Growth characteristics of NaCl-selected and nonselected cells of *Nicotiana tabacum* L. 1

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A cell line of *Nicotiana tabacum* L. var. Wisconsin 38 was selected (S-10 cells) which is capable of growth in medium containing 10 g liter⁻¹ NaCl. The fresh and dry weights of S-10 cells at stationary phase in medium containing 10 g liter⁻¹ NaCl were about 60 and 100% respectively, of that of S-10 cells grown without NaCl. When cells normally maintained in the absence of the salt (S-0 cells) were transferred to medium containing 10 g liter⁻¹ NaCl, they underwent a 14-day lag period before growth could be detected and they reached stationary phase 36 days after inoculation compared to 14 days for S-10 cells. At stationary phase, fresh and dry weights of S-0 and S-10 cells were the same in the presence of salt. The S-0 cells exhibited a reduced growth rate once growth began in medium with 10 g liter⁻¹ NaCl. The cell mass doubling time of S-0 cells in medium with 10 g liter⁻¹ NaCl was 4 days compared to 1.2 days for these cells grown in the absence of the salt and 1.6 days for S-10 cells grown in medium with 10 g liter⁻¹ NaCl. The resistance of the salt-selected cells was stable in the presence of the salt. However, after 5 cell mass doublings following transfer into medium without NaCl, these cells lost their resistance to salt and responded to NaCl like the cell population (S-0 cells) which had not been selected for growth on NaCl.

**Key words:** Cell-selection — NaCl — *Nicotiana tabacum*.

The literature regarding the use of cell culture techniques to obtain variant cell lines and in some instances plants which have agriculturally useful traits, such as disease resistance, herbicide tolerance, amino acid overproduction, salt tolerance and cold tolerance, has been reviewed recently (see 12). Since then, other investigators using in vitro culture techniques have selected plants which were reported to be herbicide resistant (2) and disease resistant (1, 7, 11). These reports illustrate the potential use of cell culture techniques to improve crops by incorporating certain agriculturally useful traits into plants which heretofore have not exhibited them.

Significant among such improvements would be increasing the ability of non-halophytes to grow under saline conditions. Nabors et al. (9), after treatment with a mutagen, obtained cells of *Nicotiana tabacum* var. ‘Samsun’ which had an increased growth rate in medium with 1.6 g liter⁻¹ NaCl. Subsequently, plants were regenerated from cells resistant to 6.4 g liter⁻¹ NaCl which exhibited a tolerance to NaCl which has been transmitted to two additional generations (10). Cells of *N. sylvestris* (4, 13) and *Capsicum annum* (4) have been selected which were capable of

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growth in 10 g liter⁻¹ NaCl. Dix and Street (4) were able to establish cell lines which were capable of growth in medium containing 20 g liter⁻¹ NaCl by using inocula from cultures on their 3rd or 4th passage in 10 g liter⁻¹ NaCl. The response of alfalfa callus selected for growth in 10 g liter⁻¹ NaCl (3), was similar to halophytic plants, i.e. optimum growth of the callus was obtained only in the presence of NaCl. Ion analyses indicated that the salt-selected alfalfa line accumulated more K⁺, Cl⁻ and NO₃⁻ than the nonselected line at varying NaCl concentrations.

In this paper we describe our attempts to obtain a cell line of tobacco capable of growth in high levels of NaCl (10 g liter⁻¹). Data regarding the relative resistance of salt-selected cells and the stability of the resistance in the presence and absence of the stress are presented.

Materials and methods

Initiation and maintenance of callus and cell cultures

Callus of Nicotiana tabacum L. var. Wisconsin 38 was initiated from stem segments as described by Hasegawa et al. (6). Under these conditions, the callus grew as a compact mass of cells and after several recultures was transferred to medium to induce friability, which contained Murashige and Skoo (MS) salts (1962) and the following, in mg liter⁻¹: thiamine·HCl, 0.4; l-inositol, 100; 2,4-dichlorophenoxyacetic acid (2,4-D), 3.0; N⁶-furfurylaminopurine (kinetin), 0.1; casein hydrolysate (enzymatic digest), 1000; sucrose, 30,000; and Bacto agar, 10,000. The pH was adjusted to 5.7 ± 0.1 prior to autoclave sterilization for 15 min at 121°C.

