



# Statistical studies on anatomical modifications in the radicle and hypocotyl of cotton induced by NaCl

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## ABSTRACT

Salinity affects extensive arid and semiarid areas all over the world, producing diminished yields of many crops. Even though there is a variety of research and reviews related to physiological and anatomical modifications produced in salinity-tolerant species, today there are different points of view in relation to this topic, and especially due to technical limitations, there are few articles in which anatomical modifications have been quantified. The aim of this research is to quantitatively and statistically evaluate the modifications that are produced in radicles and hypocotyles of cotton seedlings growing in NaCl concentrations that affect the early ontogenetic stages of this crop. Germination of two varieties of cotton at increasing concentrations (0 to 450 meq/l) of NaCl was studied as well as the growth of seedlings after their germination in water. Anatomical modifications induced in radicles and hypocotyles were analyzed quantitatively with an image analyzer. There were no differences among varieties. While the percentage of germination decreased at the 252 meq/l NaCl level, the velocity of the process and seedling growth was inhibited at lower concentrations. With increased salinity, the cortex and pith of radicle increased in width, while the xylem decreased. In the hypocotyl, the width of cortex increased, as did the number and diameter of gossypol glands. This change deserves further studies in relation to the participation of these glands in the capacity of the seedlings to tolerate salinity.

**KEY WORDS:** anatomical changes, cotton, germination, growth, salinity

## INTRODUCTION

Soil salinity is one of the most important factors that limits crop production in arid and semi arid regions (Neumann 1995). Due to the great surface area of the world that is affected by soil salinity and subsequent losses in crop yield (Kent and Läuchli, 1985), there have been numerous reviews of the effects of salinity on plant physiological processes and subsequent effects on yield (Greenway and Munns, 1980; Munns, 1993; Shannon *et al.*, 1994; Neumann, 1995, 1997). In spite of this extensive literature there is still a controversy with regard to the mechanisms of salt tolerance in plants (Neumann, 1995, 1997).

Plants have different responses to salinity depending on their stage of development (Kalaji and Pietkiewicz, 1993; Rumbaugh

*et al.*, 1993; Rogers *et al.*, 1995). It is important to know how annual crops established in the field from seeds respond to salinity, as weak plants with slow growth compete poorly with weeds and plant pathogens, etc.

Cotton (*Gossypium hirsutum* L.), a plant of worldwide importance, is moderately tolerant of salinity, as a conductivity of the order of 7.7 dS.M<sup>-1</sup> has no effect on crop yield (Mass and Hoffman, 1976). The various species and varieties of cotton that have some tolerance to salinity stress produce different morphological changes in roots, stems, and leaves to cope with salt. It is generally felt that cotton's roots are the first organ affected by high levels of salinity (Reinhardt and Rost, 1995a). Among the most cited studies related to anatomical modifications induced by salinity stress is Strogonov's classic research (1962), which

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could not detect differences in root diameter after 4 weeks of growth under saline conditions, but this author reported that salinity was associated with a greater number of small diameter xylem vessels. In contrast, Neumann (1995) found an increase in root diameter produced by salinity and suggested that a reduction in cell size, an increase in root diameter and a smaller plant size could be adaptive advantages for prolonged survival in saline or dry soils. Other workers (Kalaji and Pietkiewicz, 1993, Shannon *et al.*, 1994) suggested that salt stress resulted in increased suberization and thickening of the endodermis, which in turn resulted in an increase in the diameter of both the root and the vascular cylinder. With regard to the effect of salinity on stems, Strogonov (1962) found that salinity retarded the differentiation of xylem and phloem elements while stimulating excessive growth of the cortex parenchyma cells. Unfortunately there are fewer studies on the effect of salinity on stems than on leaves and roots (Shannon, *et al.*, 1994).

None of the previous work included quantitative and statistical analyses of the effects of salinity on changes in plant anatomy. The objective of this research was to quantify the effect of salinity on changes of plant anatomy in the radicles and hypocotyles of two cotton varieties, in concentrations that affect the early ontogenetic stages which could affect the level of adaptation to stress.

