



RESEARCH ARTICLE

Seed germination and morphological responses of cotton under model salt stress

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Abstract

Soil salinization is a major environmental stress that restricts plant growth and reduces crop yields. Developing salt-tolerant crop varieties using classical, molecular genetics methods and understanding the mechanisms of salt resistance is essential for sustainable crop improvement. This study evaluated seed germination and vegetative morphology of upland cotton (*Gossypium hirsutum* L.) under model salt stress, comparing Uzbek classical breeding varieties of AN-Bayovut-2, Tashkent-6, Namangan-77 and the Coker-312 variety with biotechnological knock-out varieties of the Porloq series viz. Porloq-1, Porloq-2, Porloq-3 and Porloq-4. An analysis of the results of seed germination parameters: relative germination (RG), average germination time (AGT), germination index (GI) and germination energy (GE) showed that the most resistant to salinity (NaCl concentrations— 50, 100, 150 and 200 mM) was the biotechnological gene-knockout Porloq-4 variety and the least resistant was the Coker-312 variety of classical breeding. A study of morphological parameters showed that 100 mM NaCl causes a slowdown in the growth of roots and shoots, as well as biomass gain in both genotypes. However, in the Porloq-4 biotechnological variety, the negative effects of NaCl were less pronounced compared to the salt-sensitive Coker-312 variety.

Keywords: cotton; morphological parameters; salt stress; seed germination

Introduction

Soil salinization is a global threat that affects 1100 million hectares of soil, which accounts for approximately 7 % of the Earth's land surface (1, 2). Soil salinization is a multifactorial process resulting from both natural geochemical processes and anthropogenic activities (2). Primary salinization phenomena caused by precipitation, sea level rise, saltwater intrusion into freshwater aquifers and rising temperatures have negatively affected a significant portion of cultivated land. In contrast, an estimated 30 % of irrigated land is affected by secondary salinization, primarily due to excessive fertilizer use, poor management and intensification of agriculture (1-3). The shortage of fresh water for irrigation and the degradation of agricultural land due to salt stress have led to significant losses in agricultural productivity, especially in arid and semi-arid areas (2-3).

Soil salinization occurs mainly due to the accumulation of water-soluble salts including sodium (Na^+), chlorides (Cl^-) and sulfate (SO_4^{2-}), causing osmotic stress and reducing the ability of plant root cells to absorb water from the soil (4-5). Among the water-soluble salts Na^+ and Cl^- are considered to be the main ions contributing to soil salinization (6).

Many agricultural crops including cotton, belongs to the category of glycophytes and cannot grow well in saline environment (7-10). The harmful effects of salinization on plants are manifested first by short-term osmotic stress and then by a prolonged accumulation of phytotoxic ions (5-8). Undesirable effects of salt stress on plants are manifested in morphological (stunting, chlorosis and seed germination disorders), physiological (inhibition of photosynthesis and nutrient imbalance) and biochemical (oxidative stress, electrolyte leakage, membrane disorganization) features of plants (7, 10).

Increasing resistance to salt stress could be one of the strategies to overcome this problem in crops. Traditional breeding methods and innovative approaches of genetic engineering have proved useful in developing salt-tolerant plants. Thus, using overexpression of several transcription factors (GhABF2, AtRAV1/2, AtABI5 and SNAC1), salinity-resistant cotton genotypes were obtained (8). The study of salt tolerance is an essential component of plant biology research as it increases our understanding related to complex mechanisms of salt tolerance in plants and explore strategies to mitigate the harmful effects of salt stress.

Thus, studying the effects of salt stress on cotton physiology

and morphology is highly relevant. Additionally, we have previously demonstrated that cotton varieties of the Porloq series, developed through biotechnological methods, have potential resistance to abiotic stress (11, 12). The present study aims to study the effect of salt stress on cotton via parameters such as seed germination morphology and identify the most resistant cotton varieties.

