# Proline Accumulation in NaCl-resistant and Sensitive Cell Lines of *Nicotiana sylvestris*

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## Summary

Cell lines of *Nicotiana sylvestris*, both sensitive and resistant to growth inhibition by NaCl, rapidly accumulated free proline on transfer from NaCl-free medium to medium containing 1.5 % (w/v) NaCl. Proline accumulated more rapidly in the sensitive cell line and the levels detected did not seem sufficient for it to act as an effective cytoplasmic osmoticum. None of the other amino acids detected exhibited comparable changes and the total of free amino acids other than proline was similar, in both lines, in the presence or absence of NaCl.

Proline accumulation is considered either to be symptomatic of salt-induced stress, or to have a protective role other than as an osmotic regulator.

*Key words: Nicotiana sylvestris, salt resistance, proline, cell lines.*

## Introduction

Interactions of plants with environmental salt are complex, involving uptake and translocation of the ions, and their differential localisation in various organs, tissues, cells and subcellular compartments. A wealth of information relating to these processes, in both halophytes and non-halophytes, has been recently reviewed in some depth (Flowers et al., 1977, Greenway and Munns, 1980) and it is clear that there are a number of strategies whereby plants may resist the deleterious effects of high soil salinity. High cytoplasmic levels of organic solutes, such as proline (Cavalieri and Huang, 1979) and glycinebetaine (Storey and Wyn Jones, 1979) accumulate in salt stressed plants, possibly either as a buffer against osmotic imbalances caused by high vacuolar ion concentrations (Field, 1976) or as a protective agent for cytoplasmic enzymes (Pollard and Wyn Jones, 1979).

The availability of cell lines of *Nicotiana sylvestris* with enhanced resistance to growth inhibition by sodium chloride (Dix and Street, 1975) suggested the possibility of investigating the basis of salt resistance at the cellular level and against the relatively uniform genetic background of different cell lines within

a single species. X-ray microanalysis and atom absorption data have indicated that $Na^+$ and $Cl^-$ ions are taken up by both sensitive and resistant cells (Dix and Toth, in preparation) and the present paper reports our observations of proline accumulation by these lines. We were also able to determine if any other free amino acids, which might afford protection (Heber and Santarius, 1973), were accumulated.

## Materials and Methods

*Cell lines and culture conditions*

Procedures for the routine initiation and maintenance of tissue and cell cultures of *N. sylvestris* have been described in detail (Dix et al., 1977). In the present work, liquid RMP medium (Maliga et al., 1977), with or without 1.5 % (w/v) NaCl, was used.

The salt-resistant cell line NCR120 was isolated in 1977, from a culture derived from a haploid plant (SH13), in the manner described previously (Dix and Street, 1975). In terms of morphology and growth in NaCl-containing medium it closely resembles the lines described in that paper.

The salt-sensitive cell line, NS, was an established culture from a normal *N. sylvestris* seedling. Microdensitometry and chromosome counts (data to be presented elsewhere) indicated that both cell lines were predominantly diploid at the time of these experiments.

*Amino acid extraction and analysis*

Samples of cell cultures were collected by filtration on to four layers of muslin, ground in a small volume of methanol (to give 80 % methanol with the tissue water), and extracted twice in 10× volume of 80 % methanol, first for 60 min at 70 °C, and second overnight at 0 °C. The two extracts were combined. Further extraction gave no appreciable increase in yield of amino acids.

Amounts of individual amino acids were measured in the extracts using a Technicon TSM automatic amino acid analyser. The standard deviation was 5 % of the individual measurements. Total free amino acid content was obtained by summing the amounts of individual amino acids.

Pure samples of amino acids were used as standards and nor-leucine was used as internal standard. Identifications were checked by thin layer chromatography (on silica gel G, developed in two directions with ethanol/$H_2O$, 97/3 by volume – first direction – and chloroform/methanol/17 % $NH_3$, 2/2/1 by volume – second direction – and detecting the amino acids with ninhydrin). Proline was also identified by the relatively much higher absorbance at 440 nm than 570 nm, of the product of its reaction with ninhydrin. An unusual fraction obtained with the amino acid analyser was tentatively identified as δ-N-acetyl ornithine (by comparison, using the amino acid analyser and TLC, with a pure sample kindly provided by Professor L. Fowden).

