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Salinity Stress**

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Mechanisms for Control of Leaf Growth during Salinity Stress

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Mechanisms for Control of Leaf Growth during Salinity Stress

Abstract

In the project "Mechanisms for Control of Leaf Growth during Salinity Stress" ionic and enzymatic changes in the cells and cell walls of the expanding region of salt-stressed maize leaves were evaluated. Conventional numerical techniques for REG estimation were reevaluated; 'Greens' method was recommended and applied throughout the project for growth intensity estimation. Salinity slowed leaf development and reduced leaf size, but increased cell development within the leaf-growing zone. Leaf elongation rate was most affected by salinity from the region of maximal growth to the distal end; the basal region was largely unaffected. Creep assays indicated that the physical properties of the cell wall were not altered. Furthermore, pH or protein concentrations in the apoplastic space were not altered. Salinity decreased in half the concentrations of putative oligosaccharides in both the apoplast and the Golgi vesicles, suggesting that salinity reduced oligosaccharide biosynthesis. Salinity significantly increased solute concentrations in the vacuoles, but the ion concentrations tested remain unchanged in the vacuole. Most importantly, salinity increased the ion concentrations in the apoplast, particularly Cl^- concentrations. The evidence obtained clearly points to the biochemical and ionic components of the apoplast as potential factors controlling leaf elongation of salt-stressed plants.

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Objectives of the original research proposal

The main objective of this research is to evaluate ionic and enzymatic changes in the cells and cell walls of the expanding region of salt-stressed leaves that may contribute to a reduced growth rate.

The specific objectives are:

- 1) To study salinity effects on growth regulating cell wall enzymes.*
- 2) To study salinity effects on ionic deposition in the cell wall and intracellular compartments of expanding cells.*
- 3) To study the basis for the greater sensitivity of younger, unemerged leaves to salt stress.*

Relevant data, methodology, results and discussion.

USA

The following are the results of the American portion of the project:

Mechanisms for Control of Leaf Growth during Salinity Stress

Determination of in vitro Cell Wall Properties of Salt-Stressed

Plants

Modification of LVDT System

We modified our LVDT system to accommodate leaf-tissue segments as opposed to intact, live maize leaves. Our initial run showed there was give in the system which could lead us to erroneous results. Possible causes were slippage of the segment within the stopper, movement of the stopper itself, or stretch in the Kevlar line. We eliminated the stopper and the plant tissue and hooked the alligator clip directly to the LVDT holder wingnut to isolate the line. Weights were added and removed, and movement was recorded. We tried to use unspun Kevlar, which had less give, but its unspun nature made it difficult to work with and we returned to using spun Kevlar.

The isolated 2-cm segment of the third leaf's growing zone of maize broke at much lower weights than the plant in vivo. Therefore, a lower range of weights was used.

In vitro Cell Wall Analysis of Salt-Stressed Plants

The first step was to determine the optimum method for removal of turgor. Changes in plant turgor will complicate the analysis, so several methods were tried that would effectively yield isolated cell walls. Initially we tried vacuum suction, 50% glycerol, DMSO, 2-20% concentrations of Triton-X, and 95% ethanol. None of these treatments were satisfactory. In fact the latter three treatments completely eliminated cell wall creep (data not shown). Finally we settled on a more detailed comparison (Table 1) of live, boiled (2 min), μ waved (on high for 30 s) and freeze-thawed tissue (in liquid N₂) over a one hour period.

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Weights of 0, 3, 6, 9, 12 g were used in a similar method of analysis to previously published work (Cramer and Bowman, 1991). The bathing solution for the segments used was 0.5 mM CaCl_2 . Distinct differences were found between the denaturing technique. Heating the tissue by boiling or μ waveing increased tissue variability and appeared to artificially increase the plasticity of the wall. When turgor is removed the elasticity of the tissue is expected to increase, but not the plasticity. We therefore concluded that freeze-thawing the tissue was the most appropriate way to analyze the cell walls in vitro.

Table 1

Comparison of denaturing techniques to remove turgor analyzed according to the methods of Cramer and Bowman (1991). E (μm) is the elastic component, P (μm) is the plastic component and slope is the rate of cell wall creep ($\mu\text{m min}^{-1}$). Values are the means $\pm$ SE; n = 17 - 23.

Denaturing			
Technique	E	P	slope
live	237 $\pm$ 34	126 $\pm$ 30	0.837 $\pm$ 0.205
freeze-thawed	360 $\pm$ 63	351 $\pm$ 59	1.132 $\pm$ 0.306
boiled	415 $\pm$ 79	715 $\pm$ 100	0.851 $\pm$ 0.173
μ waved	552 $\pm$ 178	945 $\pm$ 128	3.353 $\pm$ 0.681

When salt-treated plants (4 h before excision) were compared to control plants no differences were found and no yield threshold was detectable, the intercepts of the lines went through 0 g of weight (Fig 1).

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Figure 1

In vitro analysis of segments of leaf growing zones of maize. Segments were stretched by different amounts of weights and analyzed as before (Cramer and Bowman, 1991). Each datum is the mean $\pm$ SE; n was between 6 and 14 replications. The x-intercept represents the apparent yield threshold of the cell wall. The slope represents the growth coefficient, $\text{mL}/(\text{m}+\text{L})$, where m is the cell wall extensibility and L is the hydraulic conductance. Note that freeze-thawed segments do not have turgor.

These results were not expected, particularly the lack of yield threshold. It was decided to repeat this experiment with a new method of analysis using a large number of smaller weights to accurately determine the yield threshold. Freezing the tissue eliminated turgor.

We modified the LVDT system again, reducing the volume of bathing liquid, and gluing the plant segment in to prevent pinching or cutting of the tissue. (Syringe method). We glued segments into the syringe and LVDT, bathed them with 1 mM CaCl_2 , and applied weights of increasing mass (0 to 6 g). The weight was applied for 3 minutes then removed and the next heaviest weight is applied 10 seconds later and left on for three minutes similar to the method of Neumann

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(1993). Growth rate, plastic and elastic components were calculated for each weight applied to control and salt-treated plants (Fig. 2).

Figure 2

In vitro analysis similar to Neumann (1993). Segments were freeze-thawed in liquid nitrogen. Weights were added sequentially for a period of three minutes each and the plastic and elastic extension determined. Data are means $\pm$ SE, n were between 2 and 14 replications. The apparent yield threshold is the x-intercept. The slope represents the cell wall extensibility.

The results are consistent with Fig. 1. No differences in cell wall properties were detected between control and salt-treated plants.

Again, the latter analysis was repeated, but with one modification. Isolated excised tissue segments were not analyzed; instead the growing zones of the intact leaves were freeze-thawed with liquid N_2 before weight analysis in situ. The results (Fig. 3) were consistent with previous results, indicating that the yield threshold of this tissue without turgor is very small and that salinity has no affect on either the yield threshold of the cell wall or cell wall extensibility.

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Figure 3

Similar *in vitro* analysis as Figure 2, but growing zones of intact plants were freeze-thawed *in situ*.

Data are means $\pm$ SE, *n* were between 4 and 7 replications.

These findings are in contrast to previous findings *in vivo* where the cell wall yield threshold was found to increase with salinity and contrast with another report (Neumann, 1993) using *in vitro* analysis. However these results were obtained with three different very rigorous methods with consistent results for all three methods. These data suggest that the plant must be a living tissue to adequately determine the nature of the salinity effect. Salinity may have effects on secretion or biosynthesis of polymers that are targeted to the cell wall, have effects on pH in the apoplast, or cause ABA accumulation in the apoplast, which could then trigger solute leakage from the cell into the cell wall apoplast and disturb water relations.

To begin to assess the hypothesis that secretion or biosynthesis of the cell wall was affected several experiments were conducted. An *in vitro* cell wall

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extension assay was conducted to test the effect of the supply of cell wall polysaccharides on cell wall extension. Beta glucan from barley was added over a 6-order-of-magnitude range. No effect of beta glucan was found on cell wall extension. However, it was unclear whether the added polysaccharide was able to penetrate the tissue to the site of action in the inner cell wall layer.

In collaboration with Mathew Morell and Michael O'Shea at the Australian National University in Canberra, Australia, fluid from apoplastic free space and Golgi vesicles of growing zones of the third leaf of maize were assayed for the presence of oligosaccharides by a novel method developed there at ANU (O'Shea and Morell, 1996). Three distinct peaks were detected in both apoplastic fluid and Golgi vesicles indicating the possibility three distinct oligosaccharides between 4 and 7 monosaccharides long. Salinity decreased the concentrations of each of these peaks in both the apoplastic solution and the Golgi vesicles by about half, suggesting that the synthesis of oligosaccharides were reduced in the Golgi apparatus by salinity. These results are very exciting because oligosaccharides are known to regulate activities of cell wall enzymes such as XET, which is involved in cell wall extension.

In addition, experiments were performed with Brefeldin-A to determine its effect on the apparent yield threshold of live plants. Brefeldin-A is a fungal inhibitor which blocks secretion of noncellulosic polysaccharides to the cell wall by inhibiting the fusion of secretory vesicles from the Golgi apparatus with the plasma membrane (Schindler et al., 1994). Treatments with Brefeldin-A inhibited growth of maize leaves by increasing the apparent yield threshold in a similar manner as salinity.

Figure 4

The effect of 10 µg/ml Brefeldin-A on the growth properties of the 3rd leaf of maize. Maize plants were treated with Brefeldin-A through the hydroponic solution. Application of Brefeldin-A immediately caused inhibition of leaf elongation rates. Measurements were taken 24 h after treatment was applied when steady-state growth rates were established.

Reassessment of the stress-signal for growth reduction by salinity.

