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United States - Israel Binational Agricultural Research and Development Fund

FINAL REPORT

PROJECT No. US-239-80

Resistance to Water and Salt Stress Through Somatic Cell Selection

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1984

Standard BARD Cover Page for Scientific Reports

Date _____

BARD
P.O. Box 6
Bet Dagan, ISRAEL

BARD Project No. US-239-80

Title of Research Project:

Resistance to Water and Salt Stress Through Somatic Cell Selection

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Project's starting date: _____

Type of Report: 1st Annual _____ 2nd Annual _____ Final X

Signatures:

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Abstract

Part A

Salt-adapted cells exhibited enhanced abilities to gain both fresh and dry weight in the presence of NaCl (NaCl tolerance) compared to cells which were growing in medium without NaCl (unadapted cells, S-0). Tolerance of unadapted cells and cells adapted to 10 g L^{-1} NaCl (S-10), was influenced by the growth cycle stage. Cell osmotic potential and turgor varied through the growth cycle of unadapted cells and cells at all levels of adaptation.

Adaptation to NaCl led to a reduction in cell expansion and fresh weight gain, while dry weight gain remained largely unaffected. This reduction in cell expansion was not due to failure of the cells to maintain turgor. Tolerance, as indicated by fresh weight or dry weight gain, of the adapted cells did not increase proportionately with the increase in turgor.

Part B

In medium with 10 g/L NaCl, (+) abscisic acid (ABA) stimulated the growth of unadapted cells with an increasing response from 10^{-8} to 10^{-4} M ABA. ABA did not increase the growth of cells previously adapted to either 10 or 25 g/L NaCl unless the cells were inoculated into medium with a level of NaCl higher than the level to which the cells were adapted. ABA (10^{-8} to 10^{-4} M) did not accelerate the growth of unadapted cells exposed to water deficits induced by 5 to 20% polyethylene glycol (MW 8000). The growth of unadapted cells in medium with other salts (Na_2SO_4 , K_2SO_4) was also stimulated by ABA. These results suggest that ABA is involved in the adaptative response to exposure to excessive salts.

Part C

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 the Na^+ and Cl^- uptake, and all these cell lines take up similar or even higher 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, results in a greater loss of K^+ from the NaCl-sensitive cell line. Exposure of the NaCl-tolerant cell lines to salts other than NaCl results in even better tolerance to Na_2SO_4 , but rather poor tolerance to K^+ . The NaCl-sensitive cell line proved to be more sensitive to replacement of Na^+ by K^+ .

Part D

Nonselected and NaCl-selected cell lines of two species of Citrus were subjected to polyethylene glycol (PEG)-induced water stress. The non-selected cells of Shamouti orange (C. sinensis L. Osbeck) and Sour orange (C. aurantium) were more resistant to water stress than to salt stress when exposed to both stresses at equal osmotic potentials of the medium. However, the growth of the NaCl-selected cell lines exhibited similar degree of tolerance to both stresses. NaCl-selected cell lines of Sour orange have no advantage over the nonselected cells when exposed to water stress. However, NaCl-selected cell lines of Shamouti orange perform better than the nonselected cells in the presence of PEG. Growth in the presence of PEG resulted in a marked increase of dry matter. Analysis of intracellular concentrations of inorganic ions, free amino acids and proteins did not show any increase under these conditions.

D. Objectives

The following are the objectives of the proposed research.

1) Select cell lines from plants of several species which are resistant to water (polyethylene glycol) and salt stress.

2) Determine the stability of such resistance in culture.

A) Measure the resistance of selected and nonselected lines after prolonged intervals in culture.

B) After various time periods on selection medium, return the selected lines to nonselection medium and measure the resistance after various time periods on nonselection medium.

3) Determine the mechanism(s) of selection.

A) Evaluate the role of adaptation vs. selection of cell types in the resistance mechanism.

a) Obtain randomly selected clonal lines from the normal (non-resistant) population to determine the natural degree of variation in resistance and compare these clones to those obtained from the resistant population.

b) Evaluate the stability of the resistance of resistant clones in the presence and absence of stress.

B) Determine the effect of 1) growth stage of inoculum, 2) cell density, and 3) medium conditioning by resistant and sensitive cells on relative resistance, in order to partially evaluate the role of development and environment on the relative resistances of the cells.

4) Determine the stability of resistance through plant regeneration.

A) Regenerate plants from resistant and nonresistant populations.

a) Maintain stress throughout regeneration of resistant populations.

b) Remove stress during regeneration of resistant populations.

B) Regenerate plants from clones selected from resistant and non-resistant populations.

a) Maintain stress throughout regeneration of resistant clones.

b) Remove stress during regeneration of resistant clones.

C) After regeneration of plants by these procedures, measure the resistance of whole plants and culture cells derived from the plants.

D) Determine the genetic heritability of the resistance character.

PART A Adaptation of Tobacco Cells to NaCl

Introduction

To survive in a saline environment, plants must cope with water deficits resulting from lowered external water potentials in order to maintain turgor and grow (10,20). In addition to adjusting to the osmotic stress the plants must be able to alleviate the detrimental effects of the high external concentrations of both Na^+ and Cl^- . Activities of enzymes, protein synthesis, nitrogen metabolism, and photosynthesis have all been found to be inhibited by elevated levels of Na^+ and Cl^- (10,12,32). Furthermore, membrane functions such as the selectivity of ion transport are severely affected by high Na^+ and Cl^- levels and ratios of ions, particularly Na^+ , K^+ and Ca^{2+} (9,21). Thus the plant must use mechanisms to cope with dehydration and ion toxicity or imbalance caused by salinity. All of this must be accomplished without seriously depleting the metabolic energy available to the plant (32,42).

Plants apparently rely on a number of mechanisms with which to deal with salinity stress (10,12,32,42). Some of these mechanisms require complex cellular and tissue organization and integration and are functional only in intact plants. These include transport mechanisms which remove Na^+ and Cl^- from the xylem, or redistribute the ions into the phloem for export from the roots preventing the ions from reaching actively growing cells (10,12). Many halophytes have anatomical structures such as salt glands or bladders, or specialized trichomes which serve as repositories for accumulated salt and thus limit exposure of growing cells to NaCl (10).

Other mechanisms by which plants deal with salinity involve properties intrinsic to individual cells and include such processes as sequestering of ions into the vacuole (10,12,42), synthesis and accumulation of organic solutes like proline and glycinebetaine (41,42) and active exclusion of Na^+ and Cl^- from the cell (1,12). These cellular mechanisms are especially important to nonhalophytes which lack adaptive features such as salt glands. Furthermore, such cellular mechanisms of salt tolerance may be more amenable to genetic manipulation than more complex mechanisms involving tissue interaction or morphological structures (42).

In studies with whole plants it is difficult to separate cellular mechanisms of tolerance from those based on anatomical structures and physiological specialization requiring the cell and tissue organization which exists in the intact plant (40). The use of an in vitro system, such as cultures of callus or cell suspensions, offers a means to focus only on those physiological and biochemical processes inherent to the cell. The relative tolerances of cultured tissues of Hordeum jubatum (30), Lycopersicon peruvianum and Solanum pennellii (36) and Distichlis spicata (40) to salinity were similar to those expressed by the plants of these genotypes. Correlations of salinity tolerance of a plant with that of cultured cells and tissues occurs only if the tolerance of the plant is due predominantly to cellular based mechanisms. Cells of halophytic species such as Atriplex undulata and Suaeda australis which rely primarily on salinity tolerance mechanisms which are dependent upon whole plant organization exhibited little or no salt tolerance in vitro (34).

Cell lines with enhanced tolerance to NaCl have been isolated from many glycophyte species (1,7,8,17,28,29,31,33,38,41). It appears that these cells exhibit a number of physiological processes including proline accumulation (41), exclusion of Na^+ and Cl^- (1), as well as ion accumulation (7,41,19) which appear to contribute to adaptation against the adverse effects of salinity. Although most reports confirm the stability of the salt tolerance trait through prolonged periods of culture in the absence of salt (1,8, 28,29,41) there are reports where tolerance was only stable in some lines (33) and we have reported a lack of stability of the salt tolerance phenotype of cells isolated in medium with 10 g L^{-1} NaCl (17).

In order to evaluate cellular mechanisms of salt tolerance in a glycophyte species we have isolated cell lines of Nicotiana tabacum var. Wisconsin 38 adapted to different concentrations of NaCl. We report here the growth characteristics and NaCl tolerance of these adapted cells. We present evidence that turgor increases as cells are adapted to increasing concentrations of NaCl. Our results suggest that the relationship between turgor and cell expansion in these cells has been altered in response to NaCl.

Materials and Methods

Development and Culture of Cell Lines Adapted to NaCl. In a previous report we described the procedures for the isolation and maintenance of a cell suspension of *Nicotiana tabacum* L. var. Wisconsin 38 as well as the procedure for the isolation of a cell line which is capable of growth in medium with 10 g L⁻¹ NaCl (S-10) (17). Additional NaCl adapted tobacco cell lines were obtained by transferring S-10 cells into medium with 14 g L⁻¹, 20 g L⁻¹, and 25 g L⁻¹ NaCl in a sequential manner (Table 1). Cells were maintained in medium with the previous level of NaCl for a minimum of 50 cell generations prior to inoculation into medium containing the next highest level of NaCl. Stocks of the cell lines were maintained routinely in 500 or 1000 ml Erlenmeyer flasks as batch cultures and recultured when the cells had reached the early stationary phase of growth. Cells in the late exponential phase of growth were used for experimentation.