## Results and Discussion

Samples (0.5 g fresh weight) of NCR120 and NS cultures, grown in the absence of NaCl, were inoculated into 250 ml Erlenmeyer flasks containing 50 ml RMP medium, with or without 1.5 % NaCl, and harvested over a 10 day period.

Fresh weights of both lines in the absence of NaCl showed little or no lag phase and increased dramatically over the 10 day period (Table 1). NCR120 in the presence of NaCl showed a longer lag phase and increased in fresh weight

Table 1: Fresh weights and total free amino acids of NaCl-resistant (NCR120) and sensitive (NS) cell lines of *N. sylvestris* incubated in 50 ml cultures with (+NaCl) or without (–NaCl) the addition of 1.5 % (w/v) NaCl.

| Day | Fresh wt. (g in 50 ml culture) | | | | Total free amino acids (μmol $g^{-1}$) | | | |
|---|---|---|---|---|---|---|---|---|
| | NCR120 | | NS | | NCR120 | | NS | |
| | –NaCl | +NaCl | –NaCl | +NaCl | –NaCl | +NaCl | –NaCl | +NaCl |
| 0 | 0.62 | 0.55 | 0.52 | 0.55 | 4.6 | 4.6 | 5.5 | 5.5 |
| 1 | 0.88 | 0.52 | 1.09 | 0.62 | 8.9 | 13.3 | 9.4 | 15.5 |
| 3 | 4.57 | 1.05 | 3.78 | 0.60 | 3.4 | 9.4 | 6.2 | 11.9 |
| 10 | 15.90 | 4.48 | 17.32 | 0.52 | 5.3 | 16.4 | 9.4 | 17.6 |

more slowly (Table 1). These lower fresh weight values would not be reflected in cell numbers, which are comparable to those in the absence of NaCl (Dix and Street, 1975), and both fresh weight and cell number continue to increase until 20–25 days after subculture.

The total free amino acids other than proline showed a sharp peak at day 1 in all cases, before returning to levels similar to those on inoculation (Fig. 1 a). This peak probably results from a burst of metabolic activity associated with the transfer of cells to fresh medium (e.g. Nash and Davies, 1972) and it is of note that it even occurred on transfer of NS to NaCl-containing medium in which it did not subsequently grow.

Proline was present at low levels in both lines in the absence of NaCl (constituting 1–18 mole % of the total free amino acids present). In the presence of NaCl, however, both lines accumulated proline (Fig. 1 b). Accumulation commenced rapidly on inoculation (especially in NS) and proline levels continued to rise throughout the course of the experiment (Fig. 1 b). The highest proline level recorded in this experiment was 11.8 μmol $g^{-1}$, in NS, representing a greater than 20-fold increase compared with the proline content of NS grown without NaCl, but this only led to an approximate doubling of the total free amino acids compared with cells grown without NaCl (Table 1). Levels of up to 25 μmol $g^{-1}$ proline were found after prolonged (21 days) culture of NCR120 in medium containing 1.5 % NaCl (compared with 35 μmol $g^{-1}$ for the total of all other free amino acids).

In cells grown with or without NaCl, the other major free amino acids were α-alanine, histidine, and a fraction thought to be δ-N-acetyl ornithine. In cells grown without NaCl these accounted, respectively, for 10–35, 5–31, and 11–52 mole % of the total free amino acids present on different sample days, and twelve other amino acids collectively accounted for the remainder. The contents of these was, on average, higher in cells grown with NaCl by only 11–27 %. The largest single increase with NaCl in any of the three major amino acids on any one