In collaboration with John Passioura and Rana Munns at CSIRO in Canberra, Australia, both barley and maize plants were tested with an updated version of a pressure device first developed by John Passioura (Passioura, 1988; Stirzaker et al., 1996). This device could automatically regulate the pressure around the roots in order to maintain a zero water potential in the leaves, regardless of the water potential of the soil solution (manuscript in

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preparation). Plants were tested with or without salinity and with or without pressure applied to the roots (Fig. 5). It was found that pressure could increase growth when applied to the roots and maintained growth when plants were subjected to salinity or dehydration stress. Without a balancing pressure, leaf elongation was rapidly inhibited (Fig. 5a) and reached a steady-state growth rate several h later. When the automatic balancing pressure was used (Fig. 5 b), leaf elongation rates remained steady upon addition and removal of salt from the soil solution.

Figure 5

Effect of balancing pressure on leaf elongation of maize plants after addition and removal of 80 mM NaCl after 4 h; without (a) and with (b) balancing pressure.

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This was a rather surprising result for all of us involved, and now has been confirmed in John Boyer's lab with drought-stressed maize (J. Boyer, personal communication). These experiments were conducted in continuous light as previous experiments were. In Fig. 6, two salt-stressed maize plants were measured simultaneously. One plant had a balancing pressure applied to it; leaf elongation rates of the pressurized plant rapidly increased and eventually stabilized at control values. When plants were switched to the dark, the growth rate of the pressurized salt-stressed plant slowed below that of the unpressurized salt-stressed plant.

Figure 6

Effect of balancing pressure on leaf elongation of maize plants in 80 mM NaCl throughout the first day.

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Similar results were obtained using barley (data not shown) and with drought stress (Passioura, 1988). In other words, in the dark, despite the fact that the balancing pressure maintained the water potential of the leaf xylem, the growth rate of the pressurized salt-stressed plant was inhibited. This indicates that in the light, water relations overcome any hormonal signal, but in the dark, this signal dominates over the water relations. Light and dark can affect the pH of the xylem, which could affect ABA distribution in the apoplast (Wilkinson et al., 1998). Furthermore, they found that decreasing soil water potential causes increasing xylem pH. ABA is known to increase in salt-stressed plants and inhibit leaf elongation (He and Cramer, 1996; Cramer and Quarrie, unpublished results; Cramer et al., 1998), and thus is a plausible mechanism for the response of pressurized plants described above.

Role of cell wall proteins in growth response of salt-stressed plants

Expansins

To assess if expansins (Cosgrove, 1997) were involved in the maize leaf growth response to salinity, Dan Cosgrove suggested we first test the acid-growth response of the growing segments; if there was no acid-growth response, then it was not worth pursuing. We briefly looked at the acid-growth responses of control and salt-treated (80 mM NaCl for 4h) segments. We had 4 salt-treated and 3 control segments. Live segments were bathed in 20 mM HEPES with 1 mM CaCl_2 (pH=6.8) and a 10 g weight applied. Segments rested for 0.5 hours then the computer was started. Growth rates were recorded for 10 minutes then the solutions were changed to 20 mM sodium acetate with 1 mM CaCl_2

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(pH=4.5) and growth rates were recorded to the end of the hour. No significant acid-growth response was detected.

We briefly looked at Triton-X as a way to denature the tissue and remove turgor. Removal of turgor would allow an accurate assessment of the yield threshold in vitro. We ran one run with several treatments (control, salt, control and salt with Triton-X) at pH=6.8 and equivalent treatments at pH=4.5. We compared the live tissue treated with Triton-X to freeze-thawed tissue and looked at the acid-growth response of each. No acid response was detected.

We looked further at the acid-growth responses of live and freeze-thawed tissue. Tissue segments (live and frozen-thawed) were bathed in 20 mM HEPES with 1 mM CaCl_2 (pH=6.8) for 10 minutes. Solutions were then changed to 20 mM sodium acetate with 1 mM CaCl_2 (pH = 4.5). Growth rates were taken before the change and at various intervals after the change. We concluded that there is a highly significant difference in growth rate between tissue type, the freeze-thawed tissue showing a much lower growth rate than live tissue. We found no difference between acid treatments, no acid-growth response. The overall conclusion from these experiments was that expansin may not be involved in growth regulation of maize leaves and this avenue was not further explored.

However, I recently met with Elizabeth van Volkenburgh and Daniel Cosgrove at the Annual American Society of Plant Physiology meeting. Dr. van Volkenburgh referred me to a paper of hers (Van Volkenburgh and Boyer, 1985) in which they found clear acid-growth responses of the 5th leaf of maize and clear effects of light and drought on apoplastic pH. In addition, Dr. Cosgrove has informed me that cDNA's have been identified in maize leaves

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that are highly homologous with other expansins. Therefore, it is premature to conclude that expansins and apoplastic pH are not involved in maize leaf response to salinity.

Protein analysis in the apoplastic free-space of the cell wall

The rationale to analyze the cell wall free space for proteins rather than extract ironically bound cell wall proteins was that the proteins of the apoplastic free space would represent proteins that were newly secreted and might be a more accurate of assessment of proteins regulating growth. The apoplast extraction method was modified from Shinkle et al. (Shinkle et al., 1992). Growth zones were excised approximately 1-2 cm long under 50 mM CaCl_2 at 4° C and vacuum infiltrated for 3 min. Approximately forty zones were placed apical side down and one layer thick into 30 cc syringes for centrifugation. Samples were initially spun at 240 g for 4 min at 4°C to remove adhering surface solution. This extract was discarded. Samples were recentrifuged at 650 g for 10 minutes at 4° C, the extract collected, followed by a second spin at 1300 g for 10 minutes at 4° C and the extract collected again. Cytoplasmic leakage was determined by assaying for malate dehydrogenase activity. No activity was detected indicating no cytoplasmic leakage into the apoplast.

Protein concentration was determined using the BCA standard assay from Pierce. Protein analysis of the apoplastic extracts indicates that salinity (4h or 24 h later) had no significant effect ($p \geq 0.68$) on the protein concentration in the apoplast (Table 2). Note that extract volumes were significantly reduced by salinity after 24 h.

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Table 2

Protein concentrations in apoplastic extracts of salt-stressed growing zones of the 3rd leaf of maize. gz = growing zone segment

Treatment	Spin force	Extract Volume	Apoplastic Protein
		(μ l/ gz)	(μ g/ μ l)
control	650 g	12.3 $\pm$ 1.12	1.42 $\pm$ 0.33
	1300 g	15.5 $\pm$ 0.66	1.21 $\pm$ 0.03
salt 4 h	650 g	12.1 $\pm$ 0.24	1.04 $\pm$ 0.28
	1300 g	14.8 $\pm$ 1.00	1.29 $\pm$ 0.43
salt 24 h	650 g	7.94 $\pm$ 1.00	1.64 $\pm$ 0.04
	1300 g	9.18 $\pm$ 0.59	1.25 $\pm$ 0.11

Progress was made in identifying proteins on both SDS and cationic non-denaturing gels. Initially results were negative on both gels, however, subsequent analysis after protein was precipitated with seventy percent acetone was positive. One-dimensional SDS-PAGE assays also confirm no significant differences in different protein classes (Fig. 7).

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Figure 7

Three representative photos of one-dimensional SDS-PAGE of apoplastic proteins from the growing zone of the third leaf of maize. Protein was stained with Coomassie Blue.

A) Lane 1 is a high-speed extract (1300 g) of 24 h-salted plants. Lane 2 is a low-speed extraction (650 g) of 24 h-salted plants. Lane 3 is a high-speed extraction of 4 h-salted plants. Lane 4 is a low speed extraction of 4 h-salted plants. Lane 5 is a high-speed extraction of control plants. Lane 6 is a low-speed extraction of control plants. Lane M contains low weight SDS-PAGE molecular weight standards from Bio-Rad. Low-speed lanes were loaded with approximately 74 μ g of protein and high-speed lanes were loaded with approximately 100 μ g protein.

B) Similar to A except that all lanes were loaded with approximately 100 μ g protein

C) Similar to A except that all lanes were loaded with approximately 75 μ g protein and all lanes are high-speed extractions. Lane 1 is 24 h salt, lane 2 is 4 h salt and lane 3 is control.

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Densitometer scans of these gels indicate no significant differences in any of the bands (data not shown). These data indicate that protein secretion is not significantly affected by salinity. Ideally two-dimensional analysis should be done but the lack time and money did not permit this analysis.

These results are consistent with previously unpublished results (prior to this project) in this laboratory in which no effect of ABA or salinity on the activity of XET or cell wall peroxidases was found.

In conclusion, to date we have found no support for the hypothesis that the true cell wall yield threshold is affected by salinity. We have found no biochemical evidence that cell wall enzyme activities that could potentially modify cell wall properties are affected by salinity. However, we have found evidence to support that the secretion or biosynthesis of noncellulosic poly- or oligosaccharides could be lowered by salinity. It was also established that blockage of the supply of cell wall polysaccharides to the cell wall with the fungal inhibitor, Brefeldin-A, inhibited growth by increasing the apparent yield threshold *in vivo*.

We formulate a new hypothesis that the increase in the apparent yield threshold by salinity in intact live plants is affected by the decrease in the biosynthesis and secretion of noncellulosic polysaccharides and/or oligosaccharides to the cell wall. The effect of salinity on the cell wall would only be apparent in live tissues and would not be observed with *in vitro* assays of the cell wall; consistent with the results found in this study.

An alternative hypothesis is that ABA may act as a regulator of the apparent yield threshold in salt-stressed plants (Cramer et al., 1998) either by modulating

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in vivo cell wall properties such as secretion and biosynthesis or by solute leakage from growing cells into the apoplast.

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Israel

The following are the results of the Israeli portion of the project:

The Determination of Relative Elemental Growth Rate Profiles from Segmental Growth Rates. A Methodological Evaluation

See published article in the Appendix

Evaluations of methods for estimation of REGR profiles along grass leaves.

Winfried S. Peters and Nirit Bernstein

Introduction

Reduced rates of leaf growth are among the first detectable signs of adverse environmental conditions such as draught or salt stress (Munns & Termaat 1986; Davies & Zhang 1991). Understanding leaf growth control therefore is of interest from the viewpoint of both pure and applied research. Not surprisingly research on leaf growth under stress has frequently been performed on crops, particularly Poaceae (Acevedo, Hsiao & Henderson 1971; Cutler et al. 1980; Matsuda & Riazi 1981; Yeo et al. 1991; Bernstein, Silk & Läuchli 1995).