#### METHODS

The acid-delinted cotton seeds of the varieties, Porá-INTA and Guazuncho II-INTA, were graciously provided by the National Agricultural Technological Institute (INTA) Agricultural Experiment Station in Santiago del Estero, Argentina. The seeds were disinfected with a 50% solution of commercial sodium hypochlorite for 10 minutes. Fifty seeds were placed between filter paper in 14 mm diameter Petri dishes and 25 ml of a 0, 100, 275, 325, 375, or 475 meq/l NaCl solution added. The Petri dishes were maintained at

25°C with a 16 hr photoperiod. There were 3 replications per treatment and the entire trial was conducted twice. Daily germination was registered, and a seed was considered germinated when its root length was greater than 1 mm. Measurements were discontinued when no changes were observed in 5 days. Average time to germination was calculated according to Brar and Stewart (1994). An ANOVA was conducted on this factorial design (2 varieties, 2 experiments, 6 salt concentrations) using the arcsine of the square root of percentages of seed germination. When the ANOVA revealed significant treatment differences, a Tukey test ( $p < 0.05$ ) was used for mean separations. This data was used to calculate a regression of the effect of the salinity concentration on the threshold of germination inhibition.

For the evaluation of the effect of salinity on seedling growth, the same varieties were used. The seeds were disinfected with a 50% solution of commercial sodium hypochlorite for 10 minutes and hydrogen peroxide (10 vol.) for 10 minutes and then rinsed with distilled water. The seeds were placed between two layers of germination paper saturated with distilled water, rolled and vertically placed, and allowed to develop at 25°C with a 16-hour photoperiod until the root length reached 3 cm. These germinated seeds (25 per replicate) were then incubated with 0, 100 or 275 meq/l NaCl. The root length and hypocotyl length were measured daily for 12 days (ISTA, 1996). A factorial experimental design of 2 varieties and 3 treatments with 4 replications was used. If the ANOVA was significant, mean separations were performed with a Tukey test ( $p < 0.05$ ).

The studies on the effect of salinity on anatomical changes were conducted on plants subjected to the treatments for 7 days. The seedlings were fixed in a Carnoy solution, embedded in paraffin, sectioned transversely, and stained with safranin-fast green according to conventional techniques (Johansen, 1949). The radicle sections were made at a distance of 1.5-2.0 cm from the transition region between the radicle and hypocotyl. The hypocotyl sections were

made in the middle of the internode. For each treatment 5 seedlings were randomly selected to prepare the sections for microscopic study of radicle and hypocotyle. Sections of each seedling were considered as a replicate. The final value for each replicate was the mean of the measurements taken from the sections (varying between 3 and 10 according to the quality of the section). A MIP-4 advanced image analyzer was used to quantify the surface occupied by the cortex, xylem vessels, pith, whole vascular cylinder and width of the endodermis in the radicles. The areas were expressed as a percentage of the total area of the section. In contrast, the larger size of the hypocotyl made it impossible to scan the entire cross section and thus obtain surface areas of the cortex, pith and stele. However, it was possible to measure the width of these tissues by moving the MIP4 field of view. Then the width of the cortex, diameter of pith and width of stele, number and diameter of gossypol glands were measured in hypocotyles. Data were expressed as a percentage of the total diameter. The Statgraphics software package was used to run an ANOVA on the data using the factorial design (varieties by salinity concentration). Mean separation tests used the Tukey test ( $p < 0.05$ ).

## RESULTS

The analysis of variance for germination percentage revealed that there were no differences in either varieties or experiments, but there were significant differences in salinity treatments. There was no interaction between the salinity treatments and varieties. Therefore, data from both varieties and experiments were pooled to calculate regressions between germination percentages and salinity treatments. Germination in the control group was not significantly different from the lowest NaCl concentration (100 meq/l). However there were significant differences between the control and the other salinity concentrations with values of approximately zero percent germination being reached at 375 meq/l. The relationship

between salinity values greater than 100 meq/l and percentage of germination is presented in Figure 1. The following regression equation for this relationship was significant ( $P < 0.0000$ ):  $GR = 0.0031x^2 - 2.701x + 583.29$  ( $r = 0.92$ ), where GR is the germination rate in percentages, and  $x$  is the salinity concentration (meq/l). A threshold value of 252 meq/l NaCl was calculated where cotton germination began to decline significantly. The average time to germination (Fig. 1) did not show differences between the control (1.9 days) and 100 meq/l concentration (2.1 days). The average time to germination between 100 meq/l and 375 meq/l was found to be described by the significant ( $P < 0.0000$ ) quadratic equation shown in Figure 1.