Tolerance and Water Relations Determinations. Tolerance and water relations measurements were made using cell samples taken throughout the growth cycle of each cell line. Cells were inoculated into 2000 ml of nutrient medium contained in a 4 liter Erlenmeyer flask at a fresh weight density of 20 g L⁻¹. The nutrient medium contained either no NaCl for the S-0 cells or the level of NaCl in which the cells were being maintained routinely as stocks.

Tolerance was evaluated by determining the fresh and dry weight accumulation of cells in media with different levels of NaCl. Cells from the primary culture were obtained and the cells concentrated by either removing medium by filtration in a (coarse) fritted glass funnel or by decanting off excess medium. Care was taken when removing medium by filtration not to remove all of the excess medium from the cells. Cells subjected to harsh filtration have been found to be metabolically impaired (37). Filtered cells were resuspended into fresh medium containing the same level of NaCl as the primary culture. The resuspended cells and the cells which were concentrated by decanting were adjusted to a fresh weight density of 0.25 g ml⁻¹ and used as inoculum for determination of tolerance. Cells were inoculated into 25 ml of medium in 125 ml E. flasks containing various concentrations of NaCl at a fresh weight inoculum density of 0.02 g ml⁻¹ and harvested after 32 d. Cultures which reached stationary phase prior to 32 d were harvested when they had reached the stationary phase of growth, since cells which were allowed to remain in this phase of growth for prolonged periods of time began to senesce. Thirty-two days was the time required for the primary culture of the cell line with the slowest growth rate (S-25) to reach stationary phase.

Osmotic potentials were determined by plasmometry (4,13) and were based on the concentration of NaCl causing incipient plasmolysis in 50% of the viable cells. Water potentials of the culture media were measured by determination of the freezing point with a Precision Systems, Inc. (Springfield, MA) automatic osmometer or by the dew point method with a Wescor thermocouple psychrometer-hygrometer model HR33T, with C52 sample chambers (5). Calibration was accomplished using NaCl solutions. For the purpose of estimating turgor, cells were assumed to be in water potential equilibrium with the culture medium and cell turgor values were calculated as the difference between the water potentials and osmotic potentials.

Cell Size. Cells from stationary phase cultures were viewed with a Nikon Optiphot microscope and Nomarski interference-contrast optics.

Growth Measurements. Cells were harvested on Whatman #4 filter paper in a Buchner funnel with aspiration. Fresh weights were recorded, and cells were allowed to dry at least 24 h in an oven at 80°C before dry weights were determined.

Results

Comparison of Growth Characteristics of NaCl Adapted and Unadapted Cell Lines.

Although the lag phases appeared to be quite similar between adapted and unadapted cells, approximate fresh weight doubling times increased from 2 d for S-0 cells to 4 d for S-25 cells (Fig. 1 A) and maximum fresh weight attained declined from 10 to 6.5, 5.3, 4.2 and 3.3 g (25 ml)⁻¹, respectively, for the S-0, S-10, S-14, S-20, and S-25 cell lines (Fig. 1A, 2). The rate of dry weight accumulation (Fig. 1B) and maximum dry weight gain (Fig. 2) both appeared to be independent of external NaCl concentration. The differences in fresh weight gain maxima exhibited by the cells adapted to NaCl resulted in a decreased fresh to dry weight ratio of 28, 19, 16, 14, and 10 for the S-0, S-10, S-14, S-20, and S-25 cell lines, respectively. The water content of the cells at full expansion (stationary phase) decreased with the level of adaptation (Fig. 2).

The decrease in maximum fresh weight exhibited by the salt adapted cells was not attributable to a decrease in cell number but was due to a decrease in cell volume. The S-25 cells were 4-5 times smaller than the S-0 cells at stationary phase (Fig. 3). The cells in the S-0 population exhibited a greater degree of variability in cell volume than did cells of the salt adapted populations. This may be due to the fact that salt adapted cells undergo only radial or isodiametric cell expansion immediately following cell division, and fail to undergo extensive directional expansion or elongation before division as the S-0 cells do. This may be the result of S-25 cells having a slower rate of expansion between cell divisions and as a consequence the cells remain smaller and more spherical (Fig. 3).

S-0 cells underwent a very extended lag phase compared to adapted cells in media containing 10 g L⁻¹ NaCl (Fig. 1C-D). At higher levels of NaCl the S-0 cells exhibited little if any growth during the period observed (Fig. 1C-D). This prolonged lag phase is in part due to a significant loss of cell viability, and must represent also an adjustment period during which the cells must cope with large Na⁺, Cl⁻ and water potential gradients in order to initiate growth. Clearly one adaptive feature of the NaCl lines is the ability to exhibit a typical growth pattern without the necessity of a prolonged lag phase.

After approximately 75 generations in medium with 25 g L⁻¹ NaCl, S-25 cells did not lose the ability to grow in medium without NaCl (Fig. 1A-B). Adapted cells continually lost turgor when innoculated in medium without salt until stationary phase where these and S-0 cells have approximately the same turgor (data not shown). For several passages after inoculation into medium without NaCl the adapted cells continued to exhibit a decreased rate of fresh weight gain, suggesting that the cells are readjusting to medium without salt during this period. This restricted ability to undergo expansive growth is apparently

manifested for several generations after transfer back to medium without salt (data not shown).

Tolerance of Cell Lines to NaCl. The ability to accumulate fresh and dry weight in media with different concentrations of NaCl increased with the level of NaCl to which the cells were adapted (Fig. 4). Although the tolerance of the S-0 and S-10 cells varied during the culture growth cycle (Fig. 4A-B, E-F, I-J) the average tolerance over the entire growth cycle exhibited by the different cell lines were clearly different (Fig. 4I-L, 5A, 6). The relationship between tolerance and the level of adaptation is not proportionate (Fig. 6). The adapted cells have undergone a physiological adjustment which apparently renders them more tolerant to higher concentrations of salt and which allows them to be more able to resist dehydration when exposed to NaCl (Fig. 5B).

Despite the fact that the ability of cells to grow in higher levels of NaCl increased with the level of salt adaptation, S-25 cells were not able to gain as much dry weight at equivalent increments of NaCl above the adaptation level as did S-0 cell lines (Fig. 7). Thus it appeared as if maintenance of cells in medium with NaCl clearly adapted them to cope with that level of salinity, however, this did not necessarily make them more fit to adjust to higher increments of NaCl. However, increases in the level of NaCl above 25 g L^{-1} may not represent the same degree of stress as an equivalent increase above 0 g L^{-1} . Such evidence suggests that cells of glycophytic species have salt adaptive capabilities but that these adaptive abilities may not change much as a result of undergoing adaptation.

As noted earlier for PEG (4) and pathotoxin tolerance (15), a significant influence of the culture growth cycle on salinity tolerance was observed (Fig. 4). The tolerances of S-0 and S-10 cells were most profoundly influenced by the stage of the growth cycle. With these cell lines a several-fold difference in growth in NaCl could be observed depending on the stage of the growth cycle. Such significant influences of the stage of growth on salinity tolerance coupled with the great difficulty of obtaining cultures with synchronized series of growth cycles make accurate single point determinations of the relative NaCl tolerance of a cell line rather difficult. The influence of growth cycle stage on NaCl tolerance became less pronounced with cell lines adapted to higher levels of NaCl.

The salt adapted cells also exhibited enhanced tolerance to increased osmotic stress (Fig. 8) induced by polyethylene glycol (PEG), MW 6000, (5). At this molecular weight PEG will lower the water activity but cannot be used as a solute for osmotic adjustment (13). S-0 cells exhibited slightly greater fresh weight accumulation in media with NaCl than PEG at equivalent external water potentials, particularly at water activities higher than -14 bar (Fig. 8C). Virtually no difference in fresh weight gain was observed for S-25 cells in media with NaCl or PEG (Fig. 8C). However, S-0 and S-25 cells accumulated considerably more dry weight in media with PEG (Fig. 8B, D). This difference in dry weight gain in PEG and salt may be reflective of differences in the molecular weights of the principal solutes used for osmotic adjustment in the two instances, particularly if accumulation and compartmentation of Na^+ and Cl^- is a primary process of osmotic adjustment for cells growing in NaCl.

Water Relations Characteristics of the Cell Lines and their Relationship to Salinity Tolerance.

Analysis of the water relations of the cells indicated that they had undergone considerable osmotic adjustment in response to NaCl stress, as evidenced by the turgor increase (Fig. 9, 10). Upon inoculation into fresh medium the cells began to adjust osmotically, presumably through the influx of the numerous solutes in the nutrient medium, which resulted in an increase in turgor (Fig. 9). The changes in turgor exhibited by cells during a culture growth cycle were considerably greater for salt adapted (approximately 20 bar for S-25 cells) than for S-0 (4 bar) cells. Although it is difficult to make a precise conclusion from our data, it appears as if maximum turgor occurred at the onset of exponential fresh weight gain which is the point where cell division and isodiametric expansion are the primary processes contributing to growth, and volume of the cell is at its smallest (18). At this time osmotic adjustment appears to be maximal in preparation for cell expansion which occurs primarily during the phase of exponential fresh weight gain. It is during this phase that the turgor increase is dissipated as a result of cell expansion perhaps through regulation by some turgor sensory mechanism as described by Zimmermann (44). Turgor changes during the growth cycle were not well correlated with growth during the phase of rapid cell expansion as the cells exhibited significant decreases in turgor during periods when the growth rate remained constant (Fig. 9). This is not unexpected as the period over which growth was measured was sufficiently long to allow other factors to impinge upon the expansive properties of the cell (20). However, the adapted cells accomplished less expansion (fresh weight gain) from a given amount of turgor increase which occurred at the beginning of the growth cycle (Fig. 11A).