In Poaceae, the sheaths of the older leaves (Sharman 1942; Begg & Wright 1962; Kemp 1980) encircle leaf growing zones. Spatial growth patterns thus can only be investigated by invasive methods. An often used method is the so-called "prick-hole-experiment" (Davidson & Milthorpe 1966; Schnyder, Nelson & Coutts 1987; Bernstein, Silk & Läuchli 1993), in which a series of tiny holes is pricked through the sheaths into the enclosed growing zone. The displacement of the holes is measured some time later after dissection and Relative-Elemental-Growth-Rate (REGR)-profiles are calculated from these data. Profiles constructed by this method usually possess one, more or less pronounced, peak (Schnyder & Nelson 1988; Schnyder et al. 1990; Bernstein, Läuchli & Silk 1993; Ben-Haj-Salah & Tardieu 1995).

Alternatively, anatomical markers can be used to estimate spatial growth profiles (Brumfield 1942; Silk, Lord & Eckhard 1989; Allard & Nelson 1991; MacAdam, Nelson & Sharp 1992). The advantage is that the tissue is not wounded during the test. For

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example, wheat primary leaf growth was scrutinized using a variety of anatomical markers such as Inter-Stomatal-Distance (ISD; Keyes, Paolillo & Sorrells 1989; Paolillo, Sorrells & Keyes 1991). The result was flat profiles without pronounced peak, and it was suggested that this "flat-max-model" was typical of grass leaf growing zones in general (Paolillo & Sorrells 1992). To explain the frequent occurrence of peaked profiles supporting a "single-peak-model", two lines of argument were put forward. First, systematic analytical errors were claimed to create artificial peaks. Second, it was cautioned that single-peak-profiles might develop as a pricking-induced wound effect in leaves, which in situ grow in accord with the "flat-max-model".

The distinction between the alternative models is far from being academic. For example, a cellular parameter such as turgor, occurring at uniform quantity throughout the growing zone, would have to be suspected to control growth rates in leaves growing according to the "flat-max-model". In leaves following the "single-peak-model" this could be excluded (Tomos & Pritchard 1994). Thus, prior to a causal investigation of leaf growth control one has to establish the spatial mode of leaf growth.

We decided to unequivocally establish the spatial mode of growth in maize leaves by solving the methodical problems that have fueled the "flat-max/single-peak" controversy. In doing so, we will first study systematic errors involved in the calculation of REGR-profiles from prick-hole-experiment data, by investigating the behavior of various methods when applied to simple growing zone models (cf Peters & Bernstein 1997). In a second step, we will explore the possibility that single-peaked shapes of growing zones might be wounding-induced artifacts by comparing REGR-profiles calculated from prick-hole-experiments and from anatomical markers.

Materials and Methods

Plant Material and Growth Conditions

Caryopses of *Zea mays* L. cv "G.V.-46." (Galil Seeds, Haifa, Israel) were incubated in aerated solution (9 mM KCl, 1 mM CaCl₂) for 6 hours, and were then transferred to beds of vermiculite (12 cm deep) soaked with demineralized water in plastic boxes (30 x 35 x 15 cm). They were kept dark in a growth cabinet at 26 C. Illumination (400 μ E/s/m²) was started at a 16/8 hour day/night rhythm on the fourth day. One liter of demineralized water was added to every box on the sixth day. At the beginning of the 8th day, plants selected for uniformity (length of leaf 1 between 75 and 85 mm, and length of leaf 2 not more than 5 mm different from leaf 1) were transferred to a hydroponic growth facility in which batches of 5 plants were grown on containers with 15 l of aerated, modified

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Hoagland solution (Epstein 1972; KNO₃ 1.5 mM; Ca(NO₃)₂ and NaCl 1 mM; MgSO₄ and NH₄H₂PO₄ 0.5 mM; Fe_{III}EDTA 60 µM; Fe_{II}(NH₄)₂(SO₄) 20 µM; H₃BO₃ 12.5 µM; MnSO₄ and ZnSO₄ 1 µM; CuSO₄ and H₂MoO₄ 0.25 µM; adjusted to pH 5.7 with KOH); the light and temperature regimes were as described above. The root medium was completely renewed every other day until day 14, and from then on daily. Plants were routinely cultivated until leaf 8 emerged on day 26, without symptoms of declining viability or growth rate.

Growth measurements

Leaf lengths were measured daily to the nearest 0.5 mm with a ruler. REGR profiles in leaf 4 were determined by prick-hole experiments, which were started 4 hours after the beginning of the photoperiod on day 14. The experiment was performed 4 times with 5 pricked and 10 control plants at every occasion. A series of 20 insect-mounting pins (size 00), fixed in 5 mm intervals on a perspex holder, was aligned central and parallel to the longitudinal axis of the shoot and inserted through the older sheaths into the growing zones of the younger ones. With some practice it was possible to position the first hole between 1 and 2 mm above the insertion of leaf 4. The needles were removed and growth was allowed to continue for 5 hours. Leaf length was measured before and after this period in order to allow an assessment of pricking-induced growth reduction. After the 5 hour period, leaf 4 was isolated and the displacement of holes was measured to the nearest 12 µm with a stereomicroscope. The distance between the needle tips was also controlled to the nearest 12 µm before and after the experiments. For REGR profile calculation, relative growth rates (R) of the segments between prick-holes were determined according to Green (1976), as of eq. 1.

$$R = (\ln L_t - \ln L_0) t^{-1} \quad \text{eq. 1}$$

with L_0 and L_t being the initial and final segment length, respectively, and t the duration of the experiment. Segmental R was plotted versus segment position as defined by average segment midpoints. Depending on variation in data, fourth, fifth, or sixth order polynomial regression was applied to these data to yield a function describing the profile. Two different tests for accuracy were performed on every individual profile, as suggested previously (Peters and Bernstein 1997). First, profiles were compared to plots of segmental length increment versus initial segment position; the latter tend to yield distorted profiles, but accurate estimates of the growing zone length (Sachs 1874). In both types of plots, growing zone length was estimated and experiments exhibiting more than 15% difference

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were dismissed. Second, elongation velocity was calculated as the definite integral of the REGR profile; this value was compared to the elongation velocity measured independently during the experiment. In cases of deviations of more than 12% of the measured value (i.e. roughly twice the maximum error of a length measurement to the nearest 0.5 mm, as related to the mean of the measured values), the individual experiment was discarded. Eight out of 20 experiments failed to meet these strict criteria. Eventually, an average REGR profile was calculated as the mean of the remaining regression functions.

Alternatively, REGR profiles were determined from ISD distribution along leaf 4 at the same developmental stage. The plants were pricked as described above, and leaf 4 was isolated immediately afterwards. Distances between holes were measured, and the leaves were gently plasmolized in 0.4 Osm mannitol. After 30 minutes, they were transferred to 50% ethanol, and after one more hour to 95% ethanol for storage. Before ISD profile determination, distances between holes were measured again to determine the turgor-induced strain lost in the preparation. Anatomical measurements are now in progress and are not reported here.

REGR profile modeling

To evaluate systematic errors involved in REGR profile determination by a particular method, we used REGR profile models (as discussed in detail in Peters and Bernstein, 1997). In short, a simple function is adjusted to resemble a REGR profile of a desired shape within a defined domain of validity. The integral of this function in its domain of validity gives the displacement velocity distribution, or velocity field, along the model growing zone. Integration of the reciprocal of the velocity field function yields a trajectory function, which describes the path of a point on the model organ in a plot of position versus time; the position at time 0 can be freely chosen by adjusting the integration constant. From series of point trajectories the movement of points along the organ as governed by the known REGR profile can be calculated, allowing one to perform theoretical prick-hole experiments. Different methods of REGR profile determination can be applied to the results from these model experiments, and their reliability can be assessed by comparison with the known, "true" profile. All model calculations can be done algebraically; where necessary integrals are taken from integral lists (e.g. Bronstein & Semandjajew 1991). Thus, no numerical errors are included in such calculations.

We here use a "single-peak-model" resembling our empirical REGR profiles from leaf 4 of maize (see Fig. 3a), and a "flat-max-model" similar in shape to the profile given for seedling wheat leaves by Paolillo and Sorrells (1992; see Fig. 3b). Both models have a maximum displacement velocity of 1.9 mm h^{-1} , which is in accord with growth velocities

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(GV) we actually measured during our prick-hole experiments. The growing zone length also is identical in both models. Our "single-peak-model" is defined by the fourth order polynomial (coefficients rounded):

$$\text{REGR} = 2.639 \cdot 10^{-4} \cdot x^2 - 8.8 \cdot 10^{-6} \cdot x^3 + 7.33 \cdot 10^{-8} \cdot x^4 \quad \text{eq. 2}$$

Our "flat-max-model" consists of three consecutive functions. Between position 0 and 15 mm it is a third order polynomial (coefficients rounded):

$$\text{REGR} = 5.067 \cdot 10^{-4} \cdot x^2 - 2.251 \cdot 10^{-5} \cdot x^3 \quad \text{eq. 3}$$

Between 15 and 55 mm REGR is constant at 0.038, and between 55 and 60 mm it linearly decreases to 0.

Theoretical growth data based on the models were created for 5 mm prick-hole intervals and a 5 hour period, identical to the conditions in our experiments with maize leaves. Segment elongation data were either processed as described above for the real experiments, or by the method used by Bernstein, Silk and Läuchli (1993). In this case, segmental REGR was estimated by

$$\text{REGR} = (v_U - v_L) (z_U - z_L)^{-1} \quad \text{eq. 4}$$

with v_U and v_L denoting the displacement velocities of the two holes (*Upper* and *Lower*) confining the segment, and z_U and z_L standing for the initial positions of the two holes with respect to the leaf base. Alternatively, displacement velocities of prick-holes calculated from the model experiments under identical experimental condition were plotted versus average hole position to yield velocity fields. The Richards function (in the form discussed by Hunt 1982)

$$y = a (1 \pm e^{b-cx})^{-1/d} \quad \text{eq. 5}$$

was then fitted to these data, and REGR profiles were determined as the derivative of the fitted function:

$$y' = a b d^{-1} e^{b-cx} (1 \pm e^{b-cx})^{-(1/d+1)} \quad \text{eq. 6}$$

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All calculations, curve-fitting, and plotting was performed with standard software (QuattroPro, V 3.00, Borland; FigP, V 6.0c, Biosoft; Mathematica, V 2.2, Wolfram Research).