As was the case with seed germination, there were no significant salinity treatment

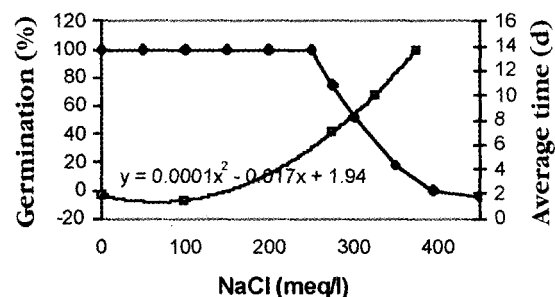


Figure 1. Idealized regression curves for the effect of NaCl concentration on cotton percentage germination (open squares) and the average time to germination (closed squares). The Porá-INTA y Guazuncho II-INTA varieties were pooled for these regressions. The results are average of the six replicas of fifty seeds

differences among varieties for seedling growth. However, the growth (Fig. 2) was more sensitive to salinity than the percentage of germination. Both salinity treatments significantly reduced the radicle and hypocotyl growth. Hypocotyl growth decreased 20-30% at 100 meq/l and 80-85% at 250 meq/l (Fig. 2A), while radicle growth decreased 10 and 35-40% respectively (Fig. 2B). The lower susceptibility of the radicle than the hypocotyl to increasing concentrations of salinity is also demonstrated by the low slope shown in Figure 3.

In relation to the anatomical differences of the radicles to salinity stress, the

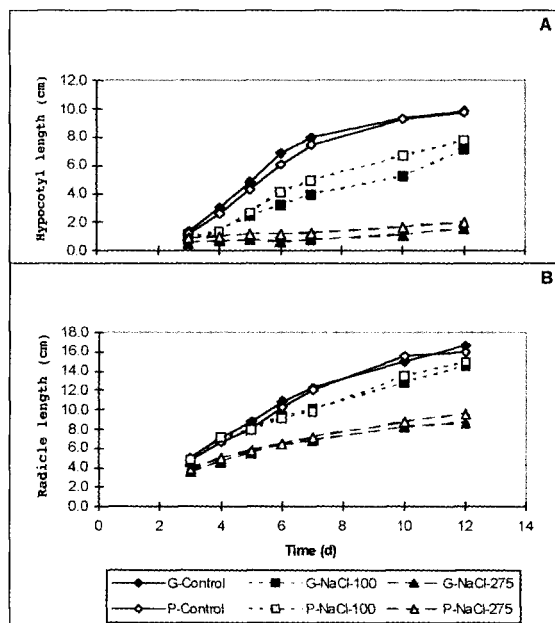


Figure 2. Time evolution of growth inhibition of hypocotyl (A) and radicle (B) of cotton seedlings growing in two levels of NaCl. The results are the average of four replicas of twenty five seedlings. P=Porá-INTA, G=Guazuncho II-INTA.

seedlings subjected to the higher salinity levels had a significantly smaller cortex (Table I), however there were no differences between varieties. There were no differences in the endodermis with increasing salinity but the Guazuncho II-INTA variety had a greater thickness of the endodermis ( $2.12\mu\text{m}$ ) than the variety Porá-INTA ( $1.84\mu\text{m}$ ). With an increase in salinity there was a decrease in the development of the xylem (Table I). In contrast, the pith was larger in the plants subjected to greater salinity. There were no differences in the volume of the stele, always being 8% of the

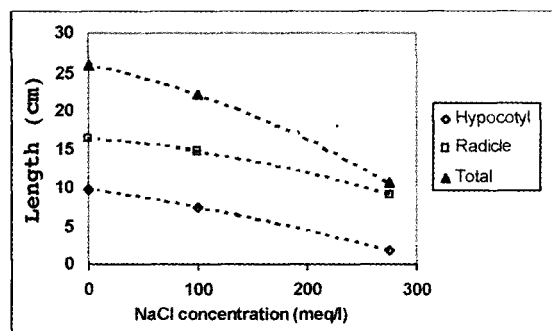


Figure 3. Effect of NaCl concentration on the growth of radicle and hypocotyle length of cotton (Varieties Porá-INTA and Guazuncho II-INTA were pooled in this figure). The results are the average of four replicas of twenty five seedlings.

total root volume, between either varieties or salt concentrations.

