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RESEARCH ARTICLE

Evaluation of salt tolerance in cotton (*Gossypium hirsutum* L.) under NaCl treatment based on chlorophyll and SPAD parameters

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Abstract

Increasing soil salinisation, exacerbated by global warming, poses a major threat to sustainable agriculture, as salinity severely impairs plant growth and development. Salinity is one of the most severe abiotic stresses affecting plant growth and development. In this study, the salt tolerance of 28 cotton (*Gossypium hirsutum* L.) cultivars was evaluated under controlled phytotron conditions through the measurement of chlorophyll a, b, total chlorophyll (a+b), carotenoids and soil plant analysis development (SPAD) index under NaCl treatments (0, 50 and 100 mM). The experiment was conducted in triplicate and measurements were taken 21 days after salt application. The obtained data revealed significant variability among the cultivars. At 100 mM NaCl, a reduction in chlorophyll a exceeding 20 % compared to the control was observed in 12 cultivars, whereas others (e.g., Baraka, Gulbahor-2, C-4727) maintained relatively high levels of total chlorophyll. A strong correlation was found between SPAD readings and laboratory-determined total chlorophyll content ($r = 0.82$; $p < 0.001$), confirming the suitability of SPAD as a rapid screening tool for identifying salt-tolerant genotypes. The findings highlight promising donor cultivars (Porloq-1, Afsona, Baraka, Kelajak, Buxoro-14) for breeding programmes and recommend an integrated approach combining chlorophyll content and SPAD measurements for early-stage selection of salt-tolerant cotton genotypes. The results may contribute to breeding for stress resistance in cotton and support the development of strategies for precision agriculture under saline conditions.

Keywords: chlorophyll; cotton; photosynthesis; salt stress; SPAD

Introduction

Climate change and global warming are contributing to soil salinisation, one of the most serious abiotic threats limiting global agricultural production. The proportion of land negatively impacted by high salinity is increasing worldwide due to both natural causes and agricultural practices. The development of salt-tolerant crop varieties is essential for the sustainable use of saline soils, land reclamation and the improvement of living conditions in arid and semi-arid regions (1–3). Salt stress represents one of the major abiotic constraints to crop productivity, particularly in regions characterised by water scarcity and secondary salinisation. Cotton (*Gossypium hirsutum* L.), an important fiber and partially food crop, is highly susceptible to the adverse effects of excessive salt accumulation in soil, which leads to reduced photosynthetic efficiency and, consequently, yield

decline.

Photosynthetic pigments such as chlorophylls and carotenoids are key indicators of the physiological status of plants and play a central role in the photosynthetic process (4). It is well established that salinity stress significantly affects chlorophyll content in cotton, which is critical for photosynthesis. Previous studies have demonstrated that increasing salinity reduces chlorophyll concentration in salt-sensitive cultivars, whereas salt-tolerant genotypes tend to maintain relatively stable pigment levels (5). This decline is associated with impaired photosynthetic activity and increased oxidative stress. However, some varieties are able to maintain the levels of these pigments, suggesting adaptive mechanisms. For example, with increasing salt levels, a significant decrease in chlorophyll content was observed, confirming the importance of these pigments for salt stress tolerance. In some cultivars, however, pigment levels remain stable, reflecting adaptive mechanisms that preserve photosynthetic integrity under salt stress (6).

The decrease in chlorophyll content under salinity may result from the disruption of chloroplast ultrastructure and photosystem II, ultimately leading to reduced photosynthetic capacity, stunted growth and yield loss. Carotenoids, on the other hand, play a protective role in mitigating oxidative damage induced by salt stress. They are involved in non-photochemical quenching (NPQ), which dissipates excess excitation energy and prevents photodamage to photosystem II. Previous studies have confirmed that salinity stress alters the structure and function of enzymes and pigments involved in photosynthesis (7). Photosynthetic pigments such as chlorophyll a (Chl_a), chlorophyll b (Chl_b) and carotenoids play a vital role in the absorption and transmission of light.

The ratio of chlorophyll a:b serves as a classic physiological and biochemical indicator of the adjustment of photosynthetic apparatus to stress conditions, light intensity and salinity (8). Higher Chl_a:b_ratios (> 3) generally indicate a predominance of chlorophyll a in reaction centers, typical of sun-exposed and stress-tolerant plants, whereas lower ratios (< 2.5) correspond to higher chlorophyll b levels, characteristic of shade-tolerant or stress-sensitive genotypes that compensate for impaired electron transport by enhancing light-harvesting capacity. In many plants, chlorophyll content in leaves consistently declines under salt stress (9). Research shows that while chlorophyll content declines in salt-sensitive cotton, it remains relatively stable in salt-tolerant varieties (10).

Under salt stress, a decrease in carotenoid content is observed in sensitive cotton varieties, which may indicate a reduction in their photoprotective capacity (4). Chlorophyll and carotenoid content often correlate, reflecting the overall photosynthetic capacity of the plant. However, in some cases, a discrepancy is observed: varieties with high chlorophyll content may have low carotenoid content (11). Salt stress leads to a decrease in carotenoid content, which is probably associated with the degradation of ß-carotene and a decrease in the photoprotective capacity of the pigment complex.

Despite the accumulated knowledge on the effects of salt stress on the photosynthetic apparatus, there remains a lack of data combining direct spectrophotometric pigment measurements with non-destructive SPAD indices across a wide range of cotton genotypes. Most studies use only one method, limiting the ability to correlate structural changes in the pigment complex with operational field indices (12). The lack of comprehensive comparative studies hinders the development of reliable physiological criteria for the early identification of salt-tolerant genotypes.

Therefore, there is a need for the parallel use of spectrophotometric analysis of chlorophyll and carotenoid content, as well as SPAD index measurements, to combine the accuracy of laboratory determinations with the advantages of rapid, non-destructive assessments. This approach provides a more comprehensive understanding of plant responses to salinity and enables the identification of informative indicators of tolerance. Based on the identified scientific gap, the aim of this study is to comprehensively evaluate the response of 28 cotton varieties to salt stress using spectrophotometric determination of pigment composition and a portable SPAD chlorophyll meter, followed by the identification of diagnostic indicators reflecting the level of salt tolerance.

Materials and Methods

Plant material and experimental design

A total of 28 local and introduced cotton cultivars were used in this study: Afsona, Baraka, C-4727, Namangan-77, Porloq-1, Ravnaq-1, Buxoro-6, Sulton, Kupaysin, Namangan-102, Nasaf, Omad, Buxoro-102, Chimboy, Buxoro-10, Buxoro-14, Navbahor-2, Kelajak, Namangan-34, Gulbahor-2, Ishonch, CGB-1, CGB-2, CGB-3, CGB-4, CGB-5 and CGB-6. The internationally recognised standard cultivar TM-1 (*G. hirsutum*) was used as a control.

The experiment was conducted in a phytotron at the Center for Genomics and Bioinformatics under controlled environmental conditions. Salt stress was simulated by applying sodium chloride (NaCl) solutions at concentrations of 0 mM (control), 50 mM and 100 mM. The experiment was arranged in a randomized design with 3 biological replicates per treatment. Ten pre-soaked seeds of each cultivar were sown per pot (each treatment consisted of 3 replicate pots, each containing 10 plants).

Plants were grown at 22–29 °C with a 16 hr light/8 hr dark photoperiod. Watering was performed every other day with 100 mL of distilled water (control) or a NaCl solution (50–100 mM), depending on the experimental design (13). The experiment lasted for 21 days. All measurements, including pigment extraction, were performed after the end of the experiment.

SPAD measurements

Relative chlorophyll content was estimated using a portable SPAD-502 chlorophyll meter (Konica Minolta, Japan). Five readings were taken per leaf at evenly distributed points and averaged. Measurements were performed on the third fully expanded leaf of 5 plants per cultivar in each treatment. SPAD values were expressed in arbitrary units. Before each measurement, the instrument was calibrated according to the instructions of the manufacturer. This was achieved by zero calibration: the instrument's measuring head was closed without a sample and the optical signal was set to zero. Instrument accuracy was verified using the included control plate. The deviation did not exceed ± 0.3 SPAD, which is within acceptable

limits. Measurements were performed on the midsection of a fully expanded leaf, avoiding large veins. Three independent biological replicates were conducted for each experimental setup.