The extent of the osmotic adjustment (averaged over the growth cycle) exhibited by the different salt adapted cell lines was greater than the change in external water potential to which the cells were exposed (Fig. 10). This high degree of osmotic adjustment occurs during the first passage in the new environment and the cells retain this high level of adjustment after the initial passage. As a consequence of the osmotic adjustment made by the cells and failure to dissipate the turgor by cell expansion, cell turgor (averaged over the growth cycle) increased. The decrease in the amount of growth resulting from a given level of turgor suggests perhaps a change in the turgor threshold necessary for growth or in the extensibility properties of the wall (Fig. 11A). However, several factors are known to influence the expansion of cells other than turgor (20,35) and changes in any of these may also be involved in adaptation.

The magnitude of the osmotic adjustment that the salt adapted cells underwent in response to NaCl followed a curvilinear type relationship such that the most dramatic increases in turgor occurred in response to very high concentrations of NaCl (Fig. 10). It is possible that the large absolute values are due in part to the limitations of the plasmometric technique for the determination of osmotic potentials (14).

The increased osmotic adjustment made by the salt adapted cells was reflected in their increased ability to grow in media with higher levels of NaCl (Fig. 11B). However, just as with growth in NaCl levels above that to which the cells are adapted, our data illustrate that the amount of tolerance per amount of turgor also decreased as the cells adapt and osmotically adjust

(Fig. 11B), indicating that turgor probably is not the factor restricting the salt adapted cells from exhibiting enhanced tolerance to NaCl above the level of adaptation (Fig. 7). The loss of the effect of increased turgor on the tolerance after adaptation can be seen also by observing the effect of turgor pressure changes during the growth cycle on tolerance changes during the growth cycle (Fig. 12). The effect of growth cycle turgor adjustment on tolerance of the cells decreased significantly when comparing S-0₁ and S-10 cells, respectively. Above the level of adaptation of 10 g L⁻¹ there appeared to be little if any effect of turgor changes (during a culture growth cycle) on the tolerance to NaCl.

Discussion

Maintenance of turgor by osmotic adjustment is not the primary factor which restricts the limits of and the rates of cell growth of the salt adapted tobacco cells. It is clear that these cells have undergone considerable osmotic adjustment to the point of substantial increases in turgor even after the possible errors in osmotic potential estimation associated with plasmometry have been considered (14). Similar observations of decreased growth have been made with plants which have been subjected to decreases in external water potential (20,25,26). From a biophysical perspective the salt adapted cells should have sufficient turgor to elicit cell expansion as observed with the unadapted cells. Growth retardation could be due to changes in hydraulic conductivity, however, it is unlikely that the cultured cells are not in water potential equilibrium with the medium and estimates of cell water potentials by direct measurement with dew point psychrometry confirm this. Consequently, it is reasonable to speculate that the restriction of expansion in salt adapted cells is a consequence of factors which either directly or indirectly influence the properties of the cell wall (20,35). Changes in the composition and/or physical characteristics of the wall would have profound effects on cell expansion either through changes in the turgor threshold or alterations in the extensibility properties of the wall (20,35). Decreased elastic extensibility properties of cell walls have been implicated in plants adjusted to desiccation conditions (22) and decreased elasticity also resulted from exposure of Alternanthera philoxeroides to saline conditions (3). Blaschek and Franz (2) reported a change in the cell wall composition of cultured tobacco cells in response to increase osmolarity of the culture medium. Changes in the wall loosening characteristics of the cells could prevent expansion and also allow turgor increases.

Under saline conditions growth may be limited also by metabolic limitations which might be caused by ion toxicity due to the accumulation of Na⁺ and Cl⁻ used for osmotic adjustment (12,42). In addition, it is possible that metabolic constraints result from adaptive changes necessary under saline conditions. The cells may be energy limited due to the diversion of metabolic carbon into processes which are required for maintenance of salinity tolerance (16,32). As cells become tolerant to NaCl, carbon is fluxed into the biosynthesis of osmotic solutes and into catabolic processes which result in respiratory energy necessary for the biosynthesis of osmotic solutes and for other processes (i.e., ion transport) which are important to osmotic adjustment. Such a situation results in a substantial partitioning of carbon away from growth processes.

An obvious advantage of such a high degree of osmotic adjustment is the ability to resist dehydration as the external water potential is being lowered even to the point of zero turgor (27). However, since the elastic modulus is typically higher for cells under high hydrostatic pressures there will be a tendency for water to move out of the cell more rapidly, that is, the half time for water loss will be shorter (44). In contrast, cells with a high elastic modulus will lose a smaller proportion of total cell water as turgor is lost (44). Meyer and Boyer (26) have suggested also that restricted cell expansion may be a requisite for osmotic adjustment under water stress.

Decreased growth under desiccation stress may be a survival mechanism important to many plants which allow water conservation. Reduced growth may be advantageous under water deficits even when osmotic adjustment has produced a water potential gradient favorable for growth, since under natural dessication conditions only a limited amount of water is available, and lowered cell water potentials will not be able to provide water to sustain growth indefinitely. This is a classic 'water saving' mechanism which is a well recognized adaptive response to desiccation stress (24). However, this necessarily should not be the case under saline stress where water availability for growth is unlimited as long as the water potential gradient favors water uptake. Plants may have evolved mechanisms to restrict their growth under dessication stress even when osmotic adjustment would allow water uptake, presumably to conserve water. However, under saline stress such growth restriction would seem unnecessary and indeed many halophytic plants exhibit little restriction of their fresh weight growth under saline conditions (11,39). Perhaps the cultured tobacco cells, being glycophytic, activate a growth restriction mechanism in the presence of both dessication and saline stress which is designed to resist primarily dessication stress. If this is true many glycophyte crop plants may exhibit restricted growth under saline irrigation which is not a natural constraint of exposure to salt but may represent the inability of these plants to discriminate between desiccation and saline stress.

Our results establish that cells arising from a salt sensitive plant can adapt to survive and grow under conditions of extreme salinity, and that adaptation involves considerable osmotic adjustment with increased turgor but reduced cell expansion. These cell lines provide a useful system in which to investigate cellular processes involved in salt tolerance. They also provide an opportunity to study further some of the fundamental questions concerning the regulation of turgor and growth.

Acknowledgements

We are grateful to Dr. C. E. Bracker for his assistance with the light microscopy and to Dr. T. K. Hodges for the use of the Optiphot Microscope. We wish to thank Drs. Yoash Vaadia and Andrew Hanson for enlightening discussions and G. McClatchy and J. Clithero for excellent technical assistance.

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Table 1. Tobacco cell lines growing in medium without NaCl (S-0) or in media with 10 (S-10), 14(S-14), 20 (S-20) or 25 (S-25) g L⁻¹ NaCl. Concentrations of NaCl (g L⁻¹, mM) and water potential (ψ_w) values are for media prior to inoculation cells. Listed also are the number of cell generations in which the respective cell lines have been maintained at that level of NaCl.

Cell Line	NaCl g L ⁻¹	NaCl mM	ψ_w bar	Cell Generations
S-0	0	0	-6	
S-10	10	171	-14	80
S-14	14	240	-17	90
S-20	20	342	-23	75
S-25	25	428	-27	75

Figure Legends

- Figure 1. Fresh (A and C) and Dry (B and D) weight accumulation of tobacco cells as a function of time. A and B, S-0 (○), S-10 (■), S-14 (□), S-20 (▲) or S-25 cells (△) in media containing the concentration of NaCl to which they are adapted; 0, 10, 14, 20, and 25 g L⁻¹, respectively and S-25 cells in medium without NaCl (◇). C and D, S-0 cells in media with 0 (○), 10 (■), 14 (□), 20 (▲), 25 (△) g L⁻¹ NaCl.
- Figure 2. Fresh (●) and dry (○) weight maxima attained by the S-0 and salt adapted cells in a culture growth cycle. The cell lines are listed by the level of NaCl to which they are adapted. Water content (■) of the cells at stationary phase was calculated as fresh weight-dry weight/dry weight.
- Figure 3. Cells from stationary phase cultures showing decreased cell size with adaptation to increasing concentrations of NaCl. Nomarski interference-contrast optics were used to view S-0 (A,B), S-10 (C), S-20 (D) and S-25 (E) cells (X250). The S-0 cells are predominantly of the form shown in (A).
- Figure 4. Growth cycle dependence of tolerance to NaCl for S-0 (A,E,I), S-10 (B,F,J), S-20 (C,G,K) and S-25 (D,H,L) cells. Relative fresh (A-D) and dry (E-H) weight gain in media with various NaCl concentrations; 2 (○), 5 (◇), 10 (●), 15 (○), 20 (■), 25 (□), 30 (▲), 35 (△) or 40 (◆) g L⁻¹ NaCl. Shown in I-L are the NaCl concentrations which inhibited 50% of the maximum dry weight gained (ID₅₀) as a function of growth cycle stage, and the fresh weight growth curves (●) for the primary cultures of S-0, S-10, S-20 and S-25 cells, respectively.
- Figure 5. Average through the growth cycle of relative dry weight gain (A) and water content (B) of S-0 (●), S-10 (○), S-20 (■) and S-25 (□) tobacco cells at various concentrations of NaCl. Values represent averages of the data in Figure 4. Water content was calculated as fresh weight-dry weight/dry weight.
- Figure 6. Tolerance to NaCl of the S-0 cells and cells adapted to varying concentrations of NaCl. Dry weight ID₅₀ values are averages of the data in Figure 4(I-L). The cell lines are listed by the level of NaCl to which they are adapted.
- Figure 7. Relative dry weight gain of S-0 (●) and S-25 (○) cells at varying NaCl concentrations above the level to which the cells are adapted.
- Figure 8. Growth of S-0 (A,B) and S-25 (C,D) cells in media with NaCl (●) and PEG (○). Relative fresh (A,C) and dry (B,D) weights are plotted as a function of the water potentials of the media containing various concentrations of PEG or NaCl.
- Figure 9. Water Relations of S-0 (A), S-10 (B), S-20 (C) and S-25 (D) cells throughout a culture growth cycle. Osmotic (●) and water potentials (■) and turgor (○) were determined at the stages of the growth cycle shown by the fresh weight growth curve (□).