Results and Discussion

Leaf elongation

Having screened the growth characteristics of the first 8 leaves of maize under the conditions chosen (data not shown), we decided to perform further experiments on the fourth leaf. Leaf four emerges from the enclosing sheaths at day nine, when it is about 10 cm long. At this stage it elongates at a growth velocity (GV) of more than 2 mm h⁻¹ (Fig. 1). Similar velocities are maintained until day 19; subsequently growth gradually ceases, and stops entirely when the leaf reaches a length of roughly 60 cm. Figure 1 also shows the Richard function (eq. 5) fitted to the elongation data, and its derivative describing the time course of the GV. The fit to the elongation data seems perfect, whereas the GV data-points appear to suggest a peak much more obtuse than shown by the derivative of the Richards fit. While the greater relative variance of the GV data as compared to the elongation measurements is one obvious explanation of this fact in the present dataset, the goodness of fit of the GV curve would certainly be considered unsatisfactory if exclusively GV data were available. The implications will be discussed in more detail below.

REGR profile

REGR profiles of leaf four were produced from prick-hole experiments on day 14, when the leaves grew at highest GV. Comparison of length increments in leaf four during the duration of the prick-hole experiments between the pricked and untreated control plants revealed a pricking induced growth reduction of about 50%. GV was 1.83 ± 0.11 mm h⁻¹ (n = 20) in the pricked, and 3.63 ± 0.35 mm h⁻¹ (n = 41) in the control plants (means $\pm$ S.D. are given). The control GV appears quite high compared to data given in Figure 1, which is explained by the pronounced diurnal GV oscillations in maize leaves (Van Volkenburgh & Boyer 1985; Ben-Haj-Salah & Tardieu 1996), which are not resolved in the Figure.

REGR profiles of 20 individual plants were subjected to rigorous checks for reliability, as detailed in the Methods section. Eight profiles (i.e. 40 %) had to be dismissed, since they did not harmonize with growth parameters (GV, growing zone length) determined independently for the same specimen. A rejection rate of 40 % might appear discouraging, but we feel that a less rigorous procedure cannot be justified given the

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fact that small random errors in the experimental data might cause greatly inaccurate results in the mathematical analysis.

The remaining, consistent data were used to determine a mean profile (Fig. 2). We calculated the mean profile as the numerical mean of the regression functions fitted to the individual datasets, instead of determining it by regression of all datapoints involved (also shown in Fig. 2), for the following reason. With the method of REGR profile determination employed here, segmental R is plotted versus segment position as defined by average segment midpoints (cf Green 1976; Peters & Bernstein 1997). Each average segment midpoint moves away from the meristem with a velocity, that depends on the displacement velocity of the lower and upper edges of the segment, and thus to some extent on the GV of the particular leaf. It follows that in plots of raw data from a number of plants, datapoints from slower growing individuals will tend to accumulate near the meristem, while datapoints from faster growing ones will dominate the opposite side of the profile. If so, regression analysis of bulk data will cause profile distortions. In our case, the results of both methods were practically indistinguishable (not shown), indicating a negligible role of this systematic error. However, since this might be different in other cases, we suggest that one generally not calculate average profiles directly from primary data.

In none of the profiles of individual leaves any indication of a flat REGR maximum was apparent. Accordingly, the mean profile exhibits a single pointed peak, which is in agreement with previous studies using the prick-hole technique (Fig. 2; Schnyder, Nelson & Coutts 1987; Bernstein, Silk & Lauchli 1993). Schnyder et al. (1990) reported that cells in the sheath/blade transition zone of *Lolium perenne* L. grow at very low rates compared to typical sheath and blade cells, causing a more or less pronounced trench passing through the REGR profile with time. In our leaves the ligule was localized at 4.6 ± 1.1 mm (mean $\pm$ S.D., $n = 12$) above the node at the time the experiments took place, so that the effect could not become apparent.

REGR profile modeling

To investigate whether the "single-peak" REGR profile we found (Fig. 2) might be an artifact caused by the analytical methods involved, we applied the methods to a model growing zone with single-peak characteristics. This model had similar features than the empirically determined REGR-profile, namely a GV (equaling the maximum DV) of 1.9 mm h^{-1} , a growing zone length of about 60 mm, and a peak REGR value of 0.06 h^{-1} . The experimental conditions in the theoretical test were also identical to the ones prevailing in the real experiments (initial segment length 5 mm, $t = 5 \text{ h}$). The model REGR- and

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DV-profiles are shown in Figure 3a. The Figure also shows REGR values calculated from the data from the theoretical experiments; as can be seen, the correspondence to the true profile is excellent.

An analogous test was carried out on the basis of the "flat-max"-model shown in Figure 3b. This model is also in full quantitative agreement with GVs and growing zone lengths measured in real prick-holed leaves. Again, our method of REGR-profile determination yields a very accurate estimate of the true profile, from which the experiment started. Thus the empirical "single-peak" REGR-profile shown in Figure 2 is unlikely to be an inaccurate image of a "flat-max" profile, which became distorted by inadequate analytical methods.

We also calculated an alternative REGR-profile from data created by the "flat-max"-model, this time using the alternative method previously employed by Bernstein, Silk & Läuchli (1993). In this case, average REGR for segments between prick-holes is not estimated as the mean relative growth rate of the segment (see eq. 1), but by an expression including the DVs of the margins of the segments (eq. 4). Figure 3b shows that this technique leads to an overestimation of REGR peak values; this effect was termed "REGR-compounding" by Paolillo & Sorrells (1992), who claimed that it was one of the artifacts responsible for the occurrence of "single-peak" REGR-profiles. However, in our special case the effect does clearly not result in such a profile, and neither does it question the qualitative conclusions drawn in the paper by Bernstein, Silk & Läuchli (1993), as recalculation of original data shows.

Nevertheless, the method should be avoided since it unnecessarily induces inaccuracy by producing "REGR-compounding". Previously (see Peters & Bernstein 1997 for mathematical details) we had suggested that "REGR-compounding" is caused by an inappropriate measure of segmental elongation, which estimates segmental R as

$$R = (L_0 \cdot L_t^{-1} - 1) \Delta t^{-1} \quad \text{eq. 7}$$

with the meaning of symbols as given for eq. 1. Although the equation does not appear to resemble eq. 4 at first sight, both equations are identical (as the reader may verify by expressing segment lengths L as differences of point positions z, and displacement velocities v as differences of z divided by t). Unfortunately this is not the only case of the "REGR-compounding" causing eq. 7 appearing in disguise. However, this single example may serve to strengthen the point made earlier (Peters & Bernstein 1997), that only mean

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relative growth rates of marked segments (as calculated by eq. 1) should serve as estimates of REGR values.

An alternative method, which does not rely on measures of segmental growth at all, is the fitting of given functions to DV profiles; the derivative of the respective function then yields an REGR profile. Usually the function fitted to a DV profile is sigmoidal, i.e. it possesses exactly one point of inflection. As a consequence, the resulting REGR profiles will show a single, more or less pronounced peak. Though mathematically elegant such sigmoidal functions are, it must be stressed that there is no reason why real REGR profiles should have to have one pronounced peak. Predefined functions always carry inherent assumptions into the empirical data, and may lead to gross misinterpretations. As an example, Figure 4 shows the Richards function fitted to DV data created by our two models under experimental conditions as discussed in the context of Figure 3. The function seems to describe the DV data reasonably well, and in the case of the "single-peak"-model the resulting REGR profile is only slightly distorted (Fig. 4a). However, the "flat-max"-model is turned into a "single-peak" one (Fig. 4b); the data have become qualitatively distorted in accordance with the mathematical nature of the analytical method. Noteworthy, very similar effects will necessarily occur with all sigmoidal functions fitted to DV profiles.

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Fig. 1: Elongation and growth velocity of maize 4th leaf. Richards function fitted to elongation data and derivative plotted with GV data.

Fig 2: Average REGR profile of maize 4th leaf (at maximum GV).

Fig 3A: Theoretical experiment:

- “true” REGR profile
- - - “true” DV profile
- oooo data calculated according to eq. 1

Fig 3B: Theoretical experiment:

- "true" REGR profile
- - - "true" DV profile
- oooo data calculated after eq. 1
- data calculated after eq. 4

Fig 4A: Theoretical experiment:

- "true" REGR profile
- DV data created by model and
Richards function fitted
- REGR profile calculated as derivative of
function fitted to DV data.

Fig 4B: Theoretical experiment:

- "true" REGR profile
- DV data created by model and
----- Richards function fitted
- REGR profile calculated as derivative of Richards
function fitted to DV data.

**SALINITY INDUCED INHIBITION OF LEAF ELONGATION IS NOT
MEDIATED BY CHANGES IN CELL-WALL ACIDIFICATION**

Nirit Bernstein; Galit B. Neves-Piestun; Winfried S. Peters

Abbreviations: FC, Fusicoccin; RGR, Relative growth rate; REG Rate, Relative elemental growth rate

Introduction

Inhibition of leaf elongation is one of the primary effects of salt stress. The physiological mechanisms underlying leaf growth maintenance or inhibition are not yet clearly understood. Alterations in the nutritional status of the leaf growing zone (Bernstein et al. 1995; Lazof and Bernstein 1998; Munns et al. 1982), growing cell wall characteristics (Cramer and Bowman 1991, 1992; Neumann 1993), growing cell's water relations (Munns and Termaat 1986; Neumann et al. 1988; Yeo et al. 1991), and metabolite availability to the growing zone (Munns et al. 1982; Shahar et al. 1997) are some of the factors inspected for their possible involvement in the growth inhibition process.