Determination of chlorophylls and carotenoids

Chlorophylls and carotenoids were extracted from leaf samples using 80 % (v/v) acetone according to the classical method (14). Fresh leaf tissue (0.2 g) was homogenised in 10 mL of chilled 80 % acetone and incubated for 24 hr in darkness at room temperature. Absorbance of the extracts was measured at 663, 645 and 480 nm using a UV-Vis spectrophotometer (Metash UV-5100, China). Pigment concentrations were calculated using the following equations (14):

Chl a (mg L^{-1} FW)=12.72×A_{663}-2.58×A_{645}

Chl b (mg L^{-1} FW)=22.87×A_{645} - 4.67×A_{663}

Carotenoids (mg L^{-1} FW)=(0.114×A_{663})+A_{480} - (0.638×A_{645})

C (mg g^{-1} FW)= C (mg L^{-1})×V/1000×W,

where V (ml) is extraction volume and W (g) - weight of leaf tissue. V= 4 ml, W= 0.2 g.

Chlorophyll a, b and total chlorophyll (a + b) contents were expressed on a fresh weight basis.

Statistical analysis

All data are presented as mean ± standard error of the mean (SEM). Statistical analyses were performed using the R software environment (15). To determine the effect of NaCl concentration on pigments and SPAD parameters, one-way ANOVA (SPSS 21 package) and two-way analysis of variance (Microsoft Excel 2010) were used. The significance of differences was tested using the F-test and critical differences (CDs) were calculated with probabilities of $p < 0.05$ and $p < 0.01$. Graphical visualisation and preliminary data processing were carried out using Microsoft Excel 2010.

Results and Discussion

Variation in chlorophyll content under salt stress

In breeding programmes aimed at improving salt tolerance, chlorophyll content is a key physiological parameter, as higher levels of photosynthetic pigments typically correlate with greater plant resistance to stress. In this study, the response of 28 cotton varieties to NaCl treatment (0, 50 and 100 mM) was assessed using 2 complementary methods: spectrophotometric analysis of chlorophyll and carotenoid content and non-destructive measurements of the SPAD index using a SPAD-502 portable chlorophyll meter. These methods provided a comprehensive characterization of changes in pigment composition under salt stress.

Changes in chlorophyll a content under salt stress

In this study, cultivar-specific variability in photosynthetic pigment content was assessed under the influence of salt stress at concentrations of 50 and 100 mM (Fig. 1 A–F). It was found that all studied cultivars exhibited varying degrees of change in pigment profile compared to the control.

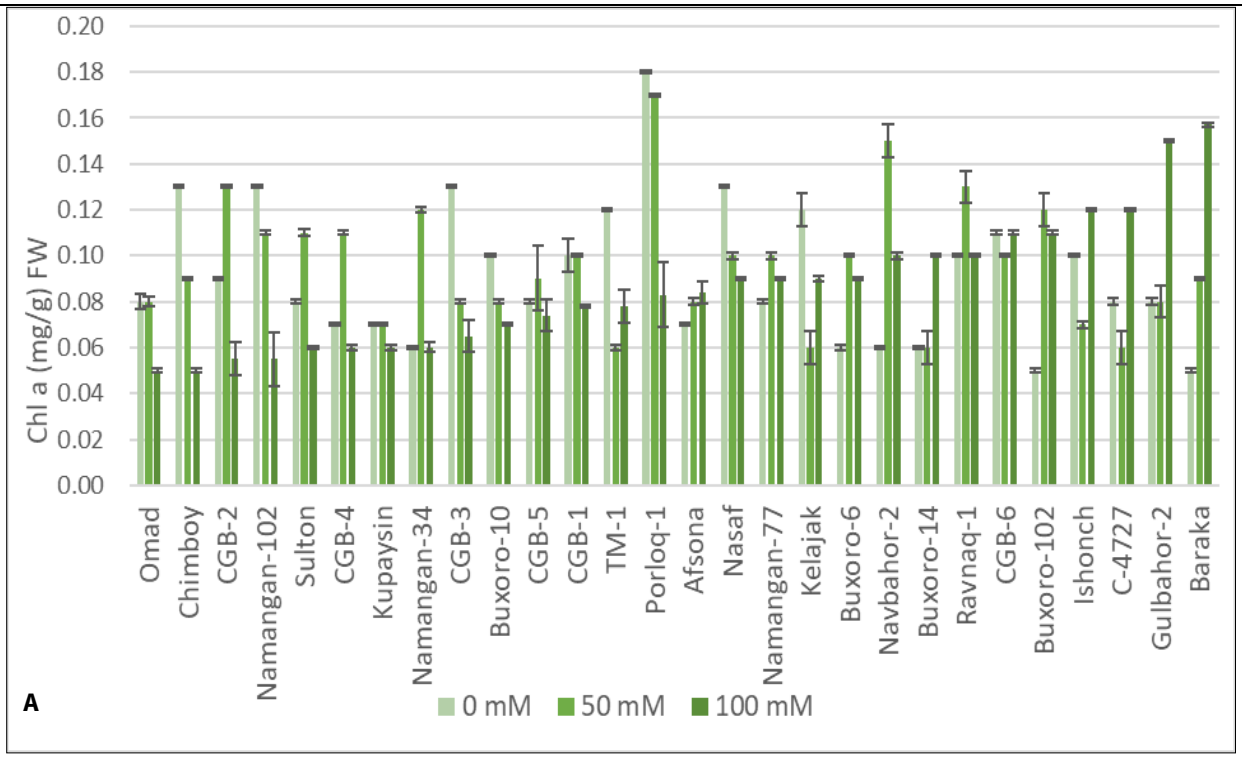
Under the influence of salt stress, the varieties were divided into 3 groups: (1) with a decrease in chlorophyll content, (2) with an increase in its content and (3) with insignificant changes compared to the control. At a NaCl concentration of 50 mM, a decrease in chlorophyll a content was observed in the varieties Chimboy, Namangan-102, CGB-3, Buxoro-10, TM-1, Porloq-1, Nasaf, Kelajak, CGB-6, Ishonch and C-4727 (Fig. 1A). In contrast, an increase in chlorophyll a content was recorded in the varieties CGB-2, Sulton, CGB-4, Namangan-34, Afsona, Namangan-77, Buxoro-6, Navbahor-2, Ravnaq-1, Buxoro-102 and Baraka. Minor deviations from the control level were found in the Omad, Kupaysin, CGB-5, CGB-1, Buxoro-14 and Gulbahor-2 varieties. The values varied within the range of 0.060–0.170 mg/g FW (Table 1; $p < 0.05$). The average values are presented in the diagram (Fig. 2A). The obtained data are consistent with the literature, according to which both a decrease and an increase in

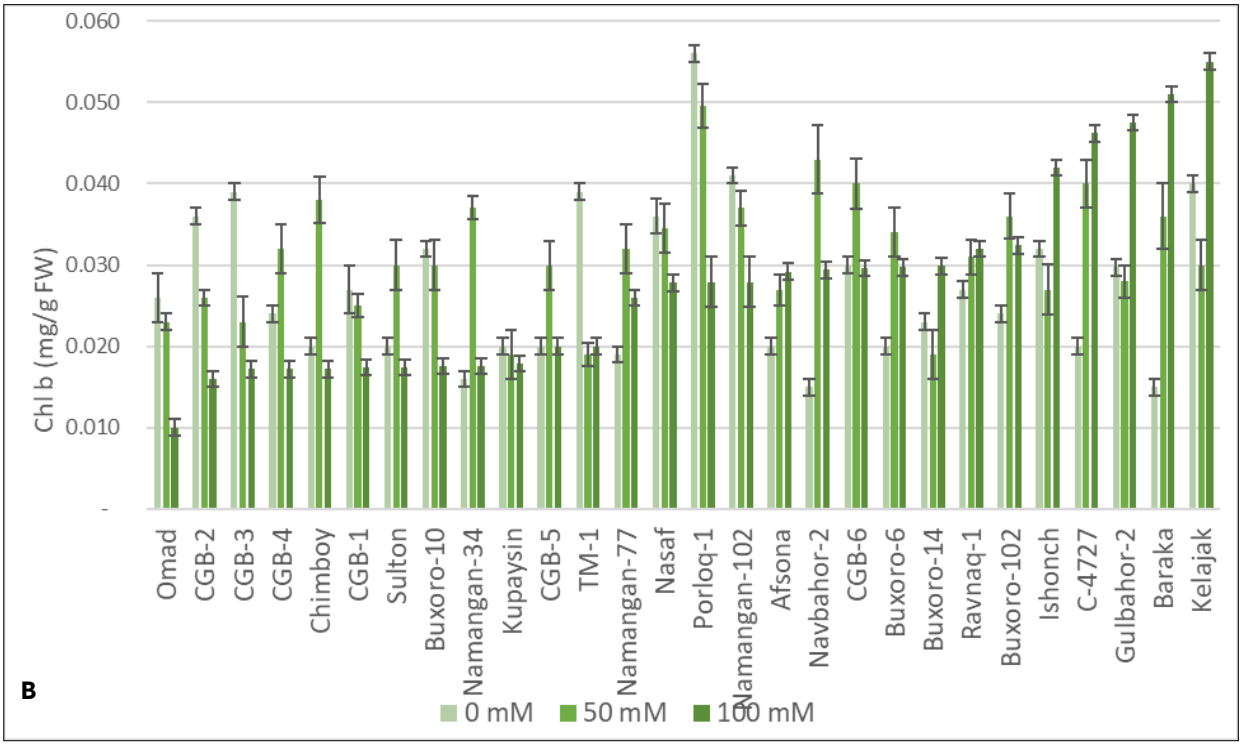
Table 1 . Descriptive statistics results for ANOVA. ($p < 0.05$)

