt tolerance. We failed to even form embryos from salt-tolerant Sour orange callus.

We followed three physiological processes for possible differences between salt-tolerant and salt-sensitive cells. 1) Sugar uptake into the cells and the nature of the carbohydrates accumulated in the cells; 2) the response of a metabolic key enzyme malate dehydrogenase to salinity; and 3) the ionic inorganic content of the cell and the ion, or ions, that are important in determining growth.

DEVELOPMENT OF PLANTS FROM THE SALT-TOLERANT CELLS

During the three years of the research we have concentrated in finding methods to develop embryos and regenerate plants from salt-tolerant callus in the presence and absence of salt.

1. Embryogenesis and regeneration of embryos from salt-tolerant callus in presence of salt

Green embryos of Shamouti orange salt-tolerant cells (R-10) were produced in the basal medium containing glycerol and 200 mM NaCl. The callus cells were grown for two weeks in suspension and were subsequently transferred for additional four week period to a solid medium. It was found that most of the callus cells underwent embryogenesis.

Embryos at their globular stage were transferred to four different media for plantlets formation. These media contained in addition to the basal medium one of the following growth factors (mg/l): (a) malt extract (ME) 1500; (b) abscisic acid (ABA) 0.1; (c) malt extract 1000 and gibberellic acid (GA_3) 1; (d) abscisic acid 0.1 and gibberellic acid 1.

The green embryos in all four different media grew in size. Those embryos that grew on the first two media (a,b) were developed to pluricotyledonary stage while the others (c,d) developed to a heart shape stage.

The developed embryos from all four treatments were transferred to a solid basal medium that contained various concentrations of the auxin NAA (0, 0.1, 0.2, 1.0 mg/l) in order to test root and shoot formation. Four weeks later the plantlets were checked and it was found that only in the highest concentration of NAA (1 mg/l) the plantlets remained green and 20% of them developed roots (50-70 mm long). The embryos on the other media were bleached, degenerated and callus was formed.

A general description of the methods used to develop embryos and plantlets from callus cells is summarized in scheme 1. The survived embryos of R-10 line, developed in the presence of 0.2M NaCl are shown in Figures 1 and 2.

Scheme 1 - Embryogenesis and regeneration of embryos
from salt-tolerant callus, in the presence of salt

2. regeneration of embryos from salt tolerant callus in the absence of salt

Stage 1 Embryogenesis

R-10 callus cells were grown for 2 weeks in liquid medium containing glycerol. Then they were transferred to solid medium containing glycerol and after 4 more weeks they were transferred again to solid medium containing glycerol + 0.2 mg/l benzyladenine. This three step protocol induced most of the callus cells to develop into globular green embryos. It should be noted that greening was obtained only in the third step, and this is in contrast to the response of salt-sensitive cells, where greening was observed concomitantly with embryo formation.

Stage 2 Embryo development

The embryos were transferred to a solid basal medium containing either malt extract 1500 mg/l or abscisic acid 0.1 mg/l for three weeks. As a result heart shaped embryos were developed.

Stage 3 Plantlet formation

The developed embryos were transferred to solid medium containing adenine sulphate (ADS) 27 mg/l and gibberellic acid (GA_3) 1 mg/l for three weeks. This treatment resulted in plantlet formation and subsequent growth was observed.

Stage 4 Plantlet development

The plantlets were transferred to solid medium in plastic containers, with addition of various concentrations of NAA. The formation of roots was

the major development during one month, and the results of stage 4 are summarized in table 1.

Stage 5 Plant development

The rooted plantlets were transferrd to test tube containing basal liquid medium, without additional growth factors. The development of the plants after six weeks is described in table 1. The R-10 plants developed during stage 5 are shown in Figure 3. For comparison, the plants developed from the salt-sensitive L-5 line are shown in Figure 4.

The roots of plants derived from the NaCl-tolerant callus, using the protocol described above, looked similar to roots obtained from NaCl-sensitive cells. However, we have not succeeded to develop normal shoot. There was no further growth of the shoot during the development of the root. Thus, we changed somewhat the strategy for regeneration trying to induce normal shoot formation prior to root induction. We tried several new media at stage 3. As a result from earlier observation that callus proliferates from shoots that are not induced to form roots (lack of auxin), we applied kinetin to antagonize possible excess of endogenous auxins. Plantlets subjected to medium containing 0.01-0.1 mg/l kinetin grew in size and callus formation was markedly reduced. Several transfers on this medium or back and forth transfers to medium without any growth hormone, resulted in green shoots, few normal-looking leaves but no internodes. Any further treatment to induce roots, as indicated in stages 4 and 5 resuled in root formation but no subsequent shoot growth.