In the hypocotyl, the cortex thickness increased significantly for the Guazuncho II-INTA variety, while for the variety Porá-INTA, a significant increase was found only at the highest concentration tested (Table II, Fig. 4). In contrast, the thickness of the pith decreased with increased salinities (Table II, Fig. 4). The width of the stele varied between 11 and 15%, but there were no statistical differences in treatments and varieties.

Although the number of gossypol glands was greater in Guazuncho II-INTA, both varieties behaved similarly in that the number and diameter of gossypol glands increased significantly with increased salinity (Table II, Fig. 4). Very marked changes, *i.e.*: greater than 100%, occurred between the control and the 275 meq/l concentration.

TABLE I

Modifications in the areas occupied by the cortex, xylem vessels, and pith measured in transverse sections of the radicles of cotton seedlings. Results are expressed in percentages, and are averages of 5 replicas.

	Cortex (%)	Xylem Vessels (%)	Pith (%)
<b>Control</b>	86.41 a	2.82 a	0.16 a
NaCl 100 meq/l	86.96 a	3.15 a	0.35 a
NaCl 275 meq/l	83.72 b	2.34 b	2.82 b

\* Means within columns followed by same letter are not significantly different ( $p < 0.05$ )

TABLE II

Modifications in the width of the cortex, pith diameter, number, and diameter of gossypol glands measured in transverse sections of hypocotyles of cotton seedlings. Results are averages of 5 replicas.

	Cortex (%)		Pith (%)		Glands number		Glands diameter (µm)	
	Guazuncho	Porá	Guazuncho	Porá	Guazuncho	Porá	Guazuncho	Porá
<b>Control</b>	39.87 a	47.45 a	45.21 a	41.38 a	0.8 a	0.8 a	31.8 a	40.8 a
NaCl 100 meq/l	49.50 b	47.44 a	38.16 b	39.99 a	1.8 b	0.6 a	55.1 b	51.2 b
NaCl 275 meq/l	55.21 c	56.67 b	32.33 c	30.75 b	3.3 c	2.2 b	64.4 c	82.1 c

\* Means within columns followed by same letter are not significantly different ( $p < 0.05$ )

DISCUSSION

The threshold value of 252 meq/l NaCl found in this study was somewhat higher than that reported by Varghese *et al.* (1995), but these authors did not include a statistical analysis of their treatments and it is not possible to know if their treatments were significantly different. Bozcuk (1981) reported more than a 50% inhibition of

germination with a 100 mM NaCl concentration. However it is possible that this high level of inhibition at this low concentration was due to poor quality seeds, as only 58 % germination was obtained in water control treatment.

Even though the final percentage of germination is unaffected up to 252 meq/l, these seedlings would be at a competitive disadvantage because the speed of the