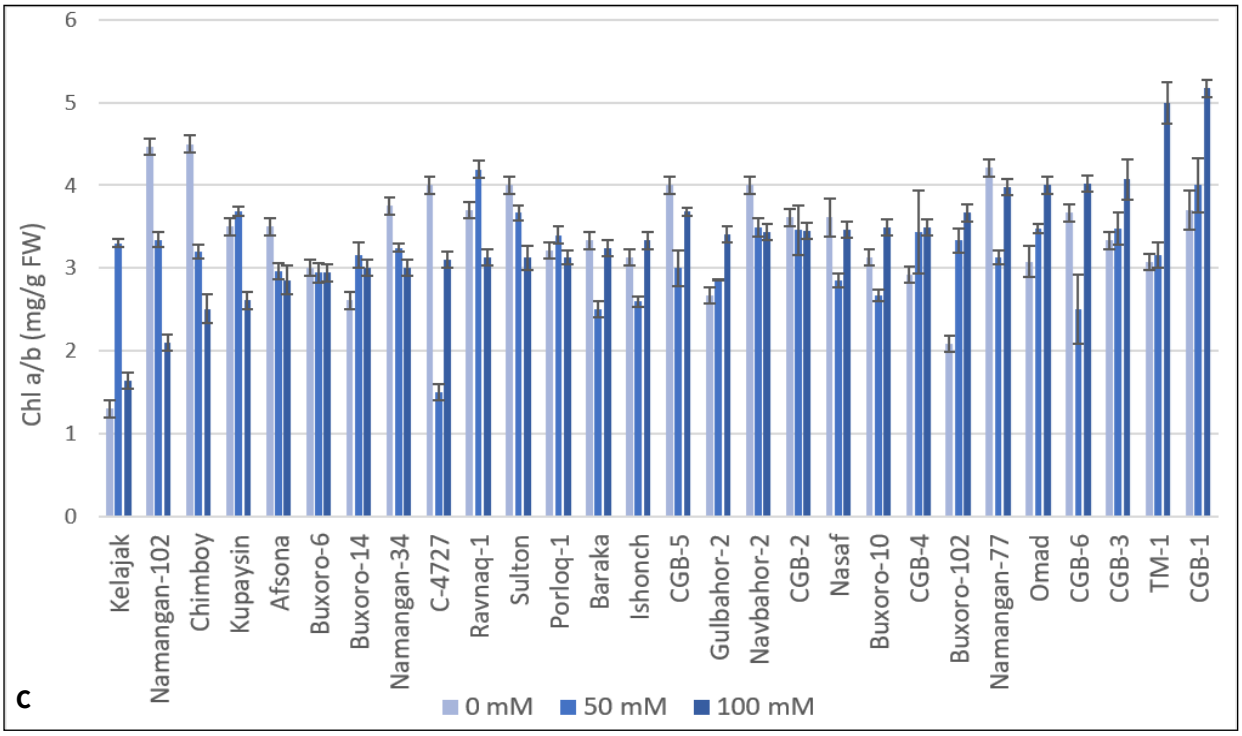
Descriptives									
		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower	Bound		
Chl_a	0 mM	28	0.09536	0.038919	0.007355	0.08027	0.11045	0.050	0.220
	50 mM	28	0.09643	0.027516	0.005200	0.08576	0.10710	0.060	0.170
	100 mM	28	0.08964	0.029905	0.005652	0.07805	0.10124	0.050	0.150
	Total	84	0.09381	0.032232	0.003517	0.08681	0.10080	0.050	0.220
Chl_b	0 mM	28	0.02821	0.012781	0.002415	0.02326	0.03317	0.010	0.070
	50 mM	28	0.03643	0.019619	0.003708	0.02882	0.04404	0.015	0.085
	100 mM	28	0.02696	0.010031	0.001896	0.02307	0.03085	0.010	0.050
	Total	84	0.03054	0.015130	0.001651	0.02725	0.03382	0.010	0.085
Chl_ab	0 mM	28	0.12518	0.050543	0.009552	0.10558	0.14478	0.080	0.290
	50 mM	28	0.12393	0.034030	0.006431	0.11073	0.13712	0.080	0.220
	100 mM	28	0.16089	0.050388	0.009523	0.14135	0.18043	0.085	0.275
	Total	84	0.13667	0.048280	0.005268	0.12619	0.14714	0.080	0.290
Cartenoids	0 mM	28	0.01311	0.00670	0.001322	0.010397	0.015823	0.013	0.0390
	50 mM	28	0.02795	0.00751	0.001419	0.025034	0.030854	0.015	0.0463
	100 mM	28	0.02366	0.00782	0.001478	0.020631	0.026698	0.014	0.0420
	Total	84	0.01498	0.00595	0.000649	0.013694	0.016276	0.015	0.0454
Chl_a/b_ratio	0 mM	28	3.52057	0.730368	0.138027	3.23736	3.80378	2.500	6.000
	50 mM	28	3.51400	1.324941	0.250390	3.00024	4.02776	2.000	6.667
	100 mM	28	3.77557	2.082838	0.393619	2.96793	4.58321	1.800	11.000
	Total	84	3.60338	1.473371	0.160758	3.28364	3.92312	1.800	11.000
SPAD	0 mM	28	35.98750	3.734877	0.705825	34.53927	37.43573	28.920	47.020
	50 mM	28	46.31893	3.351252	0.633327	45.01945	47.61841	38.460	51.620
	100 mM	28	42.84429	4.106903	0.776132	41.25179	44.43678	32.800	49.430
	Total	84	41.71690	5.685518	0.620341	40.48307	42.95074	28.920	51.620

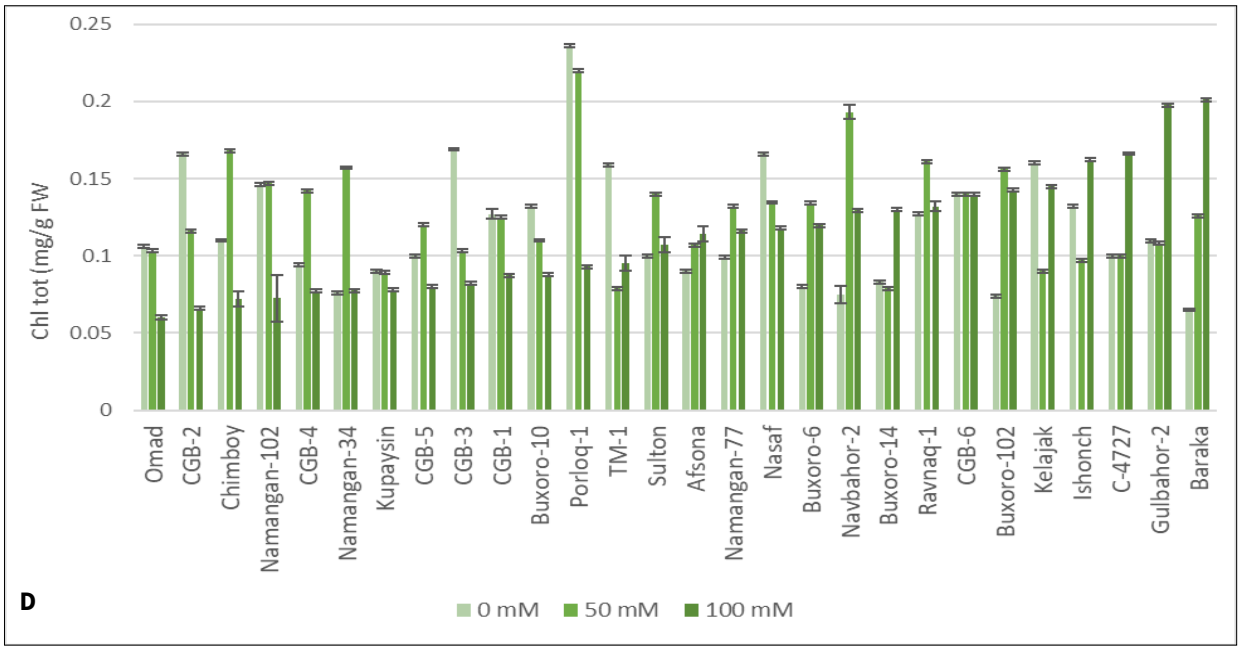
Table 2. Results of ANOVA test for the content of Chl_a, Chl_b, Chl_ab, Chl_a/b_ratio, carotenoids and SPAD value