Table 1 - Summary of developmental stages of R-10 callus in the absence of salt

<u>Stage 2</u>	<u>Stage 4</u>	<u>Stage 4</u>	<u>Stage 5</u>
additions to	NAA	percentage of	descriptions of plants
basal medium	mg/l	rooted plantlets	
malt extract	0	0	no roots, bleached plantlets.
1500 mg/l	0.1	20	3cm long single roots, green shoots, broad leaf primordia
	0.2	28	4cm long single roots, more leaves per shoot.
	0.5	33	4cm long single roots, increased number of narrow leaves.
	1.0	66	1-8 roots per plant, 5cm long, increased number of narrow leaves.
Abscisic acid	0	0	no roots, bleached plantlets
0.1 mg/l	0.1	10	3cm single roots, mainly bleached shoots
	0.2	6	2cm single roots, mainly bleached shoots.
	1.0	79	1-3 roots per plant, 5cm long, increased number of green narrow leaves.

Figure 1. Plantlets derived from salt-tolerant cells (R-10). Embryogenesis was carried out in the presence of 0.2 M NaCl. All other details are described in the text.

Figure 2. Plantlets derived from salt-tolerant and salt-sensitive cell (R-10 and L-5). Embryogenesis for R-10(0.2) was carried out in the presence of NaCl (0.2 M), and for R-10 and L-5 in the absence of NaCl.

Figure 3. Plantlets derived from salt-tolerant cells (R-10) in the absence of NaCl. All other details are described in the text.

Figure 4. Plantlets derived from salt-sensitive cells (L-5).

The number of chromosomes in dividing cells in root tips of 6 plants was normal, i.e. 18.

The inability to develop big enough shoots of R-10 plants prevented us from testing plants for salt tolerance. At present we can say that the salt tolerant callus can form embryos in the presence of 0.2M NaCl, while the salt-sensitive cells die at this concentration. Moreover, we have succeeded to develop very small shoot and normal-looking roots from these embryos. Thus, the salt tolerance trait can be retained at least during the transition from undifferentiated cells into somatic embryos, but could not be tested in further developmental stages.

Several conclusions can be drawn from the results obtained from the intensive effort trying to regenerate plants. First, selection for salt tolerance by prolonged exposure of cells to high salinity interferes with normal chlorophyll formation, even in the absence of salt during embryogenesis. The addition of various growth factors is required to restore this ability. Abscissic acid and cytokinins - plus or minus gibberellic acid - induced greening. Chlorophyll spectra and chlorophyll a/b ratio were similar to those found in salt-sensitive cells. Second, the difficulty in obtaining green embryos is not the only obstacle in regeneration of plants from salt-tolerant callus. Normal-looking torpedo-shape embryos have not yielded normal-looking shoots even though large number of treatments, including various growth factor combinations and removal of unidentified inhibitors by active charcoal and the use of special sources of agar, were employed. Thus, we have not succeeded to overcome a damage caused to these cells which is mainly manifested in lack of internode development. We cannot explain this phenomena on the basis of gross changes in

the genome, i.e. chromosome number, but it still may be a result of salt-induced chromosomal rearrangement. It should be noted that the age of the salt-tolerant callus is not a crucial factor in normal regeneration, since the salt-sensitive callus it was derived from has been cultured for longer period of time and has a capacity of normal regeneration.

SUGAR UPTAKE BY CITRUS CULTURED CELLS

The citrus calli are nonphotosynthetic cells, therefore they require an exogenous carbon source. Cells are routinely supplied with 116 mM sucrose in the growth medium.

The general proposed model for sugar uptake by plant cells involves a mechanism of symport with a proton, a transport system driven by membrane potential difference and the H^+ -ATPase activity (1).

Sugar uptake by plant cells was examined in several systems:

Komor et al (2) demonstrated hexose specific uptake mechanism (mainly for glucose) in sugar cane cell suspension. Sucrose was hydrolyzed by an extracellular invertase before the uptake of the hexose; glucose was taken up by a fast specific carrier, while fructose was taken up at a much slower rate.