Leaf longitudinal growth is dependent on irreversible expansion of cells produced by the leaf basal meristem. Inhibition of leaf growth by salinity is associated with inhibition of these cell's expansion. Salinity was shown to reduced sorghum leaf growth rate by shortening the length of the leaf basal elongation zone, and reducing the intensity of cellular growth in the central and distal portions of the growing zone (Bernstein et al. 1993a). Although duration of cell longitudinal expansion was increased by salinization (Bernstein et al. 1993a,b), the reduction in local intensities of cell elongation resulted in shorter mature cells.

According to the Lockhart growth model (Lockhart, 1965) cell and tissue expansions are generated by turgor-driven wall extension. Reduction in wall extensibility, rather than turgor, were recently suggested to be the major growth limiting factor in long term growth inhibition under salt stress (Neuman et al. 1994; Cramer and Bowman 1991, 1992; Neuman 1993) as well as water

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stress (Hsiao and Ying 1987; Nonami and Boyer 1990; Pritchard et al. 1991; Serpe and Mathews 1992; Wu et al. 1996). Cell wall yielding, which precedes wall and cell extension, is a complex process determined by the composition and physical arrangement of the polymeric components of the cell wall; activities of cell wall proteins, and regulatory processes such as Golgi secretion and cell wall acidification.

For many plant tissues it has been demonstrated that increased rates of growth are often associated with increased acidification of the cell wall solution (Moloney et al. 1981; Van Volkenburgh and Cleland 1980; Hager et al. 1971; Rayle 1973). Proton extrusion is thought to promote wall loosening events necessary for cell enlargement (Rayle and Cleland 1992; Taylor et al. 1997; Versel and Mayor 1985; Mulkey and Evans 1981). Activation of polysaccharide hydrolytic enzymes when the pH in the cell wall decreases, may increase cell wall loosening and plastic extensibility of the cell wall and therefore stimulate cell enlargement (Darvill et al., 1978). Inhibition of wall-loosening events by reduction of cell wall acidification, may therefore reduce cell growth rate.

An explanation of salt-induced growth inhibition could be that salinity reduce excretion of protons (H^+) into the growing cell wall apoplast and therefore inhibit wall loosening. There is evidence that the Indole-3-acetic acid mediated H^+ excretion is drastically inhibited by Na (Kutschera and Schopfer, 1985). This could have an affect on cell wall acidification and cell growth under salt stress conditions, since salinization is often characterized by Na uptake, translocation or accumulation. Some salinized cell walls were indeed shown to contain higher levels of Na^+ than non-stressed walls (Flowers et al. 1991; Hajibagheri et al. 1987). In some cases, cultivars that are more salt-tolerant were shown to exclude more Na from the cytoplasm and cell wall than the less salt-tolerant cultivars (Hajibagheri et al., 1987- for maize; Flowers et al., 1991- for rice; Lazof and Bernstein 1988).

Moreover, NaCl might also affect acidification and growth indirectly via an affect on the tissue nutritional status. Salt stress is known to reduce K uptake into roots and translocation to shoots of many plant species. It has long been suggested, that decreased leaf tissue contents of several inorganic nutritional

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elements, and especially of K, are associated with leaf growth inhibition under salt stress (Lazof and Bernstein 1988, and references therein). To evaluate the possibility that inhibition of leaf elongation under salinization is caused by a reduction of K (or other nutritional elements) in the growing cells, Bernstein et al. (1995) analyzed correlations between changes in the spatial distribution of elongation profile and spatial distribution of nutritional elements content profiles along sorghum leaves elongation zone. They discovered that salinity induced a decrease in K concentration and deposition rate throughout the elongation zone. The inhibition of K deposition rate due to salinity increases with distance from the leaf base as did the inhibition of growth. This suggests an inhibition of leaf growth due to progressively lower K depositions along the expanding tissue. The spatial relationships between inhibition of tissue elongation and reduction in K content may be due to possible K effect on cell wall acidification and therefore wall loosening, since K was found to be required for stimulation, and optimal activity, of the H^+ translocating ATPase (Travis and Booz, 1979), and a K^+/H^+ antiport similar to the one found in corn root protoplasts (Lin, 1981) can operate across the plasmalemma of leaf cells.

In addition to its possible effect on cell wall loosening, extracellular pH plays a regulatory role in plasmalemma transport processes. Transport of numerous organic and inorganic solutes is of course highly dependent on the pH gradient across the plasma membrane (Etherton and Rubinstein 1978; Novacky et al. 1980). If salt-stress induces changes in cell wall acidification rates or cell wall pH value, it might alter rates of membrane transport processes into the growing cell, and therefore availability of nutritional solutes for growth processes.

Several environmental conditions were shown to alter apoplast acidification. For example, water stress was shown to increase apoplastic pH (Hartung et al. 1988) and rate of acidification (Van Volkenburgh and Boyer, 1985); Cold, thermal shock, to induce an increase in the extracellular pH of the motor cells of *Mimosa pudica* L. in the lower and upper half of the leaf (Kumon and Suda, 1985); and gravitropic stimulation to enhance acid efflux along the upper surface of horizontally placed roots (Mulkey and Evans, 1981).

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The possibility that inhibition of leaf cell growth by salt stress is associated with alteration in acidification of growing cells apoplast is the subject of this report. We investigate the effect of salt stress on apoplast acidification kinetics by growing leaf tissue of maize, in relation to alterations in the spatial profile of growth intensities along the growing zone. The results provide insights regarding the possible role of proton excretion regulation in the process of cell growth inhibition under salt stress.

Materials and Methods

Plant material

Seeds of *Zea mays* L. cv Galil-Seeds 646, were soaked in tap water for 4 h and later sown on moist vermiculite in plastic boxes. The boxes were covered with aluminum foil and placed in a growth chamber at 24°C. On day 7 after the beginning of germination, when the length of leaves 1 and 2 were similar; leaf 3 was not yet visible and length of leaf 4 was < 1 mm, the plants were transferred to 0.25 concentration aerated Hoagland nutrient solution. Solution pH was adjusted to 5.7 with addition of KOH. Growth chamber temperature was kept at 24 °C, relative humidity at 60 / 80 % (day/night), and intensity of light at 400 μ E/s/m² under photoperiod of 14h.

Salinization began on day 7 after the beginning of germination and elevated in three steps, 24 h apart, to 80 mM NaCl. At the time of transfer to hydroponics, control treatment received 1mM and salt treatment 20 mM of NaCl. On day 8 after germination, NaCl level of the salinized plants was increased to 50 mM and at the next day 80 mM of NaCl was reached.

Growth measurements

Daily leaf length measurements were used for evaluation of shoot growth, calculations of leaf growth rate and selection of leaf insertion number for this study. Length of every leaf on the plant was measured daily with a ruler from the base of the plant to the tip of the leaf (Bernstein et al., 1993b). These data were used for calculation of daily leaf growth rates.

Spatial distribution of elongation was calculated from short term displacements of marks placed along the growing zone of the leaf. Leaves were marked as described previously (Bernstein et al., 1993b), except that the initial distance between the marks was 5 mm. In short, the base of the stem was pricked with a series of 13 fine pins spaced 5 mm apart at least 3 h after initiation of the light period. Growth was allowed to continue for another 5 h before leaves were carefully freed from older enclosing leaves, and the final positions of the pin marks were measured under a dissecting microscope. Data on the spatial distribution of elongation within the elongation zone of leaves were collected from 10 plants and were used for the calculations of REG rates.

Relative Elemental Growth rate (REG rate) was calculated, according to Green, (1976) as of Equation 1, where L_0 is initial distance between two marks, L_t is distance between the marks following 5 hr of displacement due to enclosed tissue segment elongation, and t is time.

Eq. 1:
$$RGR = (\ln L_t - \ln L_0) / \Delta t$$

Excised segments elongation rate, 15 days after germination, 8 days after beginning of salinization, leaf 4 was cut from the plant, and the elongation rate of 10 mm excised segments measured. A tissue segment located 20-30 mm from the control and the salinized leaf base, which was found by marking experiments (Fig 2) to include the zone of maximal growth intensity in both the control and salt treatment, were cut from the leaf and lightly vacuum infiltrated. The infiltration procedure was identical to that exercised on segments used for apoplastic acidification measurements described in the following section. Two marked points spaced 10 mm apart were placed on each segment, 5 such marked segments were placed in a vial containing aerated solution containing 1mM KCl, 0.1 mM CaCl_2 and 0.1 Mm NaCl at pH 5.5 (Peters and Felle, 1991a with modifications). After 6 hours, the length of the tissue segment enclosed by the two reference marks was measured and elongation rates calculated as the displacement rates of the marks from each other (i.e., rates of length increase of tissue segments enclosed between two marks).

Apoplastic pH measurements

15 day after germination, 8 days after commencement of salinization, leaf 4 was separated from the rest of the shoot and a 20 mm section (located 20-40 mm from the leaf base) were cut from the growing zone of both the stressed and non-stressed leaves. This tissue segment was found by marking experiments (Fig 2) to include the zone of maximal growth intensity in both the control and salt treatments. The tissue was cut along the mid vein and again perpendicular to the vein into two 1 cm sections and placed base down in a reaction chamber (6 ml vial) which was covered with parafilm. Sections from 10 replicated leaves were placed in 3 ml of reaction solution (1mM KCl, 0.1 mM CaCl_2 and 0.1 mM NaCl at pH 5.5) and lightly vacuum infiltrated. In some experiments higher Na concentrations were used, Na levels were then elevated from 0.1 mM to 5, 10 and 25mM by the addition of NaCl. The solution was constantly bubbled with air, and the pH of the solution was continuously recorded with a Russell CW711 combination pH electrode with a Mettler 340 pH meter connected to a data logger CR10X (Campbell Scientific).

In some experiments mature tissue, located 150-170 mm from the base of the leaf, was excised and prepared as described for growing tissue, except that 1 segment was used per measurement.