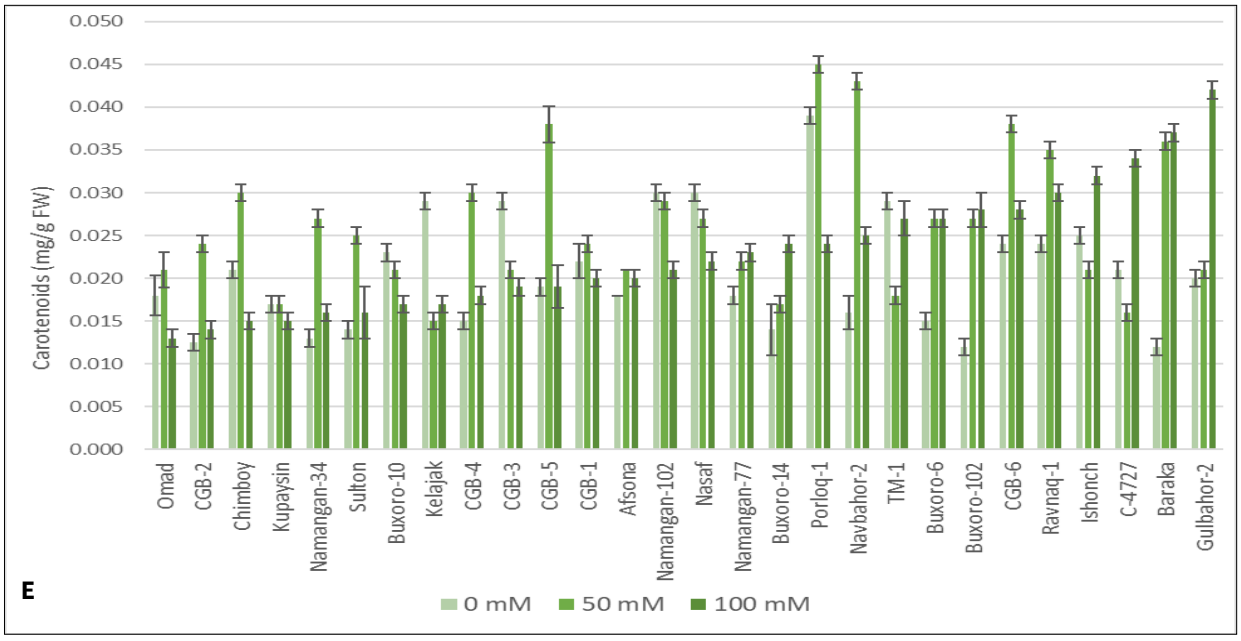
Variable	Source	Sum of Squares	df	Mean Square	F	Sig.
Chl_a	Between Groups	0.001	2	0.000	0.353	0.704
	Within Groups	0.085	81	0.001		
	Total	0.086	83			
Chl_b	Between Groups	0.001	2	0.001	3.422	0.037
	Within Groups	0.018	81	0.000		
	Total	0.019	83			
Chl_ab	Between Groups	0.025	2	0.012	5.920	0.004
	Within Groups	0.169	81	0.002		
	Total	0.193	83			
Carotenoids	Between Groups	0.001	2	0.001	1.374	0.259
	Within Groups	0.040	81	0.000		
	Total	0.041	83			
Chl_a/b_ratio	Between Groups	1.246	2	0.623	0.282	0.755
	Within Groups	178.932	81	2.209		
	Total	180.178	83			
SPAD	Between Groups	1547.719	2	773.860	55.214	0.000
	Within Groups	1135.265	81	14.016		
	Total	2682.984	83			






Chl a/b (mg/g FW)
0
1
2
3
4
5
6
Kelajak
Namangan-102
Chimboy
Kupaysin
Afsona
Buxoro-6
Buxoro-14
Namangan-34
C-4727
Ravnaq-1
Sulton
Porloq-1
Baraka
Ishonch
CGB-5
Gulbahor-2
Navbahor-2
CGB-2
Nasaf
Buxoro-10
CGB-4
Buxoro-102
Namangan-77
Omad
CGB-6
CGB-3
TM-1
CGB-1
0 mM
50 mM
100 mM
C


Chl tot (mg/g FW
0
0.05
0.1
0.15
0.2
0.25
Omad
CGB-2
Chimboy
Namangan-102
CGB-4
Namangan-34
Kupaysin
CGB-5
CGB-3
CGB-1
Buxoro-10
Porloq-1
TM-1
Sulton
Afsona
Namangan-77
Nasaf
Buxoro-6
Navbahor-2
Buxoro-14
Ravnaq-1
CGB-6
Buxoro-102
Kelajak
Ishonch
C-4727
Gulbahor-2
Baraka
0 mM
50 mM
100 mM
D


Carotenoids (mg/g FW)
0.000
0.005
0.010
0.015
0.020
0.025
0.030
0.035
0.040
0.045
0.050
Omad
CGB-2
Chimboy
Kupaysin
Namangan-34
Sulton
Buxoro-10
Kelajak
CGB-4
CGB-3
CGB-5
CGB-1
Afsona
Namangan-102
Nasaf
Namangan-77
Buxoro-14
Porloq-1
Navbahor-2
TM-1
Buxoro-6
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C-4727
Baraka
Gulbahor-2
0 mM
50 mM
100 mM
E

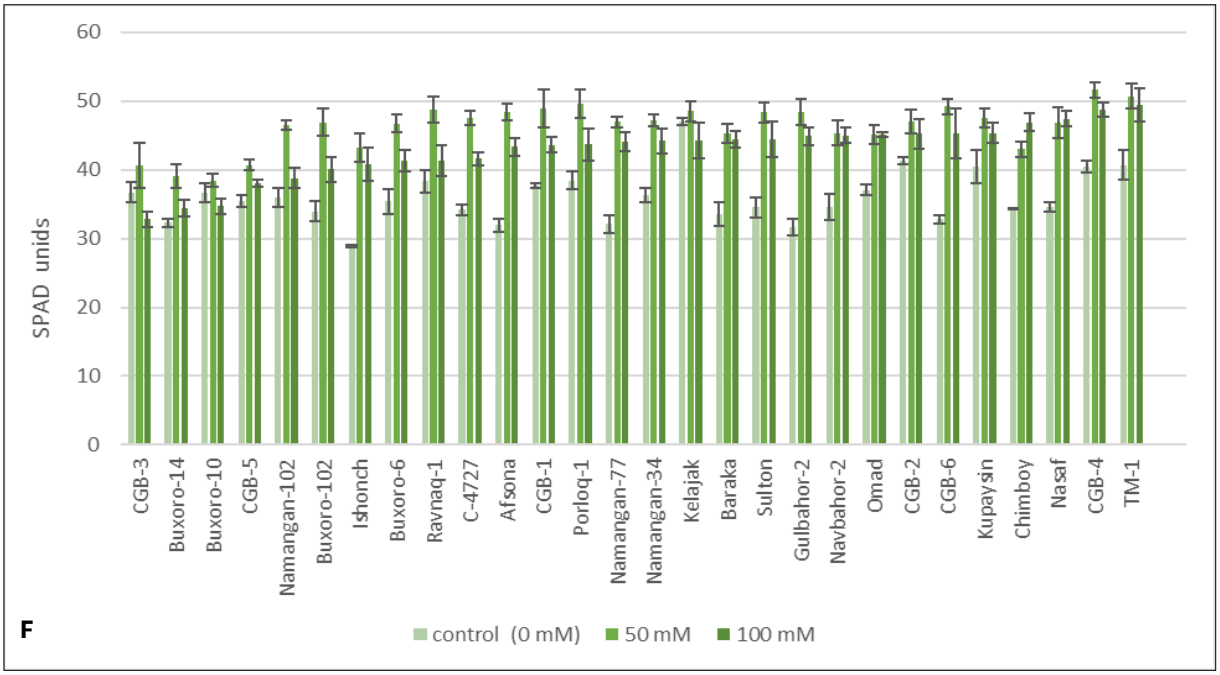

Fig. 1. Effect of salt stress (50 mM and 100 mM NaCl) on (A) chlorophyll a, (B) chlorophyll b, (C) chlorophyll a:b, (D) total chlorophyll, (E) carotenoids (F) SPAD. The data presented are the mean (±SEM). The diagrams were constructed based on the principle of increasing responsiveness of varieties to 100 mM NaCl.