Figure 10. Osmotic adjustment of S-0 cells and cells adapted to varying concentrations of NaCl. Values for turgor (○) and osmotic potential (●) represent averages of the values collected over a growth cycle (Fig. 9). The cell lines are listed by the level of NaCl to which they are adapted.

Figure 11 A. Relationship between maximum turgor difference and fresh weight gain during a culture growth cycle. The cell lines are listed by the level of NaCl to which they are adapted. B. Dry weight ID_{50} and the relationship between dry weight ID_{50} and average turgor as a function of the average cell osmotic potential of the tobacco cell lines.

Figure 12. Relationship between tolerance to NaCl and turgor through a culture growth cycle. Values for dry weight ID_{50} (Fig. 4I-L) were plotted as a function of the turgor of the cells (Fig. 9). Lines represent LS best fit of available points from S-0 (●), S-10 (△), S-20 (○) and S-25 (■) cells.

Figure 1.

Figure 2.

Figure 3

Figure 4.

Figure 5.

Figure 6.

Figure 7.

Figure 8.

Figure 9.

Figure 10.

Figure 11.

Figure 12.

PART B Absciscic Acid Accelerates Adaptation of Cultured Tobacco Cells to Salt

Introduction

Cell lines tolerant to NaCl stress can be obtained from cultured plant cells of several glycophyte species (4,12,14,20,27,33). Cell lines of tobacco (*Nicotiana tabacum* L. var. Wisconsin 38) have been isolated in our laboratory which are capable of growth in liquid medium with up to 428 mM NaCl (5). Because cultured cells must adapt to saline conditions through cellular processes, investigations of such cells may help us to elucidate the cellular mechanisms involved in NaCl tolerance.

The generally held view that abscisic acid (ABA) controls adaptation to stresses is based largely on indirect evidence. ABA accumulates in plant tissues in response to various stresses, suggesting that ABA has a role in adaptation (2,7,10,21,26). ABA accumulation, which has been shown to be heritable in some plants (22), has been found to be correlated with heritable stress tolerance (28). Moreover, ABA stimulates physiological responses believed to have adaptive value (1,13,28). More direct evidence that ABA is involved in tolerance to stress is provided by the findings that ABA can increase the tolerance of plant tissues to stresses including water deficit (26), chilling (6,10), freezing (11), and a herbicide (30). Evidence is lacking to show that ABA increases the growth of cells or tissues treated with NaCl. Furthermore, effects of ABA have not been shown to be specifically related to salt exposure and not to the water deficit coincidentally imposed. In this study, we determined that ABA increases NaCl tolerance of tobacco cells in suspension cultures by accelerating their adaptation to the salt. We show that ABA causes cells to grow sooner in the presence of elevated NaCl concentrations and to reach the capability of enhanced growth in NaCl sooner. Furthermore, we present evidence that the effect of ABA is specific to cells exposed to ions, but not water deficit caused by addition of nonionic or nonpenetrating solutes to the medium.

Materials and Methods

Cells of *Nicotiana tabacum* (cv. Wisconsin 38) in suspension culture were initiated and maintained as described by Hasegawa *et al.* (20). Cells were grown in either 1-L Erlenmyer flasks containing 250 ml medium or in 125-ml Erlenmyer flasks containing 25 ml medium. For inoculum, cells were grown to a fresh weight of ca 300 mg/ml, a density corresponding to the late exponential phase of growth. These cells were inoculated into fresh medium at a fresh weight of 10 mg/ml. To prepare stock solutions of 10^{-5} M (+) ABA (Calbiochem-Behring Corp, La Jolla CA, cat #100111), 26.43 mg ABA was dissolved in less than 100 μ l of EtOH and then mixed with medium. The ABA solution was sterilized by filtration and adjusted to a volume of 100 ml with sterile medium. ABA was added to sterile culture medium just prior to inoculation. When used as osmotica, Na_2SO_4 , KCl, K_2SO_4 , mannitol, sorbitol, sucrose, NaCl, or polyethylene glycol (PEG, MW 8000, J.T. Baker Chemical Co., Phillipsburg, N.J., Cat no. 1-U222) were added during preparation of the liquid medium and autoclave sterilized in the medium. The cell wall is impermeable to PEG at the molecular weight used and PEG does not degrade appreciably and enter cultured cells (8,17).

Fresh and dry weights were measured as described by Hasegawa *et al.* (20). The lag period is defined as the time elapsed before fresh weight of the cells exceeds the fresh weight at inoculation. Fresh weight doubling times were calculated from linear regression lines established from the data taken during the exponential phase of growth. The correlation coefficients were greater than 0.90.

Results

In this report, and from prior experiments (20,25), we conclude that tobacco cells are capable of adapting to medium with high NaCl concentrations. After adaptation these cells are able to grow more rapidly in NaCl than they could prior to initial exposure to the salt. Furthermore, the presence of ABA accelerates the process of adaptation. The cells began growing in NaCl and reach the adapted or more tolerant state sooner in the presence of ABA than in its absence (Figs. 1 and 2, Table I). ABA enhanced the growth of unadapted cells subjected to 10 g/L NaCl (Fig. 1) but did not increase final yield of cells in medium with either 0 or 10 g/L NaCl, with the possible exception of cells grown in medium containing 10 g/L NaCl and 10^{-4} M ABA. ABA concentrations from 10^{-8} to 10^{-4} M slightly inhibited the growth of cells without NaCl, resulting in slightly longer doubling times (Table I) and slightly longer lag periods. The lag period of cells treated with 10^{-4} M ABA was considerably longer. ABA concentrations from 10^{-8} to 10^{-4} M ABA stimulated the growth of unadapted cells in medium with 10 g/L NaCl in a dose-dependent fashion (Fig. 1, Table I). In other experiments (data not shown), this dose-dependence was observed when unadapted cells were transferred to medium containing 4, 6, 8, 10, and 12 g/L NaCl. ABA (10^{-5} M) also enhanced adaptation of unadapted cells to higher levels of NaCl up to 22 g/L NaCl (Fig. 2).

Tobacco cells adapted to and grown in 10 g/L NaCl for over 200 generations did not grow better with 10^{-5} M ABA in the medium (Fig. 3). As expected, growth of cells adapted to 10 g/L NaCl was inhibited in medium with 16 g/L NaCl. However, when these cells are transferred from medium with 10 g/L NaCl to 16 g/L NaCl, the presence of ABA stimulated their growth and coincident adaptation to the elevated level of salt.

The effect of ABA may be viewed in two ways. First, ABA enhances the tolerance of the cells since they grow faster when challenged with elevated concentrations of NaCl if exogenous ABA is present. Secondly, as much as the growth of the cells in elevated levels of NaCl with or without ABA leads to an increase in their tolerance, since they then can grow more rapidly in NaCl when challenged with NaCl again (20 and data not shown), ABA can be said to increase this rate of adaptation. The effect of ABA is clearly related to this adaptation or tolerance and not simply growth since ABA has no stimulating effect on the growth of unadapted cells in medium without NaCl, nor does it stimulate the growth of adapted cells in medium containing NaCl at the concentration to which they are adapted.

ABA did not accelerate the process of adaptation to water deficit induced by PEG (Fig. 4, Table II). In fact, the adjustment to water deficit induced by PEG was inhibited by ABA (Table II). ABA stimulated growth of cells subjected to water stress only in the presence of the penetrating ionic solutes NaCl, KCl, Na_2SO_4 and K_2SO_4 , but not in the presence of the slowly penetrating and

nonpenetrating, nonionic solutes mannitol, sorbitol, sucrose and PEG (Table III). This indicates that the promotive action of ABA is not effective for osmotic imbalance in general, but may be specific for excess ions e.g. Na^+ , K^+ , Cl^- , and SO_4^{2-} .

Discussion

This is the first report in which ABA enhanced growth adaptation of cells to NaCl stress has been described. The mechanism by which ABA stimulates adaptation to NaCl stress is unknown although ABA increases osmotic adjustment of pea shoots and roots in the absence of salinity (21), and it is reasonable to suspect that ABA may be acting to stimulate osmotic adjustment in the cultured cells.