Materials and Methods

Plant material

Different varieties of *G. hirsutum* L. developed through classical breeding—(AN-Bayovut-2, Tashkent-6, Namangan-77, Coker-312) and some biotechnological knock-out varieties of the Porloq series (Porloq-1, Porloq-2, Porloq-3, Porloq-4) were taken for the study. In such a case, biotechnological cotton varieties were created by RNA interference of the *Phytochrome A1* gene. Coker 312 is a traditional cotton variety developed by Coker's Pedigreed Seed Company. In this study Coker-312 was used as a control because it is a standard system for cotton biotechnology with its high amenability to genetic transformation, regeneration and tissue culture. Porloq (Porloq-1, 2, 3 and 4) were obtained by hybridization of commercial varieties of Uzbek cotton (*G. hirsutum* cv. AN-Boyovut-2, C-6524, Namangan-77 and Toshkent-6 respectively) with the RNAi line Coker-312 transformed with pHellsgate-8::PHYA1 vector.

Growing conditions

The plants were grown in a phytotron, which provides precise environmental conditions that regulate factors such as light/dark, temperature and humidity for plant research. Cotton seeds, pre-sterilized with a 15 % sodium hypochlorite solution, were planted in a sterile nutrient medium. The seeds were incubated in darkness at a temperature of 28 °C for three days. After three days, Petri dishes with sprouted seeds were exposed to photoperiodic light/dark conditions for 16/8 hr cycle respectively. The light intensity was 5000 lx. After the emergence of seedlings, the plants were transferred to soil.

Next, to simulate salt stress, the studied plants were divided into two groups: control (without salt treatment) and experimental (under salt stress). After the plants had adapted to the soil, the control plants were treated with ordinary water and the experimental plants were treated with a salt solution (NaCl) for 21 days. The concentrations of salt solutions used in the experiment were 50, 100, 150 and 200 mM respectively. The salt concentration was increased daily by 25 mM NaCl to avoid osmotic shock (or stress) to plants, until the required concentration was reached. The desired NaCl concentration was maintained till 21st day of the experiment. Morphological parameters (length of the main root and shoot, leaves and roots dry weight) were measured after 21 days of salt stress exposure. The experiments were repeated three times. Each time 10 plants were studied.

All plants were subjected to genetic verification to confirm the presence of a genetic construct by vector-specific PCR (13).

Determination of seed germination

Germination parameters were determined according to the previously known method (14). The cotton seeds were placed on filter paper soaked in NaCl solutions with concentrations of 0, 50,

100, 150 and 200 mM in Petri dishes. Each Petri dish contained 10 seeds placed equidistant. Petri dishes were placed in a growing chamber at a temperature of 29/19 °C (day/night) and a light intensity of 550 $\mu\text{m}^2\text{s}^{-1}$ 16/8 hr (light/dark) photoperiod. After 4 days, the data was recorded. If the root that appeared on the seed was about 0.5 cm, then the seed was considered to be a germinated seed (14). Petri dishes were stored for 10 days in a growth chamber and data for seed germination was recorded regularly. Seed germination data were collected from day 4 to day 14 of the experiment. Data from day 4 of the experiment were considered as day 1, day 5 of the experiment as day 2 and finally, day 14 of the experiment as day 10. All experiments were performed in three replicates. The following parameters were used to evaluate germination parameters: RG, final germination (FG), AGT, GI and GE(14). RG was calculated as follows (14):

$$RG = \frac{n}{N} \times 100 \%$$

Where,

n = number of germinated seeds under salt stress condition

N = number of germinated seeds under control condition

FG, AGT, GI and GE were estimated using the following formulas (14):

$$FG = \frac{t}{T} \times 100 \%$$

Where,

t = number of germinated seeds

T = total number of seeds

$$AGT = \frac{n1 \times t1 + n2 \times t2 + n3 \times t3 + \dots}{n1 + n2 + n3 + \dots}$$

Where,

n = number of germinated seeds

t = germination time interval, in days

$$GI = \frac{n1}{d1} + \frac{n2}{d2} + \frac{n3}{d3} + \dots$$

Where,

n = number of germinated seeds

d = 1st, 2nd and 3rd day respectively

GE =

$$\frac{\text{the number of germinated seeds on the 10th day}}{\text{total number of seeds}} \times 100$$

Determination of morphological parameters

To determine the morphological parameters, plants of each genotype were thoroughly washed with distilled water. The length of the main root and shoot was measured in cm. To determine the dry weight, the leaves and roots were kept for drying in an oven at a temperature of 70 °C for 48 hr. The dried plant samples were taken out and weighed using precision scales.