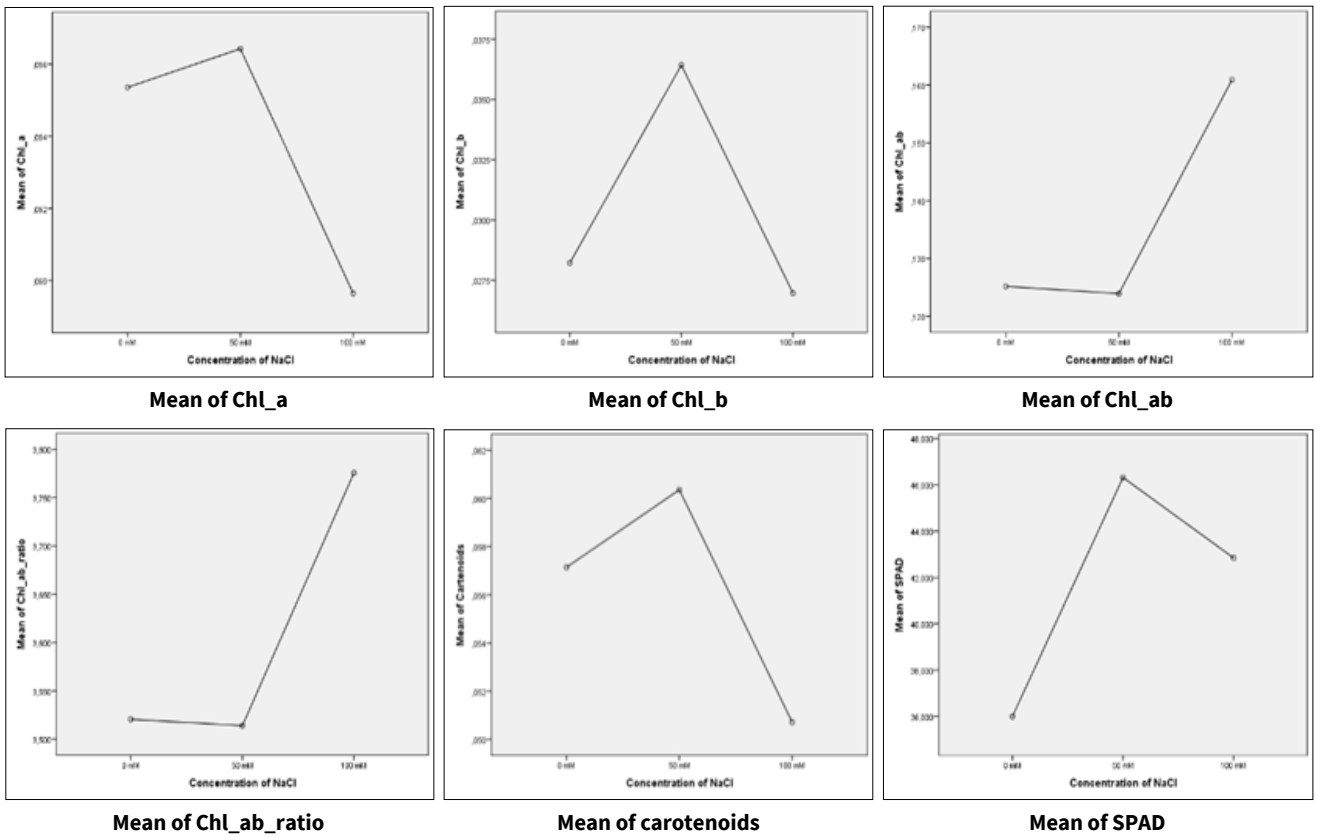

Fig. 2. Distribution of mean values of Chl_a, Chl_b, Chl_ab, Chl_ab_ratio, carotenoids and SPAD.

chlorophyll content are possible under the influence of salt stress (16, 17).

The nonparametric rank method was used to analyze the experimental data (Table 2). The results of one-way analysis of variance (ANOVA) did not reveal significant differences in the variability of the parameter under the influence of the stressor at concentrations of 50 and 100 mM NaCl ($F = 0.353$; $p = 0.704$). When treated with 100 mM NaCl, most varieties showed a tendency towards a decrease in the chlorophyll a content compared to the control, which is reflected in a decrease in the correlation strength between Chl_a and Chl_b ($r = 0.644^{**}$ versus 0.958^{**} in the control, (Table 3). Chl_a values varied within 0.050-0.150 mg/g FW. The highest values of this parameter were recorded for the Ishonch, C-4727, Gulbahor-2, Baraka and Kelajak varieties. However, according to Tukey's test, the between-group differences did not reach statistical significance (Table 4; $p < 0.05$).

Table 3. Correlation analysis (Pearson criterion)

	Chl_a	Chl_b	Chl_ab	carotenoids	Chl_a/b	SPAD
0 mM NaCl						
Chl_a	1	0.958**	0.968**	0.796**	-0.198	0.574**
Chl_b	0.958**	1	0.958**	0.780**	-0.441	0.595**
Chl_ab	0.968**	0.958**	1	0.805**	-0.238	0.623**
carotenoids	0.796**	0.780**	0.805**	1	-0.198	0.596**
Chl_a/b_ratio	-0.198	-0.441	-0.238	-0.198	1	-0.182
SPAD	0.574**	0.595**	0.623**	0.569**	-0.182	1
50 mM						
Chl_a	1	0.761**	0.922**	0.949**	-0.234	0.224
Chl_b	0.761**	1	0.782**	0.755***	-0.677**	0.157
Chl_ab	0.992**	0.782**	1	0.957**	-0.279	0.243
carotenoids	0.949**	0.755**	0.957**	1	-0.269	0.177
Chl_a/b_ratio	-0.234	-0.677**	-0.279	-0.269	1	-0.087
SPAD	0.224	0.157	0.243	0.177	-0.087	1
100 mM						
Chl_a	1	0.644**	0.858**	0.850**	0.315	0.082
Chl_b	0.644**	1	0.815**	0.691**	-0.429*	-0.033
Chl_ab	0.858**	0.815**	1	0.876**	-0.079	0.03
carotenoids	0.850**	0.691**	0.876**	1	-0.091	-0.035
Chl_a/b_ratio	0.315	-0.429*	-0.079	-0.091	1	0.199
SPAD	0.082	-0.033	0.03	-0.035	0.199	1

** - Correlation is significant at the 0.01 level (2-tailed)

* - Correlation is significant at the 0.05 level (2-tailed)

Table 4. Results of data processing on pigment content and SPAD values according to Tukey's HSD test

Dependent Variable	(I) NaCl	(J) NaCl	Mean Difference (I-J)	Std. Error	Sig.	Lower Bound	Upper Bound
Chl_a	0 mM	50 mM	-0.001071	0.008682	0.992	-0.02180	0.01966
	0 mM	100 mM	0.005714	0.008682	0.788	-0.01502	0.02644
	50 mM	100 mM	0.006786	0.008682	0.715	-0.01394	0.02752
	100 mM	0 mM	-0.005714	0.008682	0.788	-0.02644	0.01502
	100 mM	50 mM	-0.006786	0.008682	0.715	-0.02752	0.01394
Chl_b	0 mM	50 mM	0.008214	0.003931	0.098	-0.00160	0.01717
	0 mM	100 mM	0.012250	0.003931	0.046	0.00083	0.01883
	50 mM	100 mM	0.009464	0.003931	0.048	0.00008	0.01885
	100 mM	0 mM	-0.012250	0.003931	0.046	-0.01863	-0.00588
Chl_ab	0 mM	50 mM	-0.001250	0.012200	0.994	-0.02888	0.02639
	0 mM	100 mM	-0.035714	0.012200	0.012	-0.06484	-0.00659
	50 mM	100 mM	-0.036964	0.012200	0.009	-0.06609	-0.00784
	100 mM	0 mM	0.035714	0.012200	0.012	0.00659	0.06484
Carotenoids	0 mM	50 mM	-0.003214	0.005925	0.851	-0.01736	0.01093
	0 mM	100 mM	0.006429	0.005925	0.526	-0.00772	0.02057
	50 mM	100 mM	0.009643	0.005925	0.240	-0.00450	0.02379
Chl_a/b_ratio	0 mM	50 mM	-0.005671	0.397226	0.797	-0.95497	0.94358
	0 mM	100 mM	-0.255000	0.397226	0.797	-1.20340	0.69340
SPAD	0 mM	50 mM	-10.331429	1.000558	0.000	-12.72031	-7.94255
	0 mM	100 mM	-6.856786	1.000558	0.000	-9.24566	-4.46791
	50 mM	100 mM	3.474643	1.000558	0.002	1.08576	5.86352