In other experiments Fusicoccin (FC) was added to the reaction solution at a final concentration of 2 μM (in 0.00003% ethanol). It was prepared according to Blatt and Clint, (1989) with modifications; as an aqueous 1mM stock solution by adding 0.001 g of FC previously dissolved in 0.2 ml of ethanol, to the appropriate volume of d-water at 75 °C. The pH of the stock solution was 6.24. The stock was stored for a maximum period of 12 h at 4°C prior to application.

Results and Discussion

Effect of salinity on growth and development of the leaf

Figure 1A,B shows the results of nondestructive daily leaf length measurements. Salinity (80 mM NaCl) reduced leaf growth and shoot development. Maximal leaf length attained, was reduced 25-33% by

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salinization. Salt affect on leaf growth was apparent from leaf 3 which has not yet emerged from the whorl of older leaf sheaths at the time of salinization. The rate of leaf elongation was decreased as well following salinization (Fig 1B,C). Growth rate of leaves formed after leaf 2 was slower in salinized than in control plants.

Salt stress caused a decrease in the rate of leaf emergence. At the last day of measurements, 19 days after salinization, 8 leaves were visible in the control plant, but only 7 leaves in the salt-stressed plants. Control and salt plants began to differ in their apparent leaf number on day 18 after germination, 10 days after the commencement of salinization. Thus, day 15 was chosen as the day of measurements and the plants of the two treatments were than considered to be of the same developmental stage. Leaf 4, which was <1 mm long when salinization began, and was growing rapidly at the day chosen for experimentation, was chosen as the experimental system for this project. Salt-stress reduced it's growth rate by 35 % and it's maximal length by 33 %.

Salt stress effect on maize leaf and shoot growth is similar to its effect on sorghum leaf and shoot development (Bernstein et al., 1993a). Similar to maize, salinity was found to decrease rates of sorghum leaf elongation, period of leaf growth, maximal leaf length attained and plastochron duration (time period between emergence of successive leaves). Elevated concentrations of Ca in the root growing media, ameliorated part of the shoot and leaf growth reduction caused by NaCl stress (Bernstein et al., 1993b). These salt induced inhibitions of shoot and leaf growth are not unique for monocots, in the dicot lettuce, as well (Lazof et al., 1991), salt stress was shown to decrease leaf growth rate, final leaf length attained, period of leaf growth and plastochron duration.

Distribution of longitudinal expansion along the leaf axis

We evaluated the effect of NaCl on the spatial distribution of longitudinal expansion along the leaf elongation zone. In the non stressed leaf, the elongation zone was 65 mm long (Fig 2) and the maximal local elongation rate measured at 0.07 h^{-1} - corresponding to growing tissue located 20-40 mm from the base of the leaf. Salt stress shortened the length of the elongation zone by

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23 %, from 65 to 50 mm, and reduced the maximal REG Rate value to 0.05 h^{-1} . The location of maximum REG Rate is similar in salt and control plants. Intensity of growth in the basal part of the elongation zone, was not affected by salinity, REG Rates of the basal 15 mm was similar in salt and control leaves.

Salt stress effect on spatial distribution of longitudinal expansion along maize leaves is similar to its affect on sorghum leaves. Salinity (100 mM NaCl) shortened the length of the sorghum leaf growing zone by about 20%, and reduced the maximal REG Rate value by 34-70% depending on the stage of leaf development. The extent of growth inhibition varied as well with spatial location along the elongation region, and growth intensity in the basal part of elongation zone was not affected by the stress (Bernstein et al., 1993b).

Growing tissue located 20-40 mm from the leaf base was chosen as the experimental system for the study of salt-stress effect on apoplast acidification rate. Since the rate of local tissue elongation is at its maximum in this portion of the growing zone and local relative elongation rate considerably reduced by stress changes in cell wall acidification kinetics, if induced by the stress and affect cell growth, should be prominent in this location.

Effect of salt stress on the kinetics of apoplast acidification by growing leaf tissue

The objective of this work, was to evaluate correlation between tissue growth inhibition under salinity stress and possible changes in apoplast acidification rate of growing leaf tissue. We therefore compared the kinetics of external medium pH changes by control and salinized growing tissue of known local expansion rates and extent of growth reduction under stress. For reasons discussed above, the tissue analyzed was located 20-40 mm from the leaf base.

Growing leaf tissue from both salt-stressed and non-stressed plants induced acidification of the external medium (Fig 3). The pH of the external solution acidified at a nearly constant rate of 0.2 pH units per hour, until pH 4.7 ± 0.2 was reached after 11 hours. Salt stress did not induce a change in the kinetics of external pH acidification by growing leaf tissue. The rate of acidification, as well as the lowest value of pH achieved was similar for both

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control and salt-stressed growing tissues. That the acidification rate of the external solution did not change under salt stress conditions, suggests that salinity does not alter the capacity of the growing leaf cell to acidify its apoplast.

Acidification of the external medium began 2 hrs after the growing tissue was dissected and infiltrated with the reaction solution. Similar lag-phase for external medium acidification by growing tissues was characteristic also for maize roots (Moloney et al. 1981) and maize coleoptiles (Peters and Felle 1991; Kutschera and Schopfer 1985) and may in part be due to tissue growth recovery following dissection from the plant. Kutschera and Schopfer (1985) indeed reported a temporal correlation between recovery of segment elongation growth and initiation of external medium acidification. Prior to segment growth recovery, during the lag-phase, the tissue did not acidify the apoplast.

Although cell walls possess a significant buffering capacity (Pfan­z and Dietz, 1987), growing tissues are known to acidify their apoplast. Such was demonstrated for example for growing *Avena* (Rayle, 1973), and maize coleoptiles (Schopfer 1993; Peters and Felle 1991a, 1991b), growing maize leaf (Van-Volkenburgh and Boyer 1985), and roots (Versel and Mayor 1985; Moloney et al. 1981; Zidan et al. 1990). Moreover, tissue growth rates were demonstrated to correlate with apoplastic pH value, or with rates of apoplast acidification (Rayle 1973; Versel and Mayor 1985; Rayle and Cleland 1992; Kutschera and Schopfer 1985).

Several environmental conditions were shown to alter the acidification rate of the apoplast, as well as the growth rate of the tissue. For example, the apoplast is acidified in the light (Van-Volkenburgh and Boyer, 1985) following stimulation of H^+ extrusion by photosynthesis (Marre et al., 1989). Growth inhibition by water stress as well is accompanied by an increase in apoplastic pH. The growing tissue of well watered maize leaves was shown to acidify the apoplast at a rate higher than water stressed tissue and the pH of the apoplast to correlate with the growth rate of the leaf (Van-Volkenburgh and Boyer, 1985). In cotton leaves as well, dehydration caused an increase in apoplast pH (Hartung et al., 1988). Upon gravitropic stimulation, the H^+ efflux patterns from

root circumference are closely tied to geotropically induced shifts in root growth patterns. An enhanced acid efflux along the upper surface of a horizontally placed root is accompanied by an increased rate of elongation of the upper relative to the lower cells and result in downward curvature of the root (Mulkey and Evans, 1981). Lowering of the apoplastic pH on the convex side of actively bending roots by 0.4 pH units was sufficient to account for the increased growth on that side (Taylor et al., 1996). Only one reported study evaluated the effect of salt-stress on apoplastic acidification. Surface pH of maize roots growing zone was shown to decline during root growth (Zidan et al., 1990). Since the rate of acidification (during the initial 10 minutes of measurements), was similar in salt-stress and non-stressed roots while the growth rate was inhibited under stress, it was concluded that NaCl did not inhibit growth by reducing the capacity of epidermal cells to acidify their apoplast. Unlike leaf growth inhibition under water stress which might be mediated by alterations in apoplastic acidification (Van-Volkenburgh and Boyer, 1985), under salt-stress, both root (Zidan et al., 1990) and leaf (Fig 3) growing tissue retain their capacity for cell wall acidification. That the capacity of the leaf tissue to acidify the apoplast is not changed under same salt stress conditions which induced an inhibition of tissue elongation rate (Fig 2) suggests that salinity does not inhibit leaf growth by reducing capacity for cell wall acidification.

Effect of apoplastic NaCl level on the kinetics of acidification

In the experiments reported in Fig 3, we used a solution low in Na and Cl concentration as the bathing solution for apoplastic pH measurements. The reaction solution containing 0.1 mM Na and 1.3 mM Cl was used for analysis of apoplast acidification by both control and salinized growing tissue. In the intact plant, growing cells of the salt-stressed plant are exposed to apoplastic solution of different ionic composition than growing cells of the non-stressed plant. To exclude the possibility that the kinetics of acidification differs when cells are exposed to apoplastic solution of higher Na concentrations, as is typical of growing tissues under stress, we have measured the kinetics of acidification by salt-stress growing tissue segments bathed in reaction solution of different NaCl

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concentrations. 25 mM Na was selected as the highest level of salt to be examined since this was found to correlate with levels of apoplastic Na in these maize growing zone tissue (Bernstein and Neves-Piestun, unpublished).

Elevation of NaCl concentration in the external medium from 0.1 mM NaCl to 5 , 10 or 25 mM NaCl, did not change the acidification kinetics by salt stressed leaf growing tissue (Fig 4). Similar to acidification by non-stressed growing cells, the rate of external solution remained 0.2 pH unit per h and the lowest pH value reached was 4.7. Since a 250 times increase in NaCl concentration of the external medium did not alter the kinetics of acidification, we suggest that NaCl stress does not inhibit cell and leaf elongation by the affect on proton excretion to the cell wall apoplast.

Effect of Fusicoccin

Acidification of the media suggests proton extrusion. To determine in a more direct way if external media acidification was due to proton extrusion rather than leakage of acidic compounds from compartments such as the vacuole, fusicoccin (FC), a powerful enhancer of the plasmalemma H^+ -ATPase activity (Malek and Baker, 1977) was added to the external solution. FC is a toxin produced by *Fusicoccum amygdali* Del. (Marre, 1979). It has been referred to as "super-auxin" (Cleland, 1976), since its effects on cell growth and acidification are similar and often superior to those of indole-3-acetic acid (IAA). FC was added to the solution (2 μ M in the standard medium) 5 hr after the beginning of the experiment, when the acidification rate was already established at 0.2 pH units per hr. It caused an immediate, dramatic decrease in medium pH (Fig.5). Following FC application, the acidification rate increased from 0.2 to 1.5 pH units per h. About 0.8 h after FC application, the acidification rate decreased and proceeded in progressively lower rates until pH 3.8 was reached 3 h after FC application.