In Soybean cotyledons protoplasts a sucrose specific-carrier was demonstrated, that was kinetically distinct from the systems involved in hexose transport (3).

Pea stem protoplasts were shown to take up glucose more rapidly than fructose. It is suggested that the two hexoses are taken up by separate

carriers. Sucrose uptake is slower and correlated with the appearance of extracellular invertase activity (4).

Carrot cells were shown to rapidly hydrolyze extracellular sucrose to glucose and fructose, and glucose was preferentially taken up. Intracellular sugar concentrations increased during late logarithmic phase of growth and decreased during the stationary phase. (5)

Characterization of sugar uptake

The pattern of sucrose consumption during a four week growth period of citrus calli, demonstrated the consumption of about half of the sucrose during the first week, before any net growth was observed. The rest of the sugar was consumed from the medium at a slower rate, throughout the following three weeks of growth of the calli. (Fig. 1)

The sucrose consumption by the calli grown in the presence of salt, is demonstrated by fast consumption during the first week, and the appearance of glucose in the growth medium (Fig. 2). The presence of extracellular glucose could arise from two possibilities; the hydrolysis of sucrose by extracellular invertase, followed by gradual uptake of fructose and glucose, or alternatively, the glucose leaked from dead cells or from growing cells after the intensive hydrolysis of the sucrose accumulated during the first week.

We have therefore tested the uptake of sucrose, fructose and glucose; the possible competition between the uptake mechanisms of these sugars, the energetic demand for the uptake, the effect of salt, and the content of sugars in the cells.

Fig. 1 - Sucrose consumption by the cells from growth medium throughout 4 weeks of growth in the absence of salt.

Fig. 2 - Sucrose consumption by the cells from growth medium throughout 4 weeks of growth in the presence of salt.

The uptake assay system was optimized for two hours of incubation with ^{14}C -labeled sugar, at concentrations between 0 and 116 mM, which is the original concentration of sucrose in the growth medium. A comparison of the uptake activity for the three sugars, with calli of the two cell lines is shown in Table 1. In general, the uptake activities of the two cell lines are similar, fructose is taken up faster than sucrose and glucose and the rate of sucrose uptake is high enough to account for the consumption of sucrose observed during the first week of growth.

The analysis of sucrose uptake as a function of its concentration is presented in Figure 3, and demonstrates a similar pattern of uptake in both cell lines. The rest of the uptake experiments were conducted with 116 mM of the sugar. The competition between sucrose and glucose, and sucrose and fructose, is demonstrated in Figure 4. Glucose seems to compete more effectively than fructose with the uptake of sucrose. Thus, glucose may be translocated by the same carrier used for sucrose uptake, while the carrier for fructose uptake may be different.

Sodium chloride did not inhibit the uptake of sucrose into the two cell lines, as shown in Table 2. It should however be noted that the salt was present only during the two hours of incubation, which may not be sufficient for the equilibration of salt concentration inside the cells. These results do not exclude possible interference of salt with sugar consumption during growth.

Inhibitors of respiration have been introduced to study the metabolic nature of sucrose uptake. As shown in Table 3, both cyanide and azide inhibited sugar uptake, the azide being more potent than cyanide. These results are sufficient to demonstrate the metabolic nature of the sucrose uptake.

Table 1 - A comparison of Sucrose, Glucose and Fructose Uptake

The uptake experiments were conducted with 200 mg cells (3-4 weeks old) in 2 ml fresh growth medium in the presence of each of the three sugars at the defined concentration, with 0.01-0.2 microCurie uniformly labelled ^{14}C -sugar (Amersham, 350 millicurie nmol^{-1}). The cells were incubated at room temperature, for 2 hours and collected on a 0.45 u milipore filter, washed three times with growth medium. The cells were extracted with ethanol at 40°C for 1 hour, dried by evaporation in the hood and homogenized in the instagel scintillation solution, (Packard) and counted in a Beckman -counter.

Sugar	mM	Sugar uptake*	
		R-10	L-5
sucrose	11.6	2.1	1.2
	116	5.3	4.8
glucose	11.6	3.2	3.7
	116	7.7	6.6
fructose	11.6	4.6	7.9
	116	11.2	13.5

* umoles sugar. $\text{g FW}^{-1}.\text{h}^{-1}$

* Figure 3 Sucrose uptake by cells from salt-sensitive (L), salt-tolerant (R), and Salt-tolerant cells grown in the presence of 0.2 M NaCl (R 0.2).