After 4 weeks, approximately 1 g portions of friable callus were transferred to 25 ml of the same medium (minus agar, pH 5.0 ± 0.1) in 125 ml Erlenmeyer flasks to initiate cell suspension cultures. Cell suspension stocks were routinely maintained in 1000 ml Erlenmeyer flasks containing 200 ml of medium. Stock cultures were inoculated at a cell density (fresh weight) of about 8 g liter⁻¹ of medium and recultured every 12 to 15 days. Callus and cell cultures were maintained at 26°C under 16 hr daily illumination of 1500 lux (Cool White fluorescent lamps). All cell cultures were grown on either a gyratory or a reciprocating shaker (80 to 100 cycles per min).

Cell suspension culture inoculum

Experiments were performed using cells taken at the late linear phase of growth for inoculum. Cells were collected on a fritted glass funnel and unless otherwise described resuspended in basal medium to a density of about 0.2 g cells ml⁻¹. In all experiments, 0.2 g of cells were inoculated into 25 ml of medium in 125 ml Erlenmeyer flasks. Sodium chloride was added directly to the basal medium to obtain media with the desired salt concentrations.

Growth assay of cells

The fresh and dry weights of the cells were determined by harvesting them at the indicated times after inoculation. The cells were collected on a Whatman No. 4 paper filter in a Buchner funnel with an aspirator. The cells were removed from the filter paper and the fresh weight was then determined. Dry weight was measured after drying the cells in an oven (80°C) for a minimum of 24 hr.
Results

Resistance of tobacco cells at different NaCl concentrations

A comparison of growth after 14 days at varying NaCl concentrations, between cells continually maintained in the absence (S-0 cells) or in the presence of 10 g liter⁻¹ NaCl (S-10 cells) for about 50 cell doublings is shown in Fig. 1a and 1b.

![Graph showing growth of NaCl-selected Nicotiana cells](image)

**Fig. 1.** Relative growth after 14 days of tobacco cells inoculated into medium containing varying concentrations of NaCl; S-0 cells (○-○), S-10 cells (●-●) and S-10 cells after 5 cell mass doublings in medium without NaCl (△-△). A. Fresh weight of cells grown in medium with salt as a percent of fresh weight without salt. Final fresh weights in medium without NaCl attained by S-0 cells, 10.78 g; S-10 cells, 10.03 g; and S-10 cells after 5 cell mass doublings in medium without NaCl, 10.48 g. B. Dry weight of cells grown in medium with salt as a percent of dry weight without salt. Final dry weights in medium without NaCl attained by S-0 cells, 0.27 g; S-10 cells, 0.30 and by S-10 cells after 5 cell mass doublings in medium without NaCl, 0.32 g.
The fresh weights of S-0 cells at 0 and 2 g liter⁻¹ NaCl were virtually the same (Fig. 1a) but at 4 g liter⁻¹ NaCl, fresh weight decreased to about 30% of S-0 cells in salt free medium. Virtually no detectable fresh weight gain was observed at NaCl concentrations of 8 g liter⁻¹ or greater. The dry weight gain of S-0 cells grown in medium with varying concentrations of NaCl followed the same trends (Fig. 1b).

The fresh weights of S-10 cells at 0 and 2 g liter⁻¹ NaCl were the same; however, unlike S-0 cells, the fresh weight gain of S-10 cells was only slightly inhibited at 4 g liter⁻¹ NaCl (Fig. 1a). S-10 cells grown in medium containing 10 and 12 g liter⁻¹ NaCl attained about 60 and 50%, respectively, of the fresh weight of S-10 cells grown

![Figure 2](image-url)
without NaCl. Fresh weights of S-10 cells at 10 and 12 g liter\(^{-1}\) NaCl was more than 100-fold greater than S-0 cells at the same NaCl concentrations (Fig. 2a). At concentrations of 16 g liter\(^{-1}\) and above, no fresh weight increase was observed (Fig. 1b). Concentrations of NaCl up to 10 g liter\(^{-1}\) had no effect on the dry weight accumulation of S-10 cells (Fig. 1b). Dry weight accumulation at 10 g liter\(^{-1}\) NaCl of S-10 cells was about 30 times greater than that of S-0 cells (Fig. 2b). Complete dry weight suppression of S-10 cells did not occur until the NaCl concentration had reached 25 g liter\(^{-1}\).