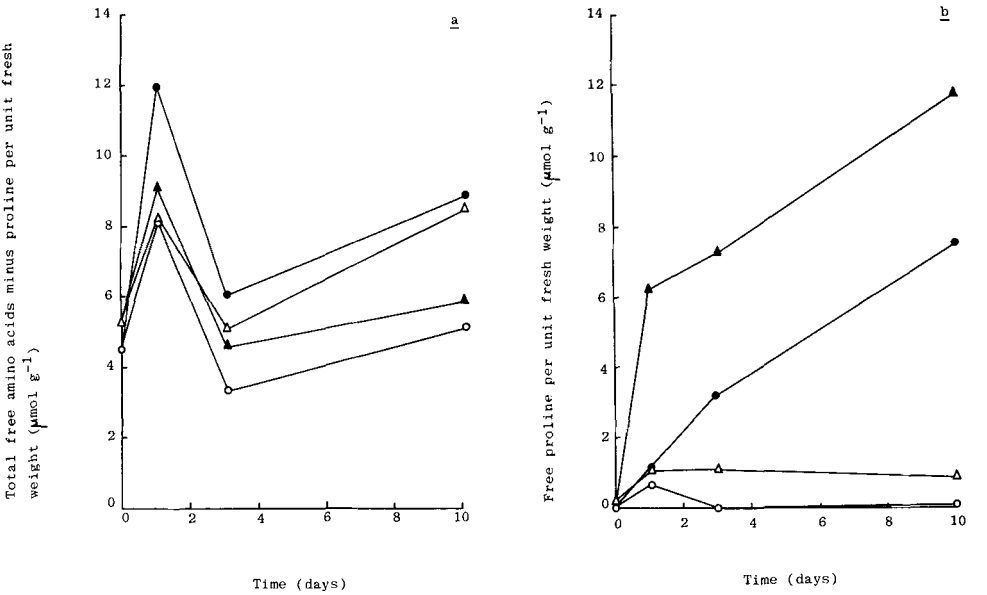


Fig. 1: Total free amino acid minus proline (a) and free proline (b) levels in NaCl-resistant (NCR120), circles, and sensitive (NS), triangles, cell lines of *N. sylvestris* incubated in the presence, solid symbols, or absence, open symbols, of 1.5 % (w/v) NaCl.

sample day was only 3-fold. The work of Heber and Santarius (1973) with a model system designed to investigate freezing effects and interactions with NaCl, suggests that not only proline but also $\gamma$-amino-butyric acid, glycine, serine, $\alpha$-alanine, sodium aspartate and sodium glutamate could give some protection against high NaCl. All these were present as free amino acids in the cultured *N. sylvestris* cells but in the presence of NaCl only proline accumulated substantially.

1.5 % NaCl corresponds to an external NaCl concentration of 256 mM and atom absorption data (Dix and Toth, in preparation) suggest comparable, or higher, intracellular levels of $Na^+$. Although the distribution of $Na^+$ and $Cl^-$ within the cells is not yet known, we find it difficult to envisage proline, particularly at the levels detected early in the growth cycle, having a significant role as an osmotic stabiliser against salt or water stress, even if confined to the cytoplasm. To suppose that proline osmotically stabilised only one type of cell organelle would leave unexplained the mechanism for survival of the remainder of the cytoplasm, and Boggess et al. (1976) concluded that proline probably was not accumulated exclusively in specific cellular organelles.

Our observation that proline accumulation is not closely related to resistance to water stress or salinity supports others obtained with whole plant systems (Hanson et al., 1979; Tymms and Gaff, 1979), and a higher rate of proline ac-

cumulation in sensitive compared to resistant species has also been reported in *Lycopersicon* (Tal et al., 1979). Proline accumulation occurs in response to other stresses, such as chilling (Yelenosky, 1979), and might simply be a symptom of severe stress (Hanson et al., 1979). Accumulation, at a lower rate, in the resistant cell line (Fig. 1 b) supports, rather than contradicts, this view since the longer lag phase and slower fresh weight increase (Table 1), together with the changed morphology of salt resistant lines in NaCl-containing medium (Dix and Street, 1975) clearly show that salt does exert a physiological stress on them. On the other hand, we favour the view (Greenway and Munns, 1980) that proline may have an ill-defined role related to survival under stress conditions. While not dividing, sensitive cells of *N. sylvestris* can retain their viability, as assessed by fluorescein diacetate staining (Widholm, 1977), for at least 10 days in medium containing 2 % NaCl (Dix, unpublished).

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