Figure 4 The effect of glucose and fructose on ^{14}C -sucrose uptake by salt-sensitive (L) and salt-tolerant (R) cells.

Table 2 - The effect of NaCl on Sucrose Uptake by the Cells

The experiment was carried out as described for table 1. The salt was present only during the 2 hours incubation of the uptake assay.

mM NaCl	Sucrose uptake*	
	R-10	L-5
0	3.4	5.2
200	4.2	6.3
400	4.3	6.0
600	3.9	8.4
0	4.0	5.5
200	5.3	5.6
400	4.9	6.8

*umoles sucrose.g FW⁻¹.h⁻¹

Table 3 - The Effect of Metabolic Inhibitors on Sucrose Uptake

Additions	Sucrose uptake*	% inhibition
none	5.3	0
1mM KCN	1.8	66
3mM KCN	1.9	65
3mM NaN_3	1.5	72
10mM NaN_3	0.3	95

*Activity is expressed in micromoles sucrose.g F W $^{-1}$.h $^{-1}$

We have also determined the intracellular content of glucose and sucrose in calli of both cell lines following the uptake of each of the sugars, for two hours. As shown in Table 4, sucrose content is higher than the content of glucose, in the L-5 cell line, when either sucrose or glucose is taken up. In contrast, sucrose is accumulated in R-10 cell line, during sucrose uptake, and glucose is accumulated during glucose uptake.

Sucrose and glucose content in the cells were determined during the 4 week growth period. Figure 5 shows that L-5 cell line contains higher amounts of either sucrose or glucose in the cells compared with R-10 cell line. Starch content was also determined being the major storage carbohydrate of these cells. L-5 cells store high amounts of starch, along the growth period and at the end of growth, reaching up to 8 mg/gr.fr.wt at the stationary stage. R-10 cells stored very low content of starch during growth, about 1 mg/gr/fr.wt. Thus, R-10 cells store less soluble sugars and starch than L-5 cells which may reflect faster metabolic activities of the sugar taken up.

The effect of salt in the growth medium on the content of glucose, sucrose and starch in the cells is shown in Figure 6. R-10 cells grown in the presence of 0.2 M salt contain more sucrose and starch as compared with the control, and there is a certain correlation between the slower growth and higher sucrose and starch content. In L-5 cells, starch accumulation was enhanced by salt mainly during the fourth week of growth.

Ultrastructural studies of the two cell lines, grown with and without salt, confirmed the analysis of starch presented in Figures 5 and 6. A comparison of starch content in a control R-10 cell (Fig. 7A) and a salt grown R-10 cell (Fig 7B) shows an obvious increase in the number of starch grains in the cell, and

Table 4 - The Effect of Sugar Uptake on Intracellular Content of

Sucrose and Glucose

The uptake experiment was conducted routinely with 116 mM sucrose or glucose and control cells were incubated in the absence of sugar. The cells were harvested, washed in fresh medium and disrupted in Thorax cell disintegrator. The homogenate was centrifuged at 35,000 x 30 min, and the supernatant was tested for glucose and sucrose content. Glucose was analysed as described by Johnson et al (6). Sucrose was hydrolysed with invertase (Sigma 200 units.ml⁻¹) for 1 hr at 37°C and its content was determined by the additional glucose content in the sample.

Extracellular sugar	Sucrose*		Glucose*	
	R-10	L-5	R-10	L-5
none	0	2.62±0.45	0	0.56±0.28
sucrose	4.52±0.74	9.32±1.30	0.21±0.046	0.44±0.40
glucose	1.84±0.08	7.6 ±0.95	2.97±0.26	1.94±0.26

* mg sugar.g FW⁻¹

Fig. 5 : The content of sucrose, glucose and starch in the two cell lines, during growth.