Similar results of FC effect on medium pH were reported for several growing plant tissues including maize coleoptiles (Peters and Felle 1991b; Kutschera and Schopfer 1985b; Schopfer 1993). The FC induced pH decrease of medium at which growing segments are incubated, is conventionally

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interpreted as a FC induced metabolism-dependent decrease in the pH value of the cell wall space (Marre, 1973). Apoplastic H^+ freely diffuse into the surrounding medium and causes external medium acidification (Marre, 1973). The major effect of FC on transport, was found to be the promotion of energy-linked H^+ extrusion (Marre 1979; Blatt and Clint 1989; Schopfer 1993) by plasmalemma ATPase, via an increase in electrical driving force of the cell membrane. It is associated with K^+ uptake or other monovalent cations. Treatments which severely decreased the intracellular ATP level, showed a suppression of the FC-promoted H^+/K^+ exchange (Marre et al, 1973). Considering the fact that in our system enhancement of acidification was observed in the presence of FC, the pH changes of the external medium reported in Fig 3 and 4 suggest that the pH changes of the external medium were due to changes in proton pumping activity to the cell wall space, and not a non-specific cellular leakage.

Kinetics of medium acidification by mature (non-growing) tissue

Unlike growing tissues, mature tissue does not necessarily acidify the external medium. If intensity of cellular growth is to correlate with rate of apoplast acidification or level of apoplast pH, than little or no such acidification is expected of mature non-growing cells. In our system, non-growing cells located 9 cm past the growing zone of the leaf, did not acidify the external medium (Fig 6). Alternatively, such mature cells alkalinized the external medium. A continuous increase of external medium pH was observed during 12 hours of measurements. Similar alkalization kinetics was typical of both non-stressed and stressed growing cells (Fig.6). Similar to non-growing leaf cells, non-growing maize root cells as well alkalinize their surface (Zidan et al., 1990). Considering the fact that in our system no acidification was observed in the non-growing tissue apoplast (Fig 6), the acidification reported in Fig 3 and 4 for growing cells suggest relation to growth processes.

Alkalization of the apoplast aqueous space by mature tissues is not a universal phenomenon. Changes in mature cells apoplast acidification rates or pH values might be tissue specific, or location specific and influenced by

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localized affects or physiological processes. For example, industrial emissions such as SO₂ and HCl are likely to alter the pH value of the apoplastic space of exposed cells. Additionally, apoplastic pH of specific mature photosynthetic source tissues might be locally changed by sucrose uptake, which is known to be mediated by a H⁺/sucrose cotransport mechanism in several tissues including phloem (Daie 1987; Giaquinta 1983, and references therein). Indeed peeled leaves of mature source leaves of *Vicia faba* were shown to acidify the surrounding medium (Aloni et al., 1988).

In summary, Our results suggest that salt-stressed and non-stressed growing cells regulate their apoplast pH endogenously and that salt stress does not alter the ability of the growing tissue to regulate its external medium pH. Large changes in apoplast Na (and Cl) concentrations, in the concentration range induced by salinization, although potentially hold the ability to inhibit H⁺ excretion (Kutschera and Schopfer, 1985) do not alter the growing tissue capacity for cell wall acidification.

That the capacity of the growing tissue to acidify the apoplast did not change under same salt stress conditions which induced inhibition of tissue elongation rate suggests that although proton excretion may mediate growth of maize leaf cells, salinity does not inhibit cell growth by impairing the acidification process or reducing capacity for cell wall acidification.

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Fig 1: Effect of salinity on maize leaf growth and shoot development. Plants were grown at 1 mM NaCl (control; A, C) or 80 mM NaCl (salt; B, D). Data represent results of non-destructive daily leaf length measurements, and RGR of individual leaves calculated from leaf length data. Each trace represents growth of one leaf. Data are means $\pm$ SD (n=10-15).

Fig 2: Spatial distribution of REG rate along the elongation zone of maize leaves growing at 1mM NaCl (control) or 80 mM NaCl (salt). Data were evaluated from prick hole-marking experiments of leaf 4 of maize. Data are means $\pm$ SD (n=6-9).

Fig 3: Effect of salt stress on acidification kinetics of external solution by growing tissue segments of maize leaves. The tissue chosen as the experimental system is located 20-40 mm from the leaf base and is characterized by highest level of REG rate in both control and salt treatments. REG rate in this growing zone portion is inhibited 25% by salinization (averaged spatially over the entire segment). Plants were grown at 1mM NaCl (control) or 80 mM NaCl (salt). pH values, recorded with a data logger, were measured with a combination pH electrode inserted into 3 ml aerated reaction solution containing 20 one cm growing leaf segments. Experiments were conducted at least 6 times for each treatment, and representative traces are shown.

Fig 4: Effect of elevated concentration of NaCl, on acidification kinetics by growing tissue segments of salt stressed maize leaves. Plants were grown at 80 mM NaCl. Reaction solution NaCl level was increased from 0.1 mM (standard medium) to 5,10 or 25 mM NaCl. 25 mM Na was selected as the highest level of salt to be examined, since this concentration was found to correlate with levels of apoplastic Na in these maize growing zone segments (Bernstein and Neves-Piestun unpublished). Experiments were conducted at least 4 times for each treatment, and representative traces are shown.

Fig 5: Effect of 2 μM fusicoccin (FC) on the acidification kinetics of external solution by growing tissue segments of maize leaf. Experiment was conducted 5 times, and representative trace is shown.

Fig 6: Kinetics of external medium pH change induced by mature cells of elongation leaf. Plants were grown at 1 mM NaCl (control) or 80 mM NaCl (salt). The tissue chosen as the experimental system is located 150-170 mm from leaf base, (90-110 mm from the end of the leaf elongating tissue) from the same growing leaves used for the growth zone study. Experiment was conducted 5 times and representative traces are shown.

Salt stress induced changes in the morphometry of growing maize leaf cells

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Introduction

Inhibition of leaf elongation is one of the primary effects of salt stress. The physiological mechanisms underlying leaf growth maintenance or inhibition are not clearly understood. Alterations in the nutritional status of the growing zone (Lazof and Bernstein, 1998 and references therein), cell wall characteristics (Cramer and Bowman 1991,1992; Neuman 1993), water relations (Munns and Termaat, 1986), and metabolite availability to the growing zone (Shahar Kafkafi and Bernstein, 1997) are some of the factors inspected for their possible involvement in the inhibition process.

In a previous section of this report we have quantified the inhibition effect of maize shoot development by salt stress. Rate of leaf initiation, final leaf length achieved and leaf growth rate were inhibited by salinization. Growth rate of the 4th leaf of maize, which was selected as the experimental system in this project, was reduced 35 % by salt stress (Neves-Piastun et al., 1997). Investigation into the leaf growing tissue, located at the base of the leaf, revealed that salt stress shortened the length of the leaf elongation zone, and reduced local magnitudes of REGR in selected locations along the elongating tissue. Leaf longitudinal growth is dependent on irreversible expansion of cells produced by the leaf basal meristem. Inhibition of leaf growth by salinity is associated with inhibition of these cells expansion. According to the Lockhart growth model (Lockhart, 1965) cell and tissue expansion are generated by turgor-driven wall extension. Reduction in wall extensibility, rather than turgor, were recently suggested to be the major growth limiting factor in long term growth inhibition under salt stress (Neuman et al., 1994; Cramer and Bowman 1991,1992; Neuman, 1993). In a previous section of this report we have demonstrated, that salt-stress did not

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change the capacity of growing tissues to acidify their apoplast, and therefore suggested, that reduction in wall extensibility and inhibition of cell expansion are not mediated via alteration in cell wall acidification.

Nutritional status of the salinized growing cells is conventionally discussed in relation to growth inhibition (Bernstein et al. 1995; Lazof and Bernstein, 1997 and references therein). Ions may be confined to various compartments within the plant cell. Microlocalization in sub-cellular compartments may explain differences in plant response to salinity and play a role in tolerance and damage mechanisms. Information on ion contents in different cell compartments, and quantitative knowledge of absolute volumes and volume fractions of such sub-cellular compartments is vital for evaluation of the role of cell nutritional status in cell growth inhibition under salt stress. No such information is available today for any growing tissue under salt stress. A stereological analysis of non growing mature mesophyll cells of the halophyte *Suaeda maritima* L., revealed that the volume fraction of vacuole increased under salinization (Hajibagheri et al., 1984). In this project, we performed a morphometric analysis of growing leaf cells, at several locations throughout the growing leaf profile to quantify the effect of salt stress on volume fractions of major cell compartments. The results obtained for the cytoplasm, the vacuole and cell wall of expanding cells will assist in interpretation of sub-cellular compartmentation results. In the following next 2 chapters of this report, we quantify changes in sub-cellular ion composition of growing cells induced under salt-stress, and evaluate their possible involvement in the localized growth inhibition process.

Materials and Methods

Plant material

Seeds of *Zea mays* L. cv GS 46, were soaked in tap water for 4 h and later sown on moist vermiculite in plastic boxes. The boxes were covered with aluminum foil and placed in a growth chamber at 27°C. After 4 days the seedling were exposed to 14-h photoperiod and were kept in that state

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for an additional 3-d. On day 7 after the beginning of germination, when the length of leaves 1 and 2 were similar (9-10 cm from leaf base.), leaf 3 was not yet visible and length of leaf 4 was < 1 mm, such selected plants were transferred to 1-L containers with 0.25 concentration modified Hoagland solution (Fe was supplied as 50 μ M Fe-EDTA, 20 μ M Fe(NH₄SO₄) and the Na level was elevated to 1 mM). The solution was aerated, changed daily and the pH adjusted every day to 5.7 with addition of KOH. Growth chamber temperature was kept at 27/23 °C and 60 / 80 % RH (day/night), intensity of light 400 μ E/s/m² and photoperiod of 14h.