From our results, we interpret that exogenous ABA supplements the synthesis of ABA in response to stress, and consequently that ABA can stimulate adaptive processes. Thus the stimulation of growth by ABA may reflect a normal process. Alternately, we cannot rule out the possibility that the stimulation of growth is pharmacological.

When cultured cells of *Nicotiana* spp. are subjected to concentrations of NaCl up to 10 g/L, the cells adapt and grow, but they grow slower than cells without NaCl present (20,27,33). The adaptation is reversible since cells transferred out of the stressful saline medium lose the ability to grow at the same rates as those maintained in the adapted condition. This reversibility of the expression of NaCl tolerance indicates that selection for stable variants is not significant under moderate NaCl stress. However, cells with apparently phenotypically stable tolerance can be recovered from cells exposed to NaCl (4,14) or PEG (18) even after they are returned to and maintained in the absence of stress. Reversible adaptation and enrichment of the cell population with stable variants probably both occur, and although these events may be related to one another there is no direct evidence for this in cultured plant cells.

ABA stimulates responses in some plant tissues that may confer tolerance to NaCl. For instance, proline accumulates in cultured tomato cells subjected to water deficit induced by PEG (19) and in tobacco cells in response to NaCl stress (5,33). Accumulation of proline in plants can be stimulated by NaCl, water deficit, or ABA treatment (1,19,29). Unique proteins, including a major 26 Kd protein, accumulate in cells adapted to NaCl stress (15,31), and the synthesis of this major 26 Kd protein appears to be stimulated by ABA (31). ABA may also influence ion fluxes and compartmentation in plant cells. Compartmental analysis of beet tissues treated with ABA grown in low salt conditions showed that ABA reduces the cytoplasmic sodium content by 50% and increases the vacuolar sodium content by 162% (16). Similar effects on the cytoplasmic and vacuolar sodium contents by ABA in barley roots have been observed (24). Net accumulation of Cl^- , K^+ , and unspecified osmotica in root tissues of various species is stimulated by ABA (3,9,32). So, it appears that ABA can stimulate physiological responses thought to be involved in adaptation to NaCl, and perhaps other stresses.

Since there appeared to be an actual decrease in tolerance to PEG caused by addition of ABA, it seems unlikely that the failure of ABA to enhance tolerance

to PEG induced water stress was due to inactivity of the ABA. This suggests that ABA in this situation protects against injuries induced by excess Na^+ , K^+ , Cl^- or SO_4^{2-} . The combination of ABA and ions may stimulate osmotic adjustment, perhaps by stimulating the uptake and compartmentation of Na^+ and Cl^- as well as other ions. This is a reasonable supposition because Na^+ and Cl^- make up a significant portion of the solutes used for osmotic adjustment to NaCl (5,23,33).

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TABLES

Table I. Growth kinetics of unadapted tobacco cells in medium with 0 and 10 g/l NaCl with various concentrations of ABA. Doubling times and lag periods were calculated from the data in Fig. 1.

<u>ABA</u>	Fresh weight doubling time	Fresh weight doubling time	Lag period	Lag period
<u>M</u>	days	% of control	days	% of control
<u>0 g/l NaCl</u>				
0	2.6	100	3.9	100
10^{-8}	2.5	96	4.2	108
10^{-7}	2.6	100	3.9	100
10^{-6}	2.5	96	4.3	110
10^{-5}	2.5	96	4.3	110
10^{-4}	2.1	81	6.4	164
<u>10 g/l NaCl</u>				
0	4.7	100	19	100
10^{-8}	4.0	85	18	95
10^{-7}	4.3	91	15	79
10^{-6}	4.3	91	15	79
10^{-5}	3.6	77	15	79
10^{-4}	3.5	74	16	84

Table II. Growth kinetics of unadapted tobacco cells in medium with 0 and 10% PEG with various concentrations of ABA. Fresh weights shown were taken when the cells without ABA (control) reached the stationary phase of growth. This data represents the average of two experiments.

ABA	Fresh weight doubling time	Fresh weight doubling time	Lag period days	% of control	Fresh weight doubling time	Lag period days	% of control	Fresh weight mg/ml	Fresh weight % of control
<u>0 g/100 ml PEG</u>									
0	2.3	-	6	-	415	-	-	-	-
<u>10 g/100 ml PEG</u>									
0	4.2	100	13	100	365	100	100	100	100
10 ⁻⁸	4.9	119	18	137	235	137	63	63	63
10 ⁻⁷	5.4	130	20	154	213	154	53	53	53
10 ⁻⁶	6.0	140	16	122	215	122	59	59	59
10 ⁻⁵	5.6	133	19	144	218	144	62	62	62
10 ⁻⁴	10.7	240	29	221	32	221	9	9	9

Table III. Growth of unadapted tobacco cells in medium with no osmoticum, or with NaCl, KCl, Na₂SO₄, K₂SO₄, mannitol, sorbitol, sucrose or PEG in the presence or absence of 10⁻⁵ M ABA. Solute concentrations were chosen to have approximately the same osmolarities or the same cation concentrations as 10 g/l NaCl except for PEG.

Solute	Fresh weight $\pm$ SE(n)	Dry weight $\pm$ SE(n)	Days to Harvest	Fresh weight days to harvest
	mg/ml	mg/ml	days	(mg/ml)/day
Control	-ABA 363 $\pm$ 22(5)	12.0 $\pm$ 0.60	12	30.3
	+ABA 370 $\pm$ 28(4)	12.4 $\pm$ 0.56	12	30.8
				102%
85.5 mM	-ABA 202 $\pm$ 27(5)	10.9 $\pm$ 0.76	14	14.4
KCl	+ABA 349 $\pm$ 16(5)	14.0 $\pm$ 0.12	14	24.9
				174%***
342 mM	-ABA 284 $\pm$ 10(5)	19.2 $\pm$ 1.46	21	13.5
Sorbitol	+ABA 296 $\pm$ 17(5)	17.8 $\pm$ 0.48	21	14.0
				103%
342 mM	-ABA 246 $\pm$ 4(5)	17.8 $\pm$ 0.48	21	11.7
Mannitol	+ABA 246 $\pm$ 4(5)	14.7 $\pm$ 0.67	21	11.7
				100%
85.5 mM	-ABA 114 $\pm$ 6(5)	8.4 $\pm$ 0.46	28	4.1
Na ₂ SO ₄	+ABA 198 $\pm$ 7(5)	12.3 $\pm$ 0.34	28	7.1
				180%***
342 mM	-ABA 145 $\pm$ 7(4)	16.1 $\pm$ 0.85	28	5.2
Sucrose	+ABA 135 $\pm$ 14(5)	15.2 $\pm$ 1.95	28	4.8
				93%
171 mM	-ABA 104 $\pm$ 18(4)	8.7 $\pm$ 0.62	28	3.7
NaCl	+ABA 151 $\pm$ 7(5)	10.3 $\pm$ 0.43	28	5.4
				145%***
85.5 mM	-ABA 24 $\pm$ 6(5)	2.1 $\pm$ 0.51	28	0.9
K ₂ SO ₄	+ABA 104 $\pm$ 17(5)	8.5 $\pm$ 0.68	28	3.7
				433%***

171 mM	-ABA	8 $\pm$ 1.3(5)	2.1 $\pm$ 0.52	28	0.3
KCl	+ABA	37 $\pm$ 12(5)	8.5 $\pm$ 0.71	28	1.3
					460%***
10 g/100 ml	-ABA	7 $\pm$ 0.8(5)	0.88 $\pm$ 0.04	28	0.3
PEG	+ABA	8 $\pm$ 0.8(5)	0.73 $\pm$ 0.16	28	0.3
					110%

*** *
p < 0.0005 by two-tailed t-test

FIGURE LEGENDS

Fig. 1. Fresh weight of unadapted cells grown in medium with 10 g/L NaCl (—), or without NaCl (---) and containing 0(○), 10^{-8} (◇), 10^{-7} (■), 10^{-6} (△), 10^{-5} (●) and 10^{-4} (○) M ABA. Points represent the average of two 4-ml samples per flask from two 1-L Erlenmeyer flasks containing 250 ml of cell suspension.

Fig. 2. Fresh weight of tobacco cells transferred to medium containing no ABA (○), or 10^{-5} M ABA (●) and A, 0; B, 10; C, 14; and D, 22 g/L NaCl. Data points represent the average mass harvested from two 125-ml Erlenmeyer flasks.

Fig. 3. Effect of 10^{-5} M ABA on relative fresh weight of unadapted tobacco cells in medium with 10 g/L NaCl (○) or without NaCl (▲) and cells adapted to 10 g/L NaCl in medium with 10 g/L NaCl (○) or 16 g/L NaCl (△). Adapted cells were maintained in medium with 10 g/L NaCl for over 200 generations. Points represent the average of two 4-ml samples per flask from two 1-L Erlenmeyer flasks containing 250 ml of cell suspension.

Fig. 4. Fresh weight of unadapted tobacco cells transferred to medium containing A, 0; B, 5; C, 7.5; D, 10; E, 12.5; F, 15; G, 17.5; and H, 20 g/100 ml PEG either with 10^{-5} M ABA (●) or without ABA (○). Points represent the average of the mass harvested from three 125-ml Erlenmeyer flasks per treatment.