Statistical analysis

All data was subjected to an analysis of variance (One-Way ANOVA) using the OriginPro 2022 software package. The data are presented as the average \pm standard error. The significance of

the differences between the averages was determined using the Tukey test. Differences in $p < 0.05$ were considered significant.

Results

Results of the study conducted on the determination of seed RG using different concentrations of NaCl showed that salt stress inhibited this parameter in most genotypes. In Porloq varieties, seed germination was only slightly affected up to 100 mM NaCl, but declined sharply at 200 mM (Fig. 1A). In the varieties developed via classical breeding i.e. AN-Bayovut-2, Tashkent-6 and Namangan-77, seed germination decreased at a NaCl concentration of 50 mM and a significant decline was noted at 150 mM and 200 mM NaCl. In the Coker-312 cotton variety, seed germination under model salt stress conditions was minimal, decreasing almost linearly over the entire range of NaCl concentrations. Thus, at 100 mM NaCl, the seed germination rate of the Coker-312 variety ($44 \pm 1.8\%$) was 2 times lower compared to the Porloq-4 variety ($88 \pm 3.7\%$). At the same time, with the same concentration of NaCl, the seed germination of the Porloq-4 variety was 63 % higher compared to the parent variety Tashkent-6.

The AGT determination showed that an increase in this parameter was observed under the influence of various

concentrations of NaCl: in the Porloq-4 variety, this raise in AGT was the lowest. At the same time, in the Coker-312 line, it was the maximum. The remaining varieties occupied an intermediate position (Fig. 1B). At the same time, in the Porloq-4 variety, the seed germination time increased from 4.5 days at a NaCl concentration of 0 mM to 6.1 days at 200 mM NaCl. A similar trend of increase in the seed germination time from 6 days to 9 days was noted in the Coker-312 line.

It was found that GI decreased with increasing NaCl concentration in all the studied varieties. The significant decrease in the GI was recorded in Coker-312 (from 3.0 to 0.6) and the minimum decrease in Porloq-4 (from 5.8 to 2.9) (Fig. 1C).

In addition, GE was significantly low in salt exposed plants of all genotypes. The maximum decrease in GE was recorded in Coker-312 (by 51 %) and the minimum in Porloq-4 (by 19 %) (Fig. 1D).

An analysis of the results showed that the biotech cotton variety Porloq-4 exhibited the highest tolerance to the negative effects of salt stress, even at the germination stage. Meanwhile, the control variety Coker-312 demonstrated minimal tolerance to salt stress. The obtained results are consistent with the available literature, which shows that salt-tolerant cotton varieties have higher germination rates under saline conditions (11-13).