The observed divergent changes in Chl_a content under salt stress are consistent with published data indicating a cultivar-specific nature of the pigment response of cultivated plants to salinity (18–20). These studies report that increased stress intensity can disrupt the integrity of thylakoid membranes, inhibit pigment biosynthesis and reduce the efficiency of photosystem II.

Changes in chlorophyll b content

The study of the dynamics of changes in Chl_b an indicator of the efficiency of antenna complexes using statistical analysis (ANOVA) revealed significant differences in the variability of the trait under the influence of salt stress in both experimental variants (50 mM and 100 mM) (Table 2, F = 3.422, p = 0.037, F = 592, p = 0.004). The distribution of mean values confirms the significance of the change in the indicator under the influence of the salt factor (Fig. 2B). Chl_b demonstrated higher sensitivity at 100 mM NaCl compared to 50 mM. The correlation of Chl_b with Chl_a decreased (r = 0.644**) and the negative relationship with Chl_a/b_ratio became statistically significant (r = –0.429*, p <0.05) (Table 3). The cultivar distribution diagram shows that the C-4727, Gulbahor-2, Baraka and Kelajak cultivars exhibited the highest values under 100 mM NaCl (Fig. 1B). Significant changes in Chl_b under the influence of abiotic stressors were also observed in other crops (20–22). Plants showed a slight decrease in this parameter compared to Chl_a. The authors attribute this process to the placement of chlorophyll on the stroma, i.e., some protection of the pigment.

Change in the chlorophyll ratio (a:b)

The Chl_a/b_ratio is an indicator of photosynthetic efficiency and the physiological state of plants. Salt stress caused significant changes in Chl_b content and, accordingly, this was reflected in the Chl_a/b_ratio (Fig. 1C). ANOVA did not confirm a significant difference in variability (F = 0.282, p = 75). At 100 mM NaCl, it demonstrated weak and inconsistent relationships with both Chl_a (r = 0.315) and Chl_b (r = –0.429*, p < 0.05) (Table 3). Under the influence of 100 mM NaCl, a significant variation in this parameter was revealed within the range of 3.1 to 3.41. High values of the Chl_a/b_ratio were found in the varieties Omad,

Table 5. Leaf pigment content and SPAD index in 28 cotton varieties. Units: Chl and Car (mg g^{-1}), SPAD (units). $p < 0.05$, $p < 0.01$

Cultivar	Chl_a (0 mM)	Chl_a (50 mM)	Chl_a (100 mM)	Chl_b (0 mM)	Chl_b (50 mM)	Chl_b (100 mM)	Chl (a+b) (0)	Chl (a+b) (50)	Chl (a+b) (100)	Car (0)	Car (50)	Car (100)	SPAD (0)	SPAD (50)	SPAD (100)
Afsona	0.07±0.001	0.08±0.001	0.09±0.003	0.02±0.001	0.03±0.002	0.03±0.001	0.090±0.001	0.107±0.001	0.114±0.005	0.018±0.001	0.021±0.001	0.020±0.001	31.93± 1.58	48.47±2.07	43.36±2.18
Baraka	0.05±0.001	0.09±0.001	0.15±0.001	0.01±0.001	0.06±0.004	0.05±0.001	0.065±0.001	0.126±0.001	0.201±0.001	0.012±0.001	0.036±0.001	0.037±0.001	33.55±2.89	45.29±2.50	44.44±1.96
Buxoro-10	0.10±0.001	0.08±0.001	0.07±0.001	0.02±0.001	0.07±0.006	0.03 ±0.001	0.132±0.001	0.110±0.001	0.088±0.001	0.026±0.001	0.021±0.001	0.017±0.001	36.68±2.29	38.46±1.74	34.720± 1.93
Buxoro-102	0.05±0.001	0.12±0.001	0.11±0.001	0.02±0.001	0.07±0.001	0.03±0.001	0.074±0.001	0.156±0.001	0.142±0.001	0.012±0.001	0.027±0.001	0.028±0.005	33.950±2.54	46.91±3.41	40.09±3.16
Buxoro-14	0.06±0.001	0.06±0.001	0.10±0.001	0.02±0.001	0.04±0.001	0.08±0.001	0.083±0.001	0.079±0.001	0.130±0.001	0.014±0.005	0.017±0.001	0.024±0.001	32.30±1.13	39.14±2.98	34.43±1.97
Buxoro-6	0.06±0.001	0.10±0.001	0.09±0.001	0.03±0.001	0.08±0.001	0.04±0.001	0.080±0.001	0.134±0.001	0.120±0.001	0.015±0.001	0.027±0.001	0.027±0.001	35.40±3.13	46.70±2.24	41.33±2.71
C-4727	0.08±0.001	0.06±0.003	0.12±0.001	0.03±0.001	0.03±0.001	0.04±0.001	0.100±0.001	0.100±0.001	0.166±0.001	0.021±0.001	0.016±0.001	0.034±0.001	34.20±1.40	47.53±1.70	41.63±1.59
CGB-1	0.10±0.007	0.10±0.001	0.07±0.001	0.07±0.001	0.03±0.001	0.05 ±0.001	0.127±0.003	0.125±0.001	0.087±0.001	0.022±0.004	0.024±0.001	0.020±0.001	37.73±0.67	48.97±4.76	43.60±0.00
CGB-2	0.13±0.001	0.09±0.001	0.05±0.001	0.02±0.001	0.03±0.001	0.02±0.001	0.166±0.001	0.116±0.001	0.066±0.001	0.125±0.001	0.024±0.001	0.014±0.001	41.37±0.86	47.08±3.03	45.22±3.77
CGB-3	0.13±0.001	0.08±0.001	0.07±0.001	0.04±0.001	0.05±0.001	0.03±0.001	0.169±0.001	0.103±0.001	0.082±0.001	0.029±0.001	0.021±0.001	0.019±0.001	36.72±2.47	40.69±5.73	40.69±5.73
CGB-4	0.07±0.001	0.11±0.001	0.06±0.001	0.02 ±0.001	0.05±0.001	0.02±0.001	0.094±0.001	0.142±0.001	0.077±0.001	0.015±0.001	0.030±0.001	0.018±0.001	40.45±1.41	51.62±2.03	48.81±1.80
CGB-5	0.08±0.001	0.09±0.001	0.06±0.001	0.02±0.001	0.03±0.003	0.03±0.001	0.100±0.001	0.120±0.001	0.080±0.001	0.019±0.001	0.038±0.0049	0.015±0.005	35.48±1.45	40.71±1.34	40.1±1.4
CGB-6	0.11±0.001	0.10±0.001	0.11±0.001	0.04±0.001	0.03±0.001	0.03±0.001	0.140±0.001	0.140±0.001	0.140±0.001	0.024±0.001	0.038±0.001	0.028±0.001	32.83±1.07	49.20±2.00	45.27±6.34
Chimboy	0.09±0.001	0.13±0.001	0.06±0.003	0.01±0.001	0.07±0.003	0.03±0.001	0.110±0.001	0.168±0.001	0.072±0.005	0.021±0.001	0.035±0.001	0.015±0.001	34.33±0.26	43.02±1.97	46.94±2.21
Gulbahor-2	0.08±0.001	0.08±0.003	0.015±0.001	0.03±0.001	0.02±0.001	0.01±0.001	0.110±0.001	0.108±0.001	0.198±0.001	0.020±0.001	0.020±0.001	0.042±0.001	31.63±2.10	48.40±3.27	44.88±2.30
Ishonch	0.10±0.001	0.07±0.001	0.12±0.001	0.05±0.001	0.09±0.001	0.03±0.001	0.132±0.001	0.097±0.001	0.162±0.001	0.025±0.001	0.021±0.001	0.032±0.001	28.92±0.32	43.24±3.57	40.83±4.29
Kelajak	0.12±0.003	0.06±0.003	0.09±0.001	0.03±0.001	0.05±0.001	0.03±0.001	0.160±0.001	0.090±0.001	0.145±0.001	0.049±0.001	0.015±0.001	0.017±0.001	47.02±0.89	48.51±2.62	44.26±4.58
Kupaysin	0.07±0.001	0.07±0.001	0.06±0.001	0.02±0.001	0.03±0.001	0.02±0.001	0.090±0.001	0.089±0.001	0.078±0.001	0.017±0.001	0.017±0.001	0.015±0.001	40.42±4.21	47.49±2.36	45.34±2.57
Namangan-102	0.13±0.001	0.11±0.001	0.06±0.001	0.02±0.001	0.06±0.002	0.02±0.001	0.146±0.001	0.147±0.001	0.073±0.0015	0.030±0.001	0.029±0.001	0.021±0.001	36.00±2.42	46.56±1.18	38.81±2.46
Namangan-34	0.06±0.001	0.12±0.001	0.06±0.001	0.04±0.001	0.02±0.001	0.02±0.001	0.076±0.001	0.157±0.001	0.078±0.001	0.013±0.001	0.032±0.001	0.016±0.001	36.37±1.76	47.21±1.57	44.23±3.10
Namangan-77	0.08±0.001	0.10±0.0001	0.09±0.001	0.04±0.001	0.02±0.001	0.02±0.001	0.099±0.001	0.132±0.001	0.116±0.001	0.018±0.001	0.022±0.001	0.023±0.001	32.12±2.21	46.98±1.29	44.11±2.32
Nasaf	0.13±0.001	0.10±0.001	0.09±0.001	0.02 ±0.002	0.04±0.001	0.02±0.001	0.166±0.001	0.135±0.001	0.118±0.001	0.030±0.001	0.027±0.001	0.022±0.001	34.55±1.23	46.89±3.94	47.44±1.89
Navbahor-2	0.06±0.001	0.15±0.003	0.10±0.001	0.03±0.001	0.04±0.004	0.03±0.001	0.075±0.001	0.193±0.006	0.129±0.005	0.016±0.002	0.043±0.001	0.025±0.001	34.55±3.35	45.34±3.19	44.99±1.94
Omad	0.08±0.003	0.08±0.002	0.05±0.001	0.02±0.003	0.03±0.001	0.02±0.001	0.106±0.001	0.103±0.001	0.060±0.001	0.018±0.004	0.021±0.004	0.013±0.001	37.10±1.38	45.10±2.32	45.12±0.63
Porloq-1	0.18±0.001	0.17±0.001	0.07±0.001	0.03±0.001	0.02±0.005	0.02±0.001	0.236±0.001	0.220±0.001	0.093±0.001	0.039±0.001	0.045±0.001	0.024±0.001	38.48±2.27	49.57±3.57	43.70±4.01
Ravnaq-1	0.10±0.001	0.13±0.003	0.10±0.001	0.04±0.001	0.02±0.002	0.02±0.001	0.127±0.001	0.161±0.001	0.132±0.003	0.024±0.001	0.035±0.001	0.030±0.001	38.33±2.80	48.77±3.17	41.39±3.88
Sulton	0.08±0.001	0.11±0.001	0.09±0.001	0.03±0.001	0.03±0.001	0.02±0.001	0.100±0.001	0.140±0.001	0.107±0.005	0.014±0.001	0.029±0.001	0.016±0.005	34.52±2.53	48.34±2.66	44.44±4.59
TM-1	0.12±0.001	0.06±0.0001	0.08±0.001	0.02±0.001	0.02±0.001	0.03±0.001	0.159±0.001	0.079±0.001	0.095±0.005	0.029±0.001	0.018±0.001	0.027±0.002	40.72±3.86	50.74±3.09	49.43±4.15