Figure 1

Figure 2

Figure 3

Figure 4

PART C Relation between ion accumulation of salt-sensitive and isolated stable salt-tolerant cell lines of *Citrus aurantium*

Introduction

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 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.

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 one year on medium containing 10g 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 days). 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 0.5mM CaSO_4 for 5 min as described by Tal et al. (16), and then dried at 85°C for 24 h. Samples of 50mg dry matter were wet ashed by H_2SO_4 . Na^+ and K^+ were determined by EEL flame photometer. Cl^- was determined according to Cotlove (5).

Results

Four stable NaCl-tolerant cell lines of Sour orange were isolated through a prolonged procedure of selection by exposing ovular callus to NaCl. The relative growth of these four 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. 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 selection pressure (15).

In order 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. 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^+ . The percent 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 (above 0.075M 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 0.025M , 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 0.05M in the case of the NaCl-sensitive cell line and above 0.075M in the case of the NaCl-tolerant cell lines is the results 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 better growth of all cell lines on medium containing Na_2SO_4 , compared with NaCl, was accompanied by a higher internal concentration of Na^+ and a slight increase in the uptake of K^+ . Secondly, partial replacement of Na_2SO_4 by K_2SO_4 resulted in an even greater increase of K^+ uptake, but with a significant inhibition of growth.

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).

Four NaCl-tolerant cell lines of Sour orange were isolated and tested for their ability to grow on increasing concentrations of NaCl. The data presented in Figure 1 indicate minor differences among the four cell lines, especially in the presence of high NaCl concentrations, and all of them perform much better than the nonselected cell line. 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 0.1M .

Ion content analysis of the NaCl-tolerant as well as the NaCl-sensitive cell lines gives a similar picture, namely, the selected cell lines resemble each other and all four of them differ from the nonselected one. 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 0.05M , 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 0.2M; 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.

Comparison of the ion content of the NaCl-sensitive cell line and one NaCl-tolerant line further indicates the lack of correlation between the extent of Na^+ or Cl^- uptake and growth (Fig. 3A). Both cell lines accumulated Na^+ and Cl^- , the NaCl-tolerant one to an even greater extent. However, growth and loss of internal K^+ seem to correlate rather well (Fig. 3B). 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, resemble 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 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.

Better insight into the relation between growth ability and internal concentrations of the major ions - Na^+ , K^+ and Cl^- - was sought by exposing Sour orange cells to various salts. From the information obtained it is evident again that internal Na^+ concentration is not a major factor in the mechanism of salt tolerance in this system. Figure 4 and Table I show that a higher level of internal Na^+ , caused by replacement of NaCl by Na_2SO_4 , even enhances growth.

It should be noted that the level of internal Cl^- concentration is also an unsatisfactory marker for salt tolerance. First, no correlation between growth and internal Cl^- concentration is found for the NaCl-tolerant and NaCl-sensitive cell lines (Fig. 3). Second, no such correlation is found when Cl^- is replaced by SO_4^{2-} , especially in the presence of K_2SO_4 (Table I). The reduced loss of K^+ , observed in the NaCl-tolerant cell lines (Figs. 2 and 3), suggests an important role for internal K^+ concentrations in the mechanism of salt tolerance for Sour orange cells. However, replacement of Na^+ salts markedly increased the internal levels of K^+ with a concomitant significant decrease of growth (Table I and Figs. 5, 6 and 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 analysed. 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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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 one month. Cells were washed and analyzed as described in "Material and Methods."

Cell line	Salt	Growth	Na ⁺	K ⁺	Cl ⁻
	M	mg	% of dry weight		
SO-A	-	408 $\pm$ 25	0.12	0.90	0.15
	NaCl, 0.05	247 $\pm$ 22	0.37	0.56	0.50
	Na ₂ SO ₄ , 0.025	357 $\pm$ 38	0.52	1.16	0.11
	NaCl, 0.1	152 $\pm$ 10	0.48	0.51	0.75
	Na ₂ SO ₄ , 0.05	309 $\pm$ 20	0.59	0.63	0.12
	Na ₂ SO ₄ , 0.025+	108 $\pm$ 11	0.46	1.38	0.13
	K ₂ SO ₄ , 0.025				
01-7-2	-	671 $\pm$ 35	0.14	1.58	0.17
	NaCl, 0.1	481 $\pm$ 28	0.56	1.40	0.96
	Na ₂ SO ₄ , 0.05	540 $\pm$ 17	0.80	1.51	0.15
	NaCl, 0.2	238 $\pm$ 16	0.60	0.47	0.59
	Na ₂ SO ₄ , 0.1	337 $\pm$ 50	0.74	0.78	0.17

	Na_2SO_4 , 0.05+	$50 \pm$	-	-	-
	K_2SO_4 , 0.05				
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+	130 ± 10	0.54	1.07	0.28
	K_2SO_4 , 0.05				

Legend to Figures

- Figure 1: Growth of NaCl-sensitive (SO-A) and NaCl-tolerant cell lines as a function of NaCl concentration. Growth was determined after one month; all other details are as described in "Materials and Methods." ●, 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.
- Figure 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.
- Figure 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.
- Figure 4: Effect of various salts on growth of two NaCl-tolerant cell lines. Cells were harvested after one month. ●, NaCl; ○, Na_2SO_4 ; ■, KCl; □, K_2SO_4 .
- Figure 5: Growth of the NaCl-sensitive (SO-A) and NaCl-tolerant (01-7-2) cell lines on Na_2SO_4 and $\text{Na}_2\text{SO}_4 + \text{K}_2\text{SO}_4$. Cells were harvested after one month. ● and ○, Na_2SO_4 ; ▲ and △, $\text{Na}_2\text{SO}_4 + \text{K}_2\text{SO}_4$ in equal molarity. Black symbols, SO-A; white symbols, 01-7-2.
- Figure 6: Growth of the NaCl-sensitive (SO-A) and two NaCl-tolerant cell lines as a function of KCl concentration. 0 KCl means 0.1M 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.
- Figure 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^- .

Fig 1

GROWTH, % OF CONTROL

Fig. 2

NaCl, M

B-5-1

01-7-2

Salt, equivalents

Fig. 6

PERCENT OF DRY WEIGHT

SO-A

01-7-4

KCl, M

PART D Relationship Between Salt Tolerance and Resistance to Polyethylene Glycol-Induced Water Stress in Cultured Citrus Cell

Introduction

Cultured somatic cells of higher plants have been used to select numerous variant cell lines (5,23,26). The selected variants exhibit a characteristic resistance, or tolerance, to the chosen desired trait achieved by prolonged exposure of the cells to a stress agent. Tolerance to certain environmental stresses like salt and drought has potentially large agricultural value. Cells exposed to either type of stress, e.g. NaCl for salt and polyethylene glycol (PEG) for water stress, yield variants possessing new properties which improve their performance under stress conditions. Salt-tolerant cells have been isolated from tobacco (8,19,20,25), alfalfa (7) and Citrus (2,3,16,24). Cells resistant to PEG-induced water stress have been isolated from tomato (4,11). While both salt and drought stress reduce the osmotic potential of the growth medium, the former has, in addition, the specific effect of the ions. Correlation between tolerance achieved to one stress and its expression when exposed to the other one, has been studied in one system only (4). Tomato cells, selected for resistance to water stress, have been exposed to increasing concentrations of NaCl. These cells showed better yield of growth in the presence of NaCl than the nonselected cells. In this study Citrus cells, isolated for better tolerance to NaCl, were examined for their resistance to PEG-induced water stress.

Materials and Methods

Plant material. Ovular callus of Shamouti orange (Citrus sinensis L. Osbeck) and Sour orange (C. aurantium) and the salt-tolerant cell lines were obtained as described previously (2,3). Callus cells were subcultured monthly.

Medium. The basal medium was that of Murashige and Tucker (18) without any growth factors. Callus of salt-tolerant cells was routinely kept on medium containing 0.2M NaCl. Polyethylene glycol 6000-8000 (Sigma P-2139) was used.

Cell growth. Cells were grown in either solid 1% agar medium for NaCl experiments, or in suspension for PEG experiments. Explants of about 50mg were plated on a petri dish and growth was determined during or toward the end of the log phase (28d). Explants of approximately 100mg were plated in 100ml Erlenmeyer flasks containing 25ml of liquid medium and growth was determined after 24d. Cells were collected and washed by vacuum filtration on Whatman no. 1 filter paper. Growth data are mean values $\pm$ standard error of 10 replications in each NaCl experiment and 3 replications in each PEG experiment. Each experiment was repeated at least 3 times. All samples of each treatment were pulled together and dried at 85°C for 24h.

Ion determination. Na^+ , K^+ , Ca^{2+} and Mg^{2+} were determined after wet ashing by HNO_3 and HClO_4 . Na^+ and K^+ were determined by an EEl flame photometer and Ca^{2+} and Mg^{2+} were determined by a Perkin Elmer 460 Atomic Absorption Spectrophotometer. Cl^- was determined according to Cotlove (6). Water-soluble NO_3^- was determined by the Technion autoanalyzer, after the dry material had been shaken in deionized water for 2h and filtered.

Protein determination. Total nitrogen was determined by the Technicon autoanalyzer after the dry material had been wet-ashed by H_2SO_4 .

Organic solutes. Free amino acids were determined by the ninhydrin method (21) using glycine standard. Soluble sugars were determined by the anthrone method (22) using glucose standard. All other details have been described previously (3).