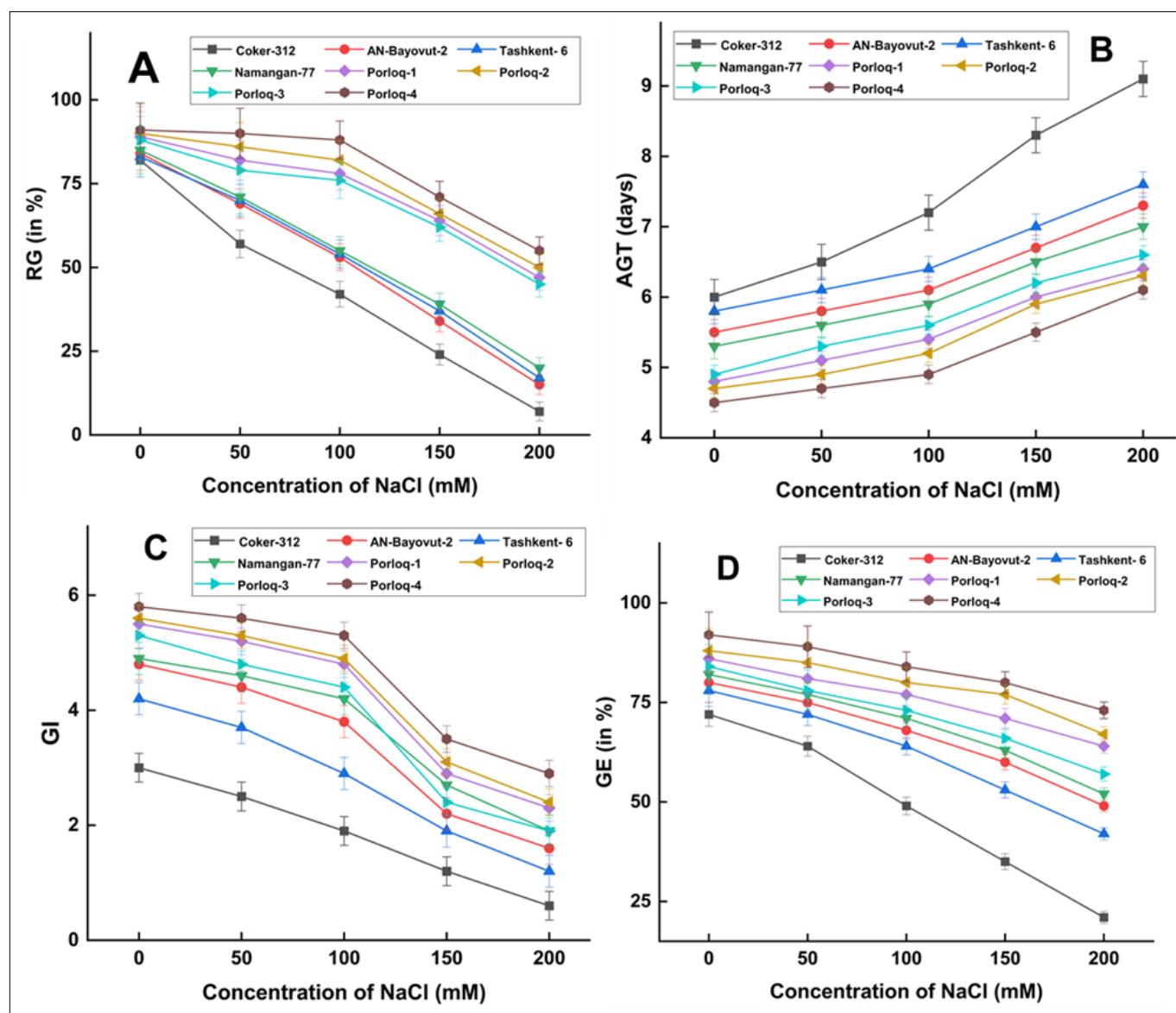


Fig. 1. The effect of different concentrations of NaCl on seed germination parameters of various cotton varieties. $p \leq 0.0001$; $n = 30$.

Further studies were conducted using 0 mM and 100 mM NaCl to simulate varying salinity levels. In this case, the Porloq-4 was used as the resistant variety, while Coker-312 was used as the sensitive variety, as these two varieties contrasted each other the most closely.

Further experiments to determine the effect of salt stress on the morphological parameters of cotton seedlings revealed that an increase in salt concentration resulted in a decrease in the length of both the primary root and shoot in both genotypes (Table 1). At the same time, in the salt-sensitive Coker-312 variety, the suppression of root and shoot growth was more pronounced than in the salt-resistant Porloq-4 variety. Root growth in the Porloq-4 variety was suppressed by 28.6 % when exposed to 100 mM NaCl and in the Coker-312 variety reduction of 56.9 % was noted. At the same time, the shoot length in the Porloq-4 variety decreased by 45 % and in the Coker-312 variety by 58.4 %. The data agree with the available literature showing that high salt concentrations can significantly inhibit root and shoot growth in cotton seedlings (14-17).