CGB-6, CGB-3, TM-1 and CGB-1 (Table 5). In general, the Chl_a/b_ratio values varied within the range from 1.64 (Kelajak) to 5.17 (CGB-1). The distribution of the mean values of the ratio differs from the distribution pattern of the mean Chl_a and Chl_b (Fig. 2C). These changes are associated with LHCII rearrangement and an increase in the proportion of Chl_b under mild (50 mM) stress, but destruction of the antennal apparatus under more severe stress. Similar studies have shown that under salt stress, the content of both Chl_a and Chl_b tends to decrease, while the chlorophyll a/b ratio tends to increase as a result of a more pronounced decrease in the content of Chl_b compared to Chl_a (23).

Total chlorophyll content

Exposure to a stressor of 100 mM NaCl resulted in a significant decrease in Chl_ab content in half of the varieties (Fig. 1D). Tukey's test showed that when comparing values between the control group (0 mM) and the experimental group (100 mM), the changes were significant (Table 4, p = 0.012). A comparison between the 2 experimental variants (50 mM and 100 mM) revealed a significant difference (Table 4, p = 0.009). The distribution pattern of mean (m) Chl _ab values is similar to the distribution of m Chl_a/b_ratio (Fig. 2D). The group with the highest values for this parameter included the Ishonch, C-4727, Gulbahor-2 and Baraka varieties. Salt stress caused noticeable changes in total chlorophyll content. At 100 mM NaCl, many plants experience significant physiological stress, leading to membrane damage, nutrient imbalance, decreased photosynthetic efficiency and enzyme inactivation (24).

Dynamics of carotenoid changes under salt stress

It is known that the role of carotenoids during photosynthesis is to transfer energy to chlorophyll molecules, providing both light harvesting and a photoprotective function by stabilizing membranes (25, 26). Of the varieties we analyzed, most demonstrated stable or increased carotenoid levels at 100 mM NaCl, indicating a protective adaptive response by plants under salt stress. When young cotton plants were exposed to 100 mM NaCl, more pronounced pigment degradation was observed, especially in the Omad, CGB-2, Chimboy, Buxoro-10, CGB-3, Namangan-102 and Nasaf varieties (Fig. 1 E). In contrast, the Ishonch, Kelajak, Baraka and Gulbahor-2 varieties exhibited relatively high chlorophyll levels, indicating increased adaptability to moderate salinity. In the Kupaysin, CGB-1, Afsona, Buxoro-6 and Buxoro-102 varieties, the carotenoid content differed insignificantly from the 50 mM solution, indicating a more stable protective function against reactive oxygen species (ROS). The distribution of mean values follows the pattern of mean Chl_a (Fig. 2E). Changes in carotenoids were not statistically significant (p > 0.05). ANOVA revealed no significant differences between the experimental groups (F=1.374, p=0.259), which is also observed in a number of studies where carotenoids show high stability or a compensatory increase under stress (26). However, visual data (graphs) indicate individual responses of varieties, namely an increase in carotenoids and a slight decrease. Determining SPAD as a Rapid Screening Tool.

To further determine the overall pigment level and assess the impact of salinity on the photosynthetic apparatus, we measured SPAD, which serves as a rapid and informative indicator of chlorophyll content. SPAD values reflect the functional integrity of the photosynthetic apparatus and allow for early plant diagnostics. Our data showed that at a concentration of 100 mM NaCl, SPAD values significantly increased in half of the studied varieties compared to the control (Fig. 1F). This group included the varieties Porloq-1, Namangan-77, Namangan-34, Baraka, Sulton, Gulbahor-2, Navbahor-2, Omad, CGB-2, CGB-6, Kupaysin, Chimboy, Nasaf, CGB-4 and TM-1. In contrast, the CGB-3 variety showed a significant decrease in this parameter, indicating high sensitivity to salt stress. ANOVA revealed significant differences (Table 2). At 0 and 50 mM NaCl, SPAD demonstrated moderate positive correlations with Chl a (r = 0.574**(in the control) and r = 0.224 (in the experiment) and Chl (a+b) (r = 0.623** and r = 0.243), but at 100 mM the dependence practically disappeared (r = 0.082 and r = -0.199). This confirms that SPAD reliably reflects the total pigment content only under mild and moderate stress, but ceases to be sensitive when the structure of the photosynthetic apparatus is destroyed. A similar loss of SPAD consistency with chlorophylls under strong NaCl has been described for other crops (27, 28).