Results

Cell lines selected for better growth on NaCl were checked for their extent of tolerance to PEG-induced water stress. These cell lines were isolated from two species of Citrus, Shamouti orange (C. sinensis) and Sour orange (C. aurantium). Figure 1 shows the data of the performance of the nonselected and three NaCl-selected cell lines of Shamouti orange grown on increasing concentrations of PEG and NaCl. All three NaCl-selected cell lines grew better than the nonselected cell line on any PEG concentration. However, it should be noted that the order of the degree of tolerance to NaCl among the selected cell lines was not retained. Thus, the best NaCl-tolerant cell line, R-10, did not exhibit the highest tolerance to PEG. The same experiment was carried out with the nonselected and three NaCl-selected cell lines of Sour orange. It is evident from Figure 2 that the nonselected cell line performed better than any of the NaCl-selected cell lines on all PEG concentrations tested. Moreover, as in the case of Shamouti orange, the order of the degree of NaCl tolerance among the NaCl-selected cell lines was not necessarily expressed in their resistance to PEG. Thus, cell line 01-7-4, which was more tolerant to NaCl than 01-7-2, was less tolerant to PEG.

A comparison was made of the relative tolerance of the various cell lines to both stresses, NaCl and water. The data presented in Table I show the relative growth of cells at the same osmotic potential of the growth medium. As shown in Table I, both nonselected cell lines of Shamouti orange and Sour orange were much more tolerant to PEG than to NaCl at equal osmotic potential of the medium. The difference observed in the case of the nonselected cell line of Sour orange was the largest. The NaCl-selected cell lines of both species showed a better correlation in their tolerance to the two types of stress, although to differing extents. The similarity in tolerance of the NaCl-selected Sour orange cell line 01-7-4 was the best, followed by the other NaCl-selected Sour orange cell line, B-5-1, and then by the two NaCl-selected Shamouti orange cell lines, R-10 and R-13.

The gain in dry weight as a function of PEG concentration, calculated as percent of gain in dry weight in the absence of PEG, is given in Figure 3. First, it is seen that better gain in fresh weight for cell lines of both species was also expressed as better gain in dry weight. However, the rate of decrease in dry weight as a function of PEG concentration was much lower than the rate of the decrease in fresh weight. This was due to a remarkable increase in percent of dry matter obtained in the presence of PEG. Thus, for example, the percent dry weight of R-10 cells increased from 11.5 to 23.5 when PEG concentration was raised from 0 to 20%. The values for the order cell lines, L-5, R-13, SO-A and 01-7-2, in the same experiment, were 20 to 28, 15 to 32, 14 to 24 and 10-22%, respectively. This increase occurred mainly in the range of 10 to 20% PEG and was rather low in the range of 0 to 10% PEG.

Preliminary analyses of inorganic and organic solutes were carried out in an attempt to identify the nature of the molecules that accumulate and cause the increase in dry weight. The data presented in Table II show the content of inorganic ions of two types of cells as a function of PEG concentrations. The concentration of none of the ions increased, and the concentration of the anions, Cl^- and NO_3^- , decreased markedly. This picture also holds for the other cell lines, with the addition that the Sour orange cells also lost K^+ when PEG concentration was increased. Thus, the content of K^+ in SO-A cells was reduced from 2.00 to 0.60% of dry weight and that of 01-7-2 cells from 2.52 to 1.17% of dry weight when PEG concentration was increased from 10 to 20%. No reduction in the content of K^+ was observed between 0 and 10% PEG.

The results of the analyses of free amino acids, protein expressed as total nitrogen, and soluble sugars are given in Table III. The protein content of all the cell lines checked in this experiment was slightly lowered with the increase in PEG concentration. The free amino acids content was slightly increased (L-5), remained rather constant (R-13), or decreased (R-10, SO-A and 01-7-2). Amino acid analysis was carried out on L-5 and R-10 cells grown in the presence of various PEG concentrations. It did not show the accumulation of proline as a response to the introduction of PEG into the growth medium. The overall pattern of amino acids was similar over the entire range of PEG concentrations tested, in both types of cells.

The response of soluble sugars to the addition of PEG varied among the different cell lines. While the content of the soluble sugars of the nonselected cell lines of Shamouti orange (L-5) and Sour orange (SO-A) remained rather constant, the amount of soluble sugar of the other cell lines increased. In the case of 01-7-2 cells it increased mainly between 10 and 20% PEG, in the case of R-13 cells it increased only between 0 and 10% PEG, and in the case of R-10 cells it increased over the range of 0 to 15% PEG and decreased at 20% PEG. It should be noted that for both species the increase in soluble sugars of the NaCl-selected cells did not exceed the level of the soluble sugars of the nonselected cells. Thus, the highest levels of R-10 and R-13 cells were about the level of soluble sugar of L-5 cells in the absence of PEG, and the same picture was true for 01-7-2 and SO-A cells. Research into other possible organic solutes that may play a role in the osmoregulation of the various types of cells grown in the presence of PEG is now underway.

Another aspect that reveals a difference between the effect of salt and water stresses is observed in the process of embryogenesis. It was reported previously (16) that NaCl induces a higher degree of embryogenesis and in some cases is even an absolute requirement for this process in NaCl-selected Shamouti orange cells. However, under the same conditions, PEG fails to induce, or even enhance, embryogenesis.

Discussion

Salt and water stresses have a common effect: both cause the lowering of the osmotic potential of the plant environment. While water stress is caused by using impermeable solutes, salt stress is caused by various ions, mainly Na^+ and Cl^- , which can be transported into and out of cells. Thus, salt stress has, in addition, the specific effect of the ions present in the environment. Similar features have been shown in plant response to both stresses (9,17).

The growth of most plants, except the halophytes, is inhibited to some extent by water or salt stress. Recently it has been shown that plants accumulate proline under both types of stresses (1,13).

Cultured plant cells have been introduced as a convenient tool for research related to the elucidation of the mechanisms operating on the cellular level by which plants survive under both types of stresses. Using this tool is justified since it has been shown that both stresses result in accumulation of proline (10,15,25). There is only one plant system where the relationship between the degree of resistance to water stress and that of tolerance to salt has been described (4). Tomato cells which have been selected for better growth on increasing PEG concentrations, also showed enhanced ability to grow in the presence of NaCl. In this study, the relationship between the two stresses has been investigated from the opposite direction, *i.e.*, cells which have been selected for better growth on increasing NaCl concentrations, have been tested for their ability to grow in the presence of PEG.

The data presented in Figures 1 and 2 and Table I show the correlation of growth capacity of nonselected and NaCl-selected cells which originated from two species of Citrus between increasing concentrations of NaCl and those of PEG. Two conclusions can be drawn (a) The discrepancy between relative growth in the presence of NaCl and PEG is largest in the case of nonselected cells. The Sour orange nonselected cells (SO-A) are more resistant to PEG than are the Shamouti orange cells (L-5), but the reason for this is not clear (b) During the prolonged selection for salt tolerance, cells have acquired tolerance to the specific ion effect. In the case of Sour orange this is the only advantage of the NaCl-selected cells, since no NaCl-selected cell line grows better than the nonselected cells in the presence of PEG. However, in the case of Shamouti orange, selection for NaCl tolerance has induced both better tolerance to specific ions as well as better adjustment to osmotic potential stress. Thus, all NaCl-selected cell lines exhibit better growth than the nonselected cells in the presence of PEG, although to a different extent. This variation may arise from the basic difference found in the mechanism for salt tolerance among the two species of Citrus. While Shamouti orange NaCl-selected R-10 cells reveal partial avoidance of NaCl (3), NaCl-selected cells of Sour orange accumulate NaCl as well as the nonselected cells (unpublished data). The mechanism of avoidance requires the ability of the cells to synthesize organic solutes as osmoregulators. No such organic solutes were identified as yet in the case of R-10 cells growing in the presence of salt (3). As will be discussed later, soluble sugars may play this role in cells growing in the presence of PEG.

An increase in percent dry weight has been observed in all cells, non-selected as well as NaCl-selected, of both Citrus species growing in the presence of PEG. This phenomenon is entirely different from previous results obtained with the same cells growing in the presence of NaCl. The percent dry weight of nonselected and NaCl-selected cells growing in the absence or presence of NaCl remained more or less constant (3). A similar observation was made by Heyser and Nabors (11): the percent dry weight of tobacco cells growing in the presence of NaCl was much lower than that of such cells growing in the presence of PEG.

An attempt was made to identify the nature of the molecule that contributes to the excess of dry matter gained in the presence of PEG. Analysis of

inorganic ions did not reveal an increase of any of the ions analyzed (Table II). On the contrary, a decrease of both anions, Cl^- and NO_3^- , and in some cases also in K^+ , has been observed. This picture is somewhat similar to that obtained for cells growing in the presence of NaCl. Except for the expected increase of internal Cl^- , NO_3^- and, in certain cell lines, also K^+ , decrease as a function of rising NaCl concentrations, in a manner similar to that described in Table II for the PEG experiment (data not shown). These results are in contrast to those described by Heyser and Nabors (12) for tobacco cells growing in the presence of NaCl or PEG, and by Handa *et al.* (10) for tomato cells growing in the presence of PEG. Both groups of researchers reported increase in the intracellular concentration of NO_3^- caused by either type of stress. The decrease in intracellular concentration of NO_3^- observed in plants growing under saline conditions is usually explained as being caused by the competition of Cl^- (14). However, this explanation does not hold in the case of PEG. More investigation into the mechanism of NO_3^- uptake and its utilization under PEG-induced water stress conditions would probably lead to an understanding of this phenomenon.