An increase in NaCl concentration to 100 mM slowed the growth of leaves and roots in both genotypes. However, the percentage decrease in dry weight of leaves and roots due to salinity compared with respect to control was lower in the Porloq-4 variety than Coker-312. This indicated that Porloq-4 is a more salt-resistant variety. In Porloq-4 variety, the dry weight of leaves and roots decreased by 14.9 % and 22.4 % while in the Coker-312 variety by 55 % and 59.5 % respectively.

Discussion

Although cotton is a moderately salt-tolerant crop, salinity levels above 7.7 dS/m negatively affect most growth parameters of cotton, including seed germination and seedling growth (14). Seed germination is an important stage of development that determines the subsequent development and growth of plants (18). Increased soil salinity leads to a decrease in the rate and energy of germination along with an increase in germination time (19). Moreover, such a negative effect of salinization on the parameters of seed germination is associated with a decrease in the osmotic potential of the surrounding soil water, which leads to a reduction in the absorption of water by dry seeds (20, 21). In addition, the effect of salinity on germination may be mediated by the toxic effects of excess Na⁺ and Cl⁻ ions, which manifests itself in the disruption of many biochemical processes, as well as changes in the balance of hormones, especially gibberellin/abscisic acid (20-22).

The results of the current study showed that in the first experiment, salt stress had a negative effect on the parameters of seed germination, which was manifested in a decrease in germination and GE, as well as an increase in germination time. However, the degree of change in the parameters of germination depended not only on the level of salt stress, but also on the genotype. At the same time, the genotype of Porloq cotton showed

good results even at the stage of severe salt stress. The obtained results are consistent with existing literature on the detrimental effects of abiotic stress, including salinity, on cotton seed germination (11-13). Furthermore, several studies have shown that salt-tolerant cotton varieties have higher germination rates under salinized conditions (8, 14, 17). Thus, studies showed that salt stress had a significant effect on the parameters of cotton seed germination and this process depended not only on the intensity of the stress exposure, but also on the genotype (14). At the same time, it has also been reported that salt-tolerant cotton genotypes (NIAB-512, NIAB-135 and FH-152) showed tolerance even to high levels of salt stress already at the germination stage (14). The greater resistance of these varieties to salt stress is attributed due to the activity of the antioxidant system (11, 12).

The effect of salt stress on seedlings is due to the fact that inside the cell, salt stress leads to the occurrence of osmotic and ionic stress, which accelerates oxidative stress mediated by the hyperproduction of reactive oxygen species (ROS) (11-13, 23). The accumulation of ROS leads to oxidative damage of proteins, DNA and lipids, destabilization of membranes and increased permeability (11-13, 23). In addition, the consequence of hyperproduction of ROS is the inhibition of energy production through photosynthesis and respiration, which causes a decrease in the length of roots and shoots, dry weight and accumulation of biomass, reducing the growth and development of cotton (23-25).

Soil salinization can lead to various morphological, physiological and biochemical changes in plant cells, causing numerous changes in their structure and functions (5, 9, 10, 15). Thereby, morphological parameters of plants, such as root and shoot length, dry weight of roots and shoots, are often used as selection criteria for salt tolerance (24). In this regard, the effect of salt stress on the key morphological parameters of cotton seedlings, i.e. root and shoot length as well as dry root and shoot weight, was analyzed. The results showed a decrease in the dry shoot and root weight, as well as shoot and root length in both varieties. Such a decrease in root length, surface area, volume and average diameter can occur due to inhibition of mitosis, decreased synthesis of cell wall components, damage to the Golgi apparatus and changes in polysaccharide metabolism under the influence of elevated salt concentrations (14-16). However, the Porloq-4 variety showed a slight decline in these parameters compared to the Coker-312 variety, indicating greater resistance of the Porloq-4 variety to salt stress. The higher tolerance of the Porloq-4 variety to salt stress can be explained by the previously demonstrated increased activity of the antioxidant system and increased content of osmoprotectants (11, 12). This is consistent with other studies that demonstrated a positive correlation between the tolerance of cotton varieties and a number of physiological parameters (14, 15, 25).