Fig. 6 : The content of sucrose, glucose and starch in the two cell lines, during growth in the presence of different salt concentrations: R-10 cells were grown with 0.2M NaCl in the medium, L-5 cells - with 0.1M NaCl.

the relative cell area occupied by the starch in the cell. The structure of the starch grains in R-10 cells grown in the presence of salt, is characterized by a different degree of crystalization as compared with the starch grains of R-10 cells in the absence of salt (Fig. 7C). The different crystalization may reflect changes in the starch chain length, as well as changes in the degree of branching of the polymer. The L-5 cells grown in the presence of salt were heavily loaded with starch grains which clearly occupied most of the cell area, as shown in Fig. 7D.

In conclusion, the R-10 and L-5 cell lines do not differ in the pattern of sugar uptake, and salt does not seem to interfere with short-term uptake experiments. L-5 cells are characterized by a higher content of stored soluble sugars and insoluble starch compared with R-10 cells. The presence of salt during growth has a striking effect on starch accumulation mainly in the salt-sensitive cell line (L-5). This major effect of NaCl may reflect interference of salt in the normal metabolism of carbohydrates, probably on the pathway of utilization of stored starch to useful metabolites.

Figure 7. Electron microscopy of the two cell lines in the absence and presence of NaCl. Cells were harvested and fixed for 2 hours at room temperature in 4% glutaraldehyde buffered with 0.1 M phosphate, pH 7.3, and postfixed in 2% buffered OsO_4 . Dehydration was carried out in ascending ethanol series followed by propylene oxide, and the samples were embedded in Epon. Ultrathin sections were cut in an LKB ultramicrotome, collected on 400-mesh grids, stained in saturated uranyl acetate and 0.3% lead citrate and observed under a Jeolco electron microscope. (A) R-10 in the absence of NaCl, x5000; (B) R-10 in the presence of 0.2 M NaCl, x5000; (C) same as (B) x15000; (D) L-5 in the presence of 0.1 M NaCl, x3300.

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DESCRIPTION OF COOPERATION

The cooperation with Prof. A. San Pietro was aimed to include the analysis of green embryos for their photosynthetic and respiratory functions. Plant material was grown and shipped from Israel to the U.S.A. The analysis conducted during the second year revealed the difficulty in sending the plant material in appropriate condition, and the poor quality of the material that arrived in the U.S.A. did not allow any further experiments there. Thus, the involvement of Prof. A. San Pietro was in annual contribution to evaluation and discussions of experiments carried out in Israel.

EVALUATION OF THE RESEARCH ACHIEVEMENTS

The development of Shamouti orange plantlets from the salt-tolerant callus was accomplished and proven to be feasible. The regeneration of plants required the establishment of a new protocol and plants were developed from embryos produced in the absence and presence of salt. This novel procedure produced plants with normal looking roots and abnormal looking shoots and is therefore incomplete for a large scale plant production needed for assessment of their salt tolerance.

The difficulties that arose during the development of the new procedure for plant regeneration indicate that selection for a specific trait may affect other properties of the cell as well. Thus, the assessment of the ability to transfer a desired important agricultural trait from cell culture into plants could not be accomplished because of what seems to us as technical difficulties. The

regeneration protocol of the wild type cells is required but not sufficient to regenerate a selected cell line.

We have found two biochemical properties that distinguish between salt-sensitive and salt-tolerant cells. The salt-sensitive cells accumulate much more starch and their malate content is drastically decreased when exposed to NaCl. On the other hand, salt-tolerant cells exhibit two-fold increase in malate dehydrogenase activity when exposed to NaCl. These phenotypic differences between the two cell lines demonstrate metabolic advantages of the salt-tolerant cell line. These properties, if established on the level of plants, may be beneficial for agriculture in serving as an easy selective markers for salt tolerance. We did not have enough regenerated plant material for testing the validity of these markers in whole plants, but it can be tested in various organs of crop plants where salt-sensitive and salt-tolerant varieties exist, such as barley.

Growth of cells as a function of the ratio of $\text{Ca}^{2+}/\text{Na}^{+}$ in the external medium followed the already described pattern in roots of whole plants. Increasing Ca^{2+} concentrations partially alleviates the detrimental effect of NaCl and may be considered as a practical tool in agronomy. The cell culture served as a very useful tool in understanding this phenomenon and may be further used to analyse effects of salinity on mineral nutrition.

List of Publications

Ben-Hayyim G, U. Kafkafi and R. Neumann-Ganome 1987 Role of internal potassium in maintaining growth of cultured Citrus cells on increasing NaCl and CaCl₂ concentrations. Plant Physiol in press.