Neither proteins nor free amino acids accumulate in the presence of PEG (Table III). Although in one case a small increase in the content of free amino acids was obtained (L-5), this cannot account for the observed increase in percent dry weight. The increase in the intracellular concentration of soluble sugars as a function of PEG concentrations may account for at least a partial increase in percent dry weight of the NaCl-selected cell lines, R-10, R-13 and 01-7-2. However, the role of soluble sugars is only a partial explanation. First, the non-selected cells of both Shamouti orange and Sour orange do not accumulate excess soluble sugars as a response to the introduction of PEG into the medium. Second, the increase in soluble sugars in those cells which do accumulate them does not necessarily parallel the increase in percent dry weight. Thus, the main increase in percent dry weight of R-13 cells occurs between 10 and 20% PEG, while the increase in the content of soluble sugars occurs between 0 and 10% PEG. It seems that most probably the increase in percent dry weight stems from an increase in insoluble sugars, the main components of the cell wall. Electron microscope studies which are now underway may perhaps provide an indication of whether this assumption is correct.

In conclusion, nonselected and NaCl-selected cells of two species of *Citrus* were subjected to saline and drought stresses. A comparison of the performance of cells on the two types of stresses revealed basic differences between the nature of the salt tolerance adapted by Shamouti orange and Sour orange cells. While Shamouti orange NaCl-selected cells acquire salt tolerance by osmotic adjustment as well as a capacity to survive specific ion effects, the Sour orange NaCl-selected cells acquire only the latter. This difference probably accounts for the two types of mechanisms for salt tolerance existing in these cells, *e.g.* partial avoidance of salt in Shamouti orange cells and accumulation of salt in Sour orange cells.

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Table I. Comparison of growth of Shamouti cell lines (L-5, R-10 and R-13) and Sour orange cell lines (SO-A, 01-7-2 and B-5-1) on medium containing equal osmolarity obtained by either NaCl or PEG. Osmotic potentials of 2,4,6 and 8 bars of NaCl were obtained by addition of 0.048, 0.095, 0.145 and 0.195M, respectively. Osmotic potentials of 2,4,6 and 8 bars of PEG were obtained by the addition of 6,10,13.5 and 16.5% (w/v), respectively.

The data of PEG were taken from Bressan *et al.* (4). Growth data were taken from Figures 1 and 2.

Osmotic Potential*	RELATIVE GROWTH											
	L - 5		R - 10		R - 13		SO - A		01 - 7 - 4		B - 5 - 1	
- bar	NaCl	PEG	NaCl	PEG	NaCl	PEG	NaCl	PEG	NaCl	PEG	NaCl	PEG
0	(100)	(100)	(100)	(100)	(100)	(100)	(100)	(100)	(100)	(100)	(100)	(100)
2	74	84	95	95	91	103	51	99	97	87	102	97
4	43	70	87	87	88	102	28	96	79	75	93	93
6	13	55	67	78	62	87	12	85	61	55	68	77
8	2	39	36	63	25	72	3	71	42	36	48	58

* In addition to the components of the growth medium.

Table II. Effect of PEG on the content of inorganic ions of nonselected (L-5) and NaCl-selected (R-10) Shamouti orange cells. Cells of the experiment described in Figure 1 were analyzed as described in "Materials and Methods."

Cell line	PEG	Na ⁺	K ⁺	Ca ²⁺	Mg ²⁺	Cl ⁻	NO ₃ ⁻
	%(w/v)	% of dry weight					
L-5	0	0.07	2.36	0.21	0.10	0.08	0.02
L-5	10	0.07	2.18	0.22	0.11	0.02	N.D.*
L-5	15	0.07	2.02	0.17	0.10	0.01	N.D.
L-5	20	0.06	1.64	0.17	0.09	0.01	N.D.
R-10	0	0.06	3.18	0.26	0.09	0.25	0.84
R-10	10	0.06	3.02	0.27	0.11	0.26	0.22
R-10	15	0.07	2.10	0.19	0.09	0.11	0.07
R-10	20	0.05	2.80	0.21	0.12	0.08	0.04

* N.D. - not detectable.

Table III. Effect of PEG on the content of free amino acids, proteins and soluble sugars of nonselected (L-5 and SO-A) and NaCl-selected (R-10, R-13 and 01-7-2) Shamouti orange and Sour orange cells. Cells of the experiments described in Figures 1 and 2 were analyzed as described in "Materials and Methods."

Cell line	PEG %(w/v)	Protein % of dry weight	Free amino acids μmoles/1g fresh weight	Soluble sugars
L-5	0	5.6	16.4	238
L-5	10	5.5	16.0	200
L-5	15	3.5	27.2	300
R-10	0	4.1	56.0	13
R-10	10	5.6	20.8	52
R-10	15	2.7	33.6	188
R-10	20	3.3	10.0	59
R-13	0	4.0	12.8	65
R-13	10	2.7	19.6	328
R-13	20	3.0	11.2	289
SO-A	0	4.7	22.4	153
SO-A	10	4.7	16.0	98
SO-A	20	3.3	4.0	198
01-7-2	0	6.1	54.8	40
01-7-2	10	5.4	32.6	60
01-7-2	20	4.8	12.0	151

Legends to Figures

- Figure 1. Growth of Shamouti orange nonselected and NaCl-selected cell lines as a function of NaCl and PEG concentrations. Growth was determined after 28 d for NaCl and 24 d for PEG. All other details are as described under "Materials and Methods." ●, L-5 cell; ○, R-4 cells; △, R-10 cells; □, R-13 cells. 100% growth for the NaCl experiment for L-5, R-4, R-10 and R-13 was 515 ± 32 , 711 ± 57 , 690 ± 28 and 618 ± 41 mg, respectively. 100% growth for the PEG experiment for L-5, R-4, R-10 and R-13 was 1589 ± 71 , 1910 ± 68 , 2105 ± 105 and 2015 ± 84 mg, respectively.
- Figure 2. Growth of Sour orange nonselected and NaCl-selected cell lines as a function of NaCl and PEG concentrations. Growth was determined after 28 d for NaCl and 24 d for PEG. All other details are as described under "Materials and Methods." ●, SO-A cells, ○, 01-7-2; △, 01-7-4; □, B-5-1. 100% growth for the NaCl experiment for SO-A, 01-7-2, 01-7-4 and B-5-1 was 369 ± 31 , 530 ± 32 , 437 ± 15 and 398 ± 19 mg, respectively. 100% growth for the PEG experiment for SO-A, 01-7-2, 01-7-4 and B-5-1 were 1886 ± 42 , 2147 ± 65 , 1191 ± 65 and 2108 ± 107 mg, respectively.
- Figure 3. Gain in dry weight of nonselected and NaCl-selected Shamouti orange and Sour orange cell lines as a function of PEG concentrations. Cells were harvested as described in Figures 1 and 2. The figures for 100% gain in dry weight are the values obtained in the absence of PEG. The values for L-5, R-4, R-10, R-13, SO-A, 01-7-2, 01-7-4 and B-5-1 were 356, 358, 217, 309, 270, 211, 191 and 215 mg, respectively. A. Shamouti orange cell lines, B. Sour orange cell lines. Symbols are those used in Figures 1 and 2.

FRESH WEIGHT, % OF CONTROL

PEG, % (w/v)

NaCl, M

Fig. 2

PEG, % (w/v)

NaCl, M

DRY WEIGHT, % OF CONTROL

F. Cooperation

The experiments were designed and planned in such a way to exploit the expertise and experience of both groups. The U.S. group has concentrated on the isolation of resistant cell lines and has characterized the cells, while the Israeli group has concentrated on regeneration procedures and the subsequent evaluation of regenerated plants. However, we have exchanged results frequently and interacted by suggesting modifications of each others experiments in order to achieve the best results. We feel that there has been important positive interaction by the complementation of the great expertise of Dr. Spiegel-Roy and his colleagues in the area of plant regeneration and our success in isolating and partially characterizing resistant cell lines. With advice from Dr. Spiegel-Roy and his colleagues, we have regenerated plants from our resistant cell lines and have provided plants to him in one of our exchange visits. Because, of the discontinuance of this project by BARD it is highly unlikely that we will be able to cooperate further in the evaluation of regenerated plants. However, we will make what attempts that we can toward this end.

G. Benefit to Agriculture

The research conducted during this project has contributed significantly to our present understanding of the potential use of somatic cell selection technique for the improvement of crop plants. Great interest in the use of these methods for crop improvement has developed in both the U.S. and Israel in recent years. The characteristics which we have studied using in vitro systems (water stress and salt stress resistance) have important agricultural and economic value to the U.S. and Israel. A large portion of the Israeli Agriculture is faced with problems related to these stresses. Vast areas of the Western U.S. are agriculturally unproductive because of arid and saline conditions. The development of cell lines and plants enhanced in their resistance to these stresses will be of great practical as well as scientific value. The careful studies of the physiological and metabolic changes associated with the increased resistance which we have conducted have furthered our understanding in general of the stress reactions of plant cells, and could thus be of great value to conventional breeding and selection techniques.

H. List of Publications

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