**Stability of salt resistance**

The growth of S-10 cells at different NaCl concentrations was determined after 5, 20, 30 and 60 cell doublings in medium containing 10 g liter\(^{-1}\) NaCl and the cells appeared to respond in a similar manner regardless of the length of time in 10 g liter\(^{-1}\) NaCl (data not shown). After 40 cell doublings in 10 g liter\(^{-1}\) NaCl, S-10 cells were transferred to medium without NaCl for 5 cell doublings and their resistance to different NaCl concentrations was subsequently determined. The ability of these cells to gain fresh and dry weights in the presence of varying concentrations of NaCl was nearly identical to that of the S-0 cells (Fig. 1a, b and 2a, b).

**Growth of tobacco cells with and without 10 g liter\(^{-1}\) NaCl**

Growth over time of S-0 and S-10 cells in medium with and without 10 g liter\(^{-1}\) NaCl is shown in Fig. 3a and 3b. The patterns of fresh weight increase during the culture period for S-0 and S-10 cells in medium lacking NaCl were virtually identical (Fig. 3a). The cells underwent a 3 to 6 day lag period and then rapidly increased their fresh weight (cell mass doubling time of about 1.2 days) between the 6th and the 12th day at which point stationary phase was reached at a final weight of about 10 g per culture flask.

S-10 cells in medium with 10 g liter\(^{-1}\) NaCl had a similar initial 6-day lag period as S-0 and S-10 cells in medium without NaCl. They then gained fresh weight rapidly between the 6th day and the 14th day reaching a final fresh weight of about 6 g per flask, i.e. about 60% of fresh weight of cells grown without NaCl (Fig. 3a). Fresh weight gain of these cells was somewhat slower than the cells grown without NaCl. Their cell mass doubling time was about 1.6 days.

The fresh weight growth pattern of S-0 cells in medium with 10 g liter\(^{-1}\) NaCl was different than the patterns described above (Fig. 3a). There was an initial lag of about 14 days followed by a period of fresh weight increase from day 14 to 36 at which point stationary phase was reached. Although the final fresh weight of these cells (about 6 g per flask) was about the same as S-10 cells in NaCl-containing medium, the rate of fresh weight accumulation was considerably slower than for S-10 cells in medium with NaCl. During the period of rapid growth in salt medium, S-0 cells doubled their fresh weight every 4 days compared to 1.6 days for S-10 cells.

No initial lag period in dry weight gain was observed for S-0 and S-10 cells in medium without NaCl or for S-10 cells in 10 g liter\(^{-1}\) NaCl (Fig. 3b). The pattern of dry weight increase for these cells was very similar with a maximum of about 0.25 to 0.30 g per flask after 12 to 14 days. The S-0 cells grown in medium with 10 g liter\(^{-1}\) NaCl exhibited a lag period of 14 days followed by an exponential increase in dry weight to a maximum of about 0.28 g per flask after 36 days. Although the
total dry weight accumulation of S-0 and S-10 cells in medium with and without NaCl was the same, the rate of dry weight gain of S-0 cells in NaCl-containing medium was considerably slower. During exponential growth, the dry weight of these cells doubled every 4 days compared to 2 days for S-0 and S-10 cells in the absence of NaCl or S-10 cells in the presence of 10 g liter⁻¹ NaCl.
The final dry weight to fresh weight ratios appeared to be a function of the culture medium regardless of the initial cell type that was cultured. S-0 and S-10 cells cultured in medium without NaCl had a final dry weight to fresh weight ratio (after 12 days) of about 0.03 and was equivalent to that of the S-0 cells at the time of inoculation. The dry weight to fresh weight ratios of S-0 and S-10 cells grown in medium with 10 g liter⁻¹ NaCl were about 0.05 which was essentially the same as that for S-10 cells at the time of inoculation. Therefore the S-0 cells eventually attain (after 36 days in the presence of salt) a final dry weight to fresh weight ratio which is typical of S-10 cells.

Discussion

*Nicotiana tabacum* L. var. Wisconsin 38 cells maintained in a medium containing 10 g liter⁻¹ NaCl for 50 cell mass doublings (S-10 cells) were considerably more resistant to NaCl than cells grown without NaCl (S-0 cells). The fresh weight gain of S-10 cells on 10 g liter⁻¹ NaCl during one culture passage was about 60% of that obtained in the absence of the salt, while the final dry weights were virtually identical. Evaluation of the S-10 cells for resistance to NaCl after increasing numbers of generations (5-60 cell mass doublings) in the presence of salt provided evidence that the resistance character was stable in the presence of the salt. However, after one passage or 5 cell doublings in the absence of the salt, the S-10 cells exhibited the same resistance to salt as the S-0 cell population (Fig. 1a and 1b). Thus, in the absence of the salt, resistance was not retained and the cells quickly reverted back to the sensitivity of the original population, indicating that the resistance is unstable in the absence of the salt.