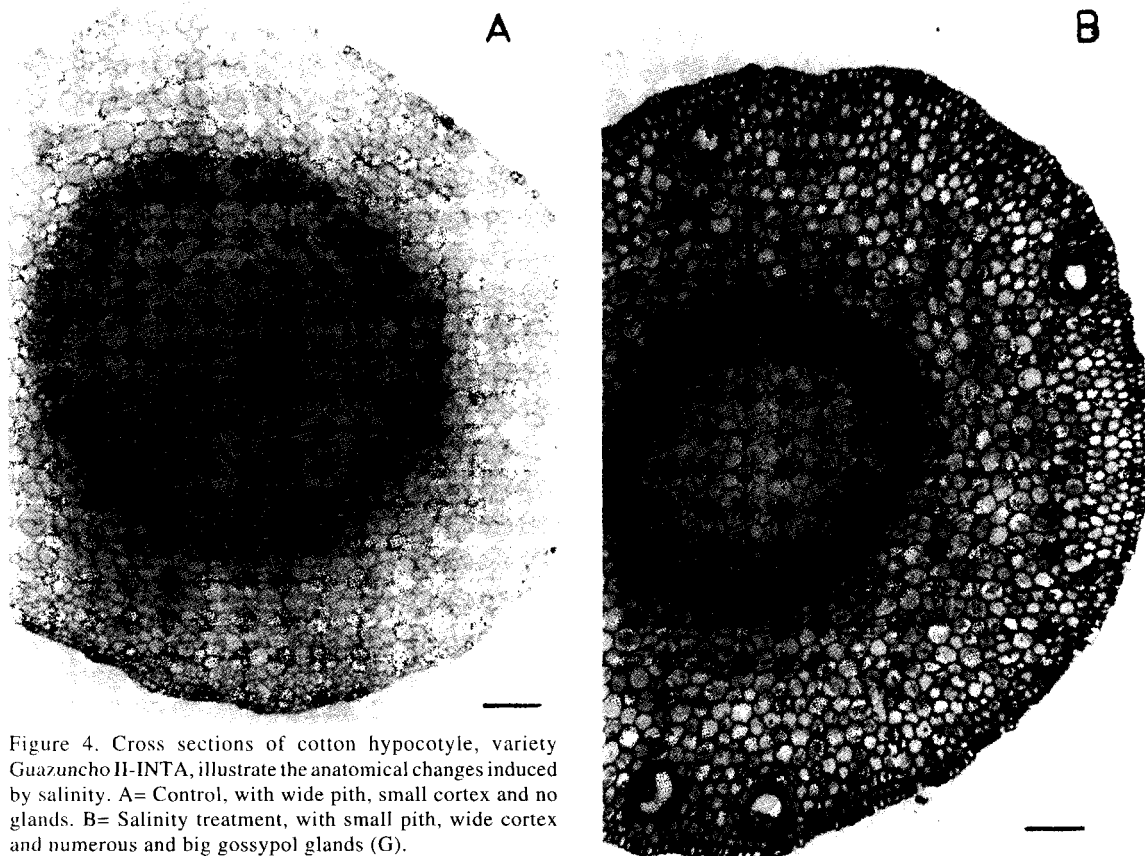


Figure 4. Cross sections of cotton hypocotyle, variety Guazuncho II-INTA, illustrate the anatomical changes induced by salinity. A= Control, with wide pith, small cortex and no glands. B= Salinity treatment, with small pith, wide cortex and numerous and big gossypol glands (G). Scale bars = 133 µm.

germination process is significantly slowed, as shown by the increase in the average time of germination in a quadratic equation for concentrations between 100 meq/l and 450 meq/l.

As reported by earlier researchers (Abul-Naas and Omran, 1974), germination was less susceptible to salinity than was the growth of the seedling. Maas and Hoffman (1976) reported that the first yield reduction occurred at a concentration of 7.7 dS.M<sup>-1</sup>. The growth of the radicle was less susceptible to increasing concentrations of salinity than the hypocotyl. Our finding of the lower susceptibility of the cotton radicle to NaCl as compared to the hypocotyl was also reported by Varghese *et al.* (1995). In contrast, Huang and Redmann (1995) and Shalhevet *et al.* (1995) found an inhibitory effect on the radicle instead of the hypocotyl for other species. This difference could be based on the fact that cotton is a species tolerant to salinity (Maas, 1984) and that the osmotic adjustment first occurs in the root, declining as the plant tissues become more distant from the location of stress (Shalhevet *et al.*, 1995). This type of mechanism would permit the root to isolate itself from the soil surface where the salinity is greatest because of the high rate of evaporation, thereby increasing the establishment of plants with this strategy.

The lower development of the xylem was probably caused by a repression in the development of metaxylem vessels. Similar results were reported by Reinhardt and Rost (1995b) who also examined different concentrations of salt on the growth of cotton. These authors hypothesized that the reduction in the production of metaxylematic vessels was due to a reduction in the cellular expansion produced by salt, which resulted in fewer vessels of smaller diameter. Huang and Redmann (1995) found that in various species the root thickness increased as the plants were subjected to salt stress. The only increase we found, however, was in the thickness of the pith, while the cortex decreased with increasing salt concentrations.

Strogonov (1962) suggested that halosucculence could be defined as the

hydration of plant tissues by a chloride ion-specific effect. Therefore our observation that the pith and/or cortex increase in diameter with increasing salinity could fit with the halosucculence Strogonov's concept. Similarly, Poljakoff-Mayber (1975) defined halosucculence as an increase in cell size even though cell division is interrupted. Therefore our pattern of hypocotyl modification could be termed halosucculence.

There is an agreement between the less-affected growth in the radicle and the gradual changes in the radicle anatomy with increased salt stress, and the greatest effect of the inhibitory effect of salinity on the hypocotyl was associated with drastic modifications in the hypocotyl anatomy. The marked induction of production of the gossypol glands with increased salinity deserves further study to help elucidate the mechanism for adaptation to salt stress.

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