This growth retardation may be due to osmotic damage or specific ion toxicity caused by salt uptake (5, 8, 14, 15). Respiratory depression is the main reason for growth

Table 1. The effect of salinity on the morphological parameters of contrasting cotton varieties

Variety	NaCl (mM)	Root length (cm)	Shoot length (cm)	Dry weight of leaves (g)	Dry weight of roots (g)
Coker-312	0	7.2 ± 1.8	12.5 ± 2.8	1.51 ± 0.37	0.42 ± 0.16
	100	3.1** ± 1.2	5.2*** ± 1.6	0.68*** ± 0.22	0.17** ± 0.1
Porloq-4	0	10.5 ± 2.2	19.1 ± 3.1	1.61 ± 0.43	0.58 ± 0.21
	100	7.5* ± 1.9	10.5** ± 2.3	1.37* ± 0.39	0.45* ± 0.18

* $p \leq 0.01$; ** $p \leq 0.001$; *** $p \leq 0.0001$; n = 30

retardation during stress caused by salinity (9, 14, 15). A smaller decrease in photosynthetic leaf tissue during salt adaptation makes it possible to maintain energy synthesis, which can then be redirected to support the leaf growth process (14, 15, 19, 21). Thus, a significant decline in dry weight of leaves of the salt-sensitive Coker-312 variety indicates that under salinization conditions, this line cannot synthesize enough energy necessary for cell growth and development, compared with the more salt-resistant Porloq-4 variety. The results are consistent with data obtained from the studies on the effect of salinity on cotton morphological parameters (14, 15, 25).

Thus, the results of the assessment of salinity on morphological parameters showed that an increase in NaCl concentration to 100 mM causes a slowdown in root and shoot growth, as well as a reduction in biomass in both genotypes. However, in the biotechnological Porloq-4 variety obtained by RNA interference of the *PhyA1* gene, this negative effect of NaCl was less pronounced than in the salt-sensitive Coker-312 variety, which may be due to the peculiarities of expression of salt tolerance genes, as well as the physiological and biochemical mechanisms that determine the resistance of this variety to salinity. The biotechnological varieties of the Porloq variety have potential salt tolerance (11-13, 23), which the present result is consistent with.

Conclusion

Experiments were conducted to determine the effect of salt stress (NaCl solution concentrations of 50, 100, 150 and 200 mM) on cotton varieties. Eight cotton varieties were assessed and results showed that highest seed germination, AGT and maximum GI were observed in the cotton variety Porloq-4. The minimum values were recorded in Coker-312 variety. The sensitivity of Porloq-4 and Coker-312 to salt stress was evaluated through further experiments. The study of morphological parameters such as root and shoot length, leaf growth, dry weight of roots and leaves proved that these traits did not change significantly under salt stress (NaCl salt solution at a concentration of 100 mM) indicating high salt resistance in Porloq-4. The resistance of Porloq cotton varieties to salinity may be due to the expression of salt tolerance genes and the physiological as well as biochemical mechanisms that determine the resistance of this variety to salinity.

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Authors' contributions

VSK, ASI, IBS and NRR carried out the experiments, performed

statistical analysis, wrote and revised the manuscript. FSR, SHM, OLI, DEU, JKN, KAU, ANA, SOK, SBK, NNN, MMK, FIB and BKR collected the data, participated in the experiments and preparation of the manuscript. ZTB edited and approved the manuscript. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: The authors declare that they have no competing interests.

Ethical issues: None

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