Salinization commenced on day 7 after beginning of germination and elevated in three steps 24 hrs apart to 80 mM NaCl. At the time of transfer to hydroponics, control treatment received 1mM and salt treatment 20 mM of NaCl. On day 8 after germination, NaCl level of the salinized plants was increased to 50 mM and at the next day 80 mM of NaCl was reached.

Growth measurements

Daily leaf length measurements were used for evaluation of shoot growth, calculations of leaf growth rate and selection of leaf insertion number for future study. Length of every leaf on the plant was measured daily with a ruler from the base of the plant to the tip of the leaf (Bernstein et al., 1993). The leaf growth rate was calculated from the daily length measurements as the slope of the linear regression during the phase of rapid leaf growth.

Spatial distribution of elongation was calculated from short term displacement of marks along the growing zone of the leaf. Leaves were marked as described previously (Bernstein et al., 1993), accept that the initial distance between the marks was 5 mm. In short, the base of the of the stem was pricked with a series of 13 fine pins spaced 5 mm apart at least 3 h after initiation of the photoperiod. Growth was allowed to continue for another 5 h before leaves were carefully freed from older enclosing

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leaves, and the final positions of the pin marks were measured under a dissecting microscope. Data on the spatial distribution of elongation within the elongation zone of leaves were collected from 6-8 plants and were used for the calculations of REG Rate.

Relative Elemental Growth rate (REG rate) was calculated, according to Green, (1976), as of equation 1,

eq. 1:
$$RGR = (\ln L_t - \ln L_0) / \Delta t$$

When L_0 is distance between the two prick marks at the time of marking, L_t is distance between the two marks in time t and Δt is the time period in h from marking to final measurement.

Preparation for Transmission Electron Microscopy

One mm transverse sections of 4th leaf of maize, were excised from three replicates of control (1mM NaCl) and salt-treated (80mM NaCl) plants. Plants were sampled 15 days after germination (8 days after commencement of salinization). Specific leaf number and leaf developmental stage for sampling and analysis, was selected following an assessment of the kinetics of salt stress affect on shoot and leaf development (as described in the previous chapter of this report). Sections were excised from 3 locations, 1.3, 2.3 and 6.0 cm from the leaf base.

The cut sections were immediately placed in a fixative solution containing 5% glutaraldehyde in sodium cacodylate/HCl buffer (0.05M, pH 7.0) and kept for 3 hours at room temperature (the appropriate culture solution was added to this fixative to maintain the relevant osmolarity for each treatment). The samples were then thoroughly rinsed in buffer and post-fixed in 1% buffered osmium tetroxide overnight at 4° C. After thorough rinsing in distilled water (2% Triton -X100), the specimens were pre-stained in 0.5% aqueous uranyl acetate overnight at room temperature. They were then rinsed in distilled water and subsequently dehydrated in an ethanol series (50%, 75% and 3 X 100% for 20min each) and left overnight in 50:50 propylene oxide/Spurr resin. After several days infiltration with complete resin the samples were polymerised overnight at 65° C.

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Thin sections were cut on an LKB Ultratome III ultramicrotome, post-stained in lead citrate and examined on a Hitachi-7100 transmission electron microscope at 100kV.

Image Acquisition and Analysis

Digital images were acquired with a charge-coupled device (CCD) camera (Digital Pixel Advanced Imaging Systems, Brighton, U.K.). Ten images each of complete epidermal and mesophyll cell profiles were taken for each replicate of each leaf region from the control and salt-treated plants. These cell profiles were systematically selected by choosing the most central from each of 10 different grid mesh squares for each section examined.

The acquired digital images were then 'thresholded' for the grey level of the region of interest and the areas quantified utilising the image analysis software associated with the camera (Kinetic Imaging Ltd., Liverpool, U.K.). This automated 'thresholding' was complemented by manual painting where necessary (this was required especially for the cell walls).

The areas of the vacuole, cytoplasm, cell wall and total cell were determined in this way and the results expressed as A_{comp}/A_{cell} (area of the compartment of interest/total cell area), which is equivalent to volume/volume, i.e. the relative volume fraction (V_v) within the cell.

Results and Discussion

Effect of salinity on leaf growth

Salinity (80mM NaCl) inhibited leaf growth. Final leaf length attained by salinized leaves was 32 % lower than by non-stressed leaves (Fig 1A). Salt stress reduced final leaf length by shortening the period of rapid leaf elongation by 1 day (Fig 1A) and reducing leaf elongation rate throughout the period of rapid growth (day 13-21 after germination) (Fig 2). Day 15 after germination (8 days after commencement of salinization) was chosen for further

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experimentation in this project. At this time leaf 4 was in the stage of rapid elongation, 3 days post emergence from the whorl of older leaf sheaths and its growth rate was reduced 35% by the stress.

Effect of salinity on distribution of growth throughout the leaf elongation zone

Salt stress reduced the length of the basal leaf elongation zone by 23% from 65 to 50 mm. The maximal value of REG rate was reduced 23% by salinization and the tissue demonstrating maximum elongation growth was located 23 mm from the leaf base in both stressed and unstressed leaves (Fig 2).

The growing zone of the leaf can be divided into 3 distinct zones in reference to the tissue growth response to salt-stress:

Zone a. The tissue nearest the leaf base (0-8 mm), where salt-stress has no effect on cell elongation.

Zone b. 8-50 mm from the leaf base, where cell elongation is reduced under salt stress.

Zone c. 50-65 mm from the leaf base, where cell elongation is fully inhibited by salt stress.

Four locations in the growing tissue were accordingly chosen for the presented investigations of salt-stress effect on cell morphometry (Fig 2):

- a. 0.5 cm from the leaf base: (zone a).
- b. 1.3 cm from the leaf base: (zone b).
- c. 2.3 cm from the leaf base: (zone b, location of maximal value of REG rate).
- d. 6.0 cm from the leaf base: (zone c).

Effect of salinity on morphometry of growing leaf mesophyll cells

Morphometric analysis of cells throughout the development gradient revealed that the relative volume fraction of the cytoplasm of developing mesophyll leaf cells decreases with cell development (Fig 3) i.e., with increased distance from the node. Such trend is expected along a gradient of developing

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cells, where cytoplasm volume fraction decreases with vacuole volume increase during growth. Salt stress induced an increase in the relative volume fraction of the cytoplasm throughout the growing zone. Growing cells of salinized leaves therefore have higher cytoplasm volume fraction during their development than cells of non-stressed leaves.

Vacuole relative volume fraction increased with increased distance from the leaf base, i.e., with cell development. Such trend is of course expected along a gradient of developing cells, where vacuole takes progressively higher volume fraction of the cell. Salt stress induced a decrease in the relative volume fraction of the vacuole throughout the growing zone (Fig 4).

Salt stress induced an increase in the relative volume fraction of the cell-wall in youngest cells closest to the leaf base (zone a) (Fig 5). Throughout the remaining developmental gradient, relative volume fraction of the cell-wall of the stressed and non-stressed leaves was not significantly different. The reduced relative vacuolar fraction in the salt-stressed leaf cells might affect tissue capacity for compartmentation. Work is now in progress to quantify absolute volume fractions of the cellular compartments.

The relative volume fraction of the cytoplasm and vacuole from growing epidermis cells, were not affected by salt stress (Fig 6,7). This is unlike same compartments of mesophyll cells, which were altered under salinization (Fig 3,4). As expected, the spatial trend of vacuolar and cytoplasm relative volume fractions are similar in mesophyll and epidermis tissues. In both tissues, cytoplasmic relative volume fraction decreases with cell development, and vacuolar relative volume fraction increases with cell development.

Salt stress induced an increase in the relative volume fraction of the cell-wall in growing cells only during last stages of leaf development. During initial stages of cell development (zone a,b,c) the relative volume fraction of the wall was not affected by salt-stress (Fig 8).

At each location along the growing zone, the salt stressed cells are more developed because they mature closer to the leaf base than in control leaves and their displacement from the leaf base is slower. The reduced relative vacuolar fraction in salt-stress mesophyll is therefore opposite to that which

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would be expected on the basis of development. The alteration in relative volume fractions of sub-cellular compartments in the salt-stressed leaf, certainly affect cell capacity for compartmentation, and therefore might affect tissue resistance/sensitivity to the stress. Work is now in progress to quantify absolute volume fractions of these cellular compartments.

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Fig 1: Effect of 80mM NaCl on length (A) and growth rate (B) of leaf 4 of maize throughout its development. Data are average $\pm$ SE (n=10-15).

Fig 2: Effect of 80 mM NaCl on the spatial distribution of Relative Elemental Growth rate (REG rate) along the leaf. Results of marking experiments. Data are average $\pm$ SE ($n=4-7$). Zones a-d represent spatial division of the growing profile according to local tissue growth sensitivity to salt-stress (see text for details). Tissue growth sensitivity to the stress was used as a criteria for selection of locations for the morphometric analysis.

Fig 3: Effect of salt-stress on cytoplasm relative volume fraction of growing mesophyll leaf cells. Data are average $\pm$ SE ($n=10-30$).

Fig 4: Effect of salt-stress on vacuole relative volume fraction of growing mesophyll leaf cells. Data are average $\pm$ SE (n=10-30).

Fig 5: Effect of salt-stress on cell wall Relative Volume Fraction of growing mesophyll leaf cells. Data are average $\pm$ SE (n=10-30).

Fig 6: Effect of salt-stress on relative volume fraction of cytoplasm of growing epidermis leaf cells. Data are average $\pm$ SE (n=10-30).

Fig 7: Effect of salt-stress on relative volume fraction of vacuoles of growing epidermis leaf cells. Data are average $\pm$ SE (n=10-30).

Fig 8: Effect of salt-stress on relative volume fraction of cell walls of growing epidermis leaf cells. Data are average $\pm$ SE (n=10-30).