The enhanced ability to grow in the presence of NaCl that is exhibited by S-10 cells (Fig. 1a and 1b) is characteristic of cells obtained after one growth cycle (5 cell mass doublings) of S-0 cells in medium containing 10 g liter⁻¹ NaCl (data not shown). It is therefore clear that the mechanism by which the variant cell population is obtained occurs sometime during this initial growth cycle. Whether this cell population arises through the selection of variant cell types or by a cellular adaptation phenomenon or by a combination of both processes is unknown. It should be pointed out that the growth rate difference between S-0 cells and S-10 cells growing in the presence of 10 g liter⁻¹ NaCl is highly suggestive of an adaptive process. If the selection of a particular resistant cell type during the first growth cycle had occurred, the growth rates of S-0 cells after the 14 day lag period and of S-10 cells in medium containing 10 g liter⁻¹ NaCl should have been similar. In addition, it is unlikely that selection of variant cells could occur in only 5 generations unless the variants are already present at a high frequency. In fact, if it is assumed that the observed growth pattern of S-0 cells in 10 g liter⁻¹ NaCl (Fig. 3a) is the result of the growth of resistant variants within the S-0 population, such variants would have to occur at a frequency of about 9%. This is assuming a lag period of 0 days. An increasing lag period necessitates even higher frequencies of variants. However, the occurrence of adaptable cells at high frequencies in the S-0 cell population, and the selection of these cells by the presence of NaCl, could explain the observed growth pattern changes.

Regardless of the nature of the genetic mechanism by which the variant cell
population arises, it is very likely that during the first growth cycle in NaCl the cells are osmotically adjusting to the salt environment. This is suggested by the fact that the dry weight to fresh weight ratio of S-0 cells changes to that of S-10 cells after one growth cycle in medium containing 10 g liter⁻¹ NaCl. It is likely that this increase in density under saline conditions is due in part to an increased accumulation of ions and/or organic solutes in the cells similar to that observed with halophytes grown under saline conditions (5). However, as it is clear (from microscopic examination) that S-10 cells are somewhat smaller than S-0 cells, the involvement of a reduction of cell volume in the osmoregulatory process cannot be precluded.

Our observation that S-10 cells grown in medium with 10 g liter⁻¹ NaCl attain the same dry weight as S-0 cells in the absence of salt is contrary to the results of Croughan et al. (3) and Zenk (13) for alfalfa and N. sylvestris, respectively. In the latter two reports the final dry weight gain of the selected cell population in 10 g liter⁻¹ NaCl medium was less than that of the nonselected cell population in medium without salt even though the selected cells had been continually maintained in medium with 10 g liter⁻¹ NaCl. Stability of resistance in the presence of the salt was also observed by Croughan et al. (3) with alfalfa callus. Dix and Street (4) have reported that cells of C. annuum and N. sylvestris progressively improved their growth in 10 g liter⁻¹ NaCl in the first 10 passages of culture. Contrary to our results, Dix and Street (4) reported that N. sylvestris cells which were maintained for 3 growth cycles in medium with 10 g liter⁻¹ NaCl and then transferred to medium without NaCl for 3 growth cycles, were able to attain the same level of maximum growth in 1% NaCl as cells which had been maintained previously for 6 growth cycles in the presence of the salt. Their results may indicate that stability of salt resistance in cell populations is not always lost in the absence of the selection pressure.

The agricultural potential of using cell selection techniques to obtain plants capable of withstanding high saline environments has been alluded to by other researchers (3, 9). However, despite this very significant potential, emphasis should be placed also on the potential usefulness of cell selection techniques to develop systems for studying fundamental cellular phenomena such as osmoregulation and the effects of stress on nutrient utilization. Not only is it possible to compare divergent populations which exhibit different degrees of tolerance to salt but it is also possible to examine the biochemical changes which occur as the cells are subjected to the selection pressure.

References

Growth of NaCl-selected *Nicotiana* cells  