Two-way ANOVA of SPAD values

A two-way ANOVA of SPAD values revealed a significant effect of the "Salt" factor on SPAD values (F = 1486.75, p < 0.001), indicating variety-specific, i.e., genetically determined, differences between varieties (Table 6). The first factor contributed significantly to the total variance (F = 2192.80; p < 0.01), indicating significant differences in SPAD values between groups. The second factor also had a statistically significant effect (F = 5051.49; p < 0.01), indicating that changes in chlorophyll accumulation occur depending on the exposure conditions. The significant interaction of factors (F = 1486.75; p < 0.01) indicates that plant response to SPAD levels is determined by both individual and combined effects of the factors. The results are consistent with the earlier reports (29, 30).

Overview of quantitative trends

Summary data for all 28 varieties:

- Chl_a: decrease >20 % in 12 varieties; average decrease across varieties = 7.4 %.
- Chl_ab: decrease >20 % in 11 varieties; average decrease = 8.4 %.
- Carotenoids: decrease >20 % in 9 varieties.
- SPAD: average decrease = 19.3 %.

These results confirm that SPAD-based assessment is a reliable, flexible, non-destructive method for assessing pigment stability and identifying salt-tolerant genotypes.

Based on the data, varieties with a relatively stable reaction and varieties with high sensitivity to salt stress were identified. Resistant varieties, consistently demonstrating high values of Chl_a, Chl_b, Chl_ab and SPAD, even at 100 mM NaCl, included Ishonch,

Table 6. Two-way analysis of variance of 28 cotton varieties for variety-salinity interaction

Factor	SS	df	MS	F	P- value	F-critical
Varieties	426150.97	83	5134.35	2192.79567	0	1.2908274
Salt Factor Level	11827.88	1	11827.88	5051.492134	0	3.85533386
Varieties x Salinity	288936.97	83	3481.17	1486.749497	0	1.2908274
Total	728489.29	839	2.34		-	-

Note: All main effects and the variety × salinity interaction were significant for SPAD (p < 0.01).

C-4727, Gulbahor-2, Baraka and Kelajak. Sensitive varieties, showing a decrease in chlorophyll and carotenoid content, as well as a decrease in SPAD at 100 mM NaCl, included Chimboy, Namangan-102, CGB-3, Buxoro-10 and Omad.

Salt stress causes damage to plants at the cellular level, altering the structure of cellular organelles such as chloroplasts and mitochondria (31). Damage to these organelles is primarily caused by reactive oxygen species (ROS) produced in the leaves as a result of salinity. The literature notes that salt-sensitive plants, such as potato and pea, exhibit decreased chlorophyll content under salt stress, while salt-tolerant plants such as mustard and wheat, cotton and others, exhibit increased chlorophyll content (31–35). Among the cotton varieties we studied, changes in Chl_a and Chl_b content under the influence of NaCl reflect the differential sensitivity of the photosynthetic complex of plants and cotton in particular. Such variability in cotton is reflected in the scientific literature and is explained by genetic differences in the mechanisms of ion homeostasis, antioxidant defense and photosynthesis regulation (1, 2, 4, 6).

Tolerant genotypes from the 28 varieties examined in our study demonstrated more stable chlorophyll content at 50 mM NaCl and a moderate decrease at 100 mM NaCl. This is consistent with the earlier reports which revealed that tolerant cotton lines maintain chlorophyll synthesis and keep photosystems active even under high salt pressure (2, 6).

The strong decrease in Chl_b at high salt concentrations is also consistent with classical models of damage to LHCII light-harvesting complexes under salt stress (7, 24, 25). Chl_b is a more sensitive antenna component and its degradation is considered an early marker of photosystem II dysfunction. Reports are there indicating indicating the decrease in the proportion of Chl_b and the relative predominance of Chl_a are part of a broader photoadaptation strategy that allows plants to reduce excitation flux to damaged reaction centers (8, 18).

The dynamics of the Chl_a/b_ratio increase at 100 mM NaCl revealed in our study reflects the transition of the plant to a more "chlorophyll a-dominant" state. A similar restructuring is characteristic of cotton under saline conditions (6, 27) and is considered an adaptive mechanism aimed at reducing the size of the LHCII antenna and reducing photodestruction under excess Na^+ and Cl^- (7, 31).

A decrease in the total chlorophyll content Chl_ab in most of the studied varieties under 100 mM NaCl is consistent with the mechanisms of thylakoid damage described in numerous studies of cotton and other crops (6, 10, 24). As per an earlier work, disruption of ion homeostasis under salt conditions leads to Mg^{2+} deficiency and membrane destabilisation, which directly affects the integrity of chlorophyll-protein complexes (30). Similarly, there are works revealing that a decrease in total chlorophyll content is often used as a diagnostic indicator of salt stress severity in cotton (4, 5).

One of the key results of our study was the relative preservation or even increase in carotenoid content in a number of varieties. This is fully consistent with current understanding of the role of carotenoids in salt defense mechanisms. There are several reports which emphasize that carotenoids are not only a tool for photoadaptation but also active components of antioxidant defense, stabilizing membranes and preventing ROS-induced pigment degradation (7, 25, 26). Based on all these results, it should be concluded that the ability of some cotton varieties to maintain high carotenoid levels can be considered an important marker of salt tolerance. The results of SPAD analysis confirm that under mild and moderate stress, this parameter is a good predictor of chlorophyll content, consistent with the seminal works of (36, 37).

However, at 100 mM NaCl, the correlation values with Chl a and Chl_ab decreased sharply, which is consistent with the previous data of showing that SPAD loses accuracy under conditions of severe photosystem damage (19, 38). Nevertheless, a two-way ANOVA revealed a significant contribution of both stress level and varietal origin, which is fully consistent with the data of the recent studies , where in SPAD is also recommended as a rapid marker of salt tolerance (27).

These results are consistent with previous reports that salt-tolerant cotton genotypes tend to maintain higher chlorophyll and carotenoid levels under stress conditions due to more efficient antioxidant defenses and osmotic regulation mechanisms (38, 39). Carotenoids (Car) are essential for photoprotection of photosynthesis and play an important role as signal precursors during plant development under abiotic conditions. A close relationship between Car-Chl electron interactions and the regulation of photosynthesis has been experimentally demonstrated (40). Overall, the present study highlights that chlorophyll degradation serves as a sensitive indicator of salinity-induced damage to the photosynthetic apparatus. Conversely, carotenoid stability is a key component defense response of the plant. Thus, varieties exhibiting limited chlorophyll loss combined with carotenoid retention can be classified as salt-tolerant and are promising candidates for breeding programs aimed at improving cotton tolerance to salinity.

Integration of rapid SPAD-based screening with biochemical pigment analysis provides a practical approach to the early identification of salt-tolerant genotypes. Such combined methods enable efficient phenotyping and selection as part of breeding processes, especially under field or semi-controlled conditions.

Conclusion

Salt stress damages the photosynthetic apparatus at the cellular level, primarily through reactive oxygen species (ROS), leading to changes in chloroplast structure and pigment degradation (31). In our study, Chl_a and Chl_b demonstrated cultivar-specific responses to NaCl, reflecting differential photosystem sensitivities. Tolerant cultivars maintained stable chlorophyll content at 50 mM and demonstrated a moderate decrease at 100 mM NaCl, whereas sensitive cultivars showed a significant decrease in Chl_a and Chl_b. These results are consistent with data on genetic differences in ion homeostasis, antioxidant defense and photosynthesis regulation in cotton and other crops (1, 2, 4, 6).

Carotenoids remained relatively stable or increased in a number of cultivars, confirming their role in photoprotection and antioxidant defense (7, 22, 23). Changes in the Chl_a/b_ratio at 100 mM NaCl reflect adaptive restructuring of LHCII aimed at reducing photo destruction. Results of SPAD measurements

showed that under mild and moderate stress, this indicator reliably reflects chlorophyll content; however, under severe damage to the photosystem, the accuracy decreases (29, 32). Thus, varieties with minimal chlorophyll loss and preservation of carotenoids can be considered salt-tolerant and the combination of SPAD and biochemical analysis of pigments is an effective tool for their early identification and selection.

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Authors' contributions

ASI, VSK, IBS and NRR carried out the experiments and wrote and revised the manuscript, performed statistical analysis. DBU, FSR, KAU, SES, SOK, SBK, DKB, DAM, SSA, BMS, AKM, VVU, AAB, ZZY, RMA, AM, MRZ, AAA and BKR participated in the experiments, collected the data and preparation of the manuscript. ZB edited and approved the manuscript. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interest to declare.

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