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## **RESPONSES OF ANTIOXIDANT SYSTEM OF VARIOUS COTTON GENOTYPES TO HEAT STRESS AT THE JUVENILE STAGE OF ONTOGENESIS**

### **SUMMARY**

Extremely high temperatures affecting cotton plants at various stages of ontogenesis. Oxidative damage of cell structures inflicted by moderately high temperatures is a main cause for the deleterious effect of heat stress. However, the status of the protective antioxidant system (AOS) in cotton plants has been studied mainly at the generative stages of development, while peculiarities of its functioning in the juvenile plants of various genotypes remains underexplored. The work was done to study the reaction of growth and the AOS response to the heat stress in the 7-8-day seedlings of various cotton cultivars obtained by various selection methods differing in economically valuable features. Surkhan-103 and Bukhara-102, the cultivars generated by classical selection, as well as those generated by biotechnological methods with higher fiber quality, to cultivars Porlok-1, Porlok-2, Ravnak-1 and Ravnak-2, were the objects of experiments. The 7-day seedlings were exposed to the 6-hour effect of high temperature (45°C) to subsequently assess the increment of control and stress-inflicted plants and to determine parameters of the AOS state and development of oxidative damages. Surkhan-103, Porlok-4 and Ravnak-2 cultivars were found to distinguish by significantly lower post-stress inhibition of growth of both seedlings and roots than those of Bukhara-102, Ravnak-1 and Porlok-1. The sensitive cultivars affected by heat stress demonstrated higher post-stress contents of hydrogen peroxide and malondialdehyde (MDA), a product of lipid peroxidation, in the leaves. At the same time, the resistant cultivars demonstrated higher activity of catalase and superoxide dismutase. The higher post-stress

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content of proline were typical of the resistant cultivars. A close direct correlation between inhibition of growth of seedlings and higher contents of hydrogen peroxide and MDA was demonstrated. In addition, high-level inverse correlation was demonstrated between the growth inhibition and higher catalase activity and proline content. The juvenile cotton plants were concluded to be promising in assessment of heat resistance and its dependence on the AOS state.

**Keywords:** *Gossypium*, heat resistance, reactive oxygen species, antioxidant enzymes, proline

## INTRODUCTION

Cotton (*Gossypium*) is relatively well adapted to growing in moderately arid regions. However, the plants are vulnerable to high temperatures both at the early stages of development (James *et al.*, 2005) and during flowering and development of bolls (Saini *et al.*, 2023). Due to recent changes in the climate, waves of heat in the regions of cotton growing are being more intensive and less predictable (Saleem *et al.*, 2021). That is the reason why the screening of resistibility of the basic material intended for generation of improved-yield heat resistant cotton cultivars with plastic phenotypes is the main task for up-to-date program of selection of the culture (Zafar *et al.*, 2021). Resistance assessment of novel cultivars including those generated using biotechnological methods is not less significant.

Effects of temperatures significantly exceeding physiological optimum, as a rule, result in development of oxidative stress. Fluidization of lipid basis of cell membranes, including membranes of chloroplasts and mitochondria, is a main cause for the damaging effect of high temperatures (Yoshioka, 2016; Choudhury *et al.*, 2017). The temperature rises due to increase in fluidity of thylakoid membranes, electron-transport chain of chloroplasts may disunite; that is believed to be a main cause for increase of generation of reactive oxygen species (ROS) under heat stress (Asthir, 2015). Photosystem II and its Mn-containing oxygen-releasing complex is the most sensitive to effects of high temperatures (Kreslavski *et al.*, 2012; Bernfur *et al.*, 2017).

However, generation of ROS under heat stress increases not only in chloroplasts. Stochastic generation of ROS increases in the membranes of mitochondria, as well, possibly associated both with changes in the status of membrane lipid phase and with the overrecovery of electron transport chain due to insufficient consumption of pool of reductants (Choudhury *et al.*, 2017). This state of mitochondrial complexes leads to the formation of radical ROS and, in particular, superoxide anion-radical in them.

Excessive accumulation of ROS is known to result in pro-/antioxidant imbalance and damage of redox-regulation. That may be an underlying cause of cell damage and death (Kumar, Rai, 2014; Kolupaev *et al.*, 2023a).

To prevent damages of redox-regulation processes and development of stress-induced oxidative damages, plants in evolution generate powerful multicomponent antioxidant system (AOS). The system is represented by

enzymatic and low molecular antioxidants (Kolupaev *et al.*, 2019; Hasanuzzaman *et al.*, 2020). Among the enzymes, there are ROS - neutralizing catalyzers, to name superoxide dismutase (SOD), catalase (CAT) and various peroxidases (POX), enzymes of lipid detoxication including glutathione S-transferase, glutathione reductase, and regenerators of active forms of antioxidants, such as monodehydroascorbate reductase, dehydroascorbate reductase and glutathione reductase. In addition, plants have complex system of thioredoxins and peroxiredoxins which in combination with other redox-active proteins participate in the regulation of thiol groups state (Hasanuzzaman *et al.*, 2020).

The complex of low-molecular antioxidants is represented by many chemically heterogeneous compounds (Das, Roychoudhury, 2014). Recently, some compounds with antioxidant function as non-basic one, but having marked antioxidant properties and accumulating as the response to stress-factors, particularly, proline, are believed to belong to antioxidants (Liang *et al.*, 2013).

Attempts to find associations between the AOS function and resistance of specific genotypes of plants to one or other stress-factors have being made for many years. At the same time, the strategies for plant adaptation to oxidative stress depend on their taxonomic belonging (Kolupaev *et al.*, 2016; 2022; Pržulj *et al.*, 2020). For example, peculiarities of functioning of the AOS of wheat and other cereals under stress conditions were studied in enough details. The correlation between some AOS parameters and heat- and drought resistance in the wheat cultivars was demonstrated (Kirova *et al.*, 2021; Kolupaev *et al.*, 2023b).

At the same time, peculiarities of responses of various cotton genotypes to high temperatures are underexplored. Herewith, in several works, the AOS status under heat stress at the generative stages of plant development was focused on. Thus, activity of SOD, CAT and POX, as well as content of proline was found higher in the heat resistant cultivars on the stage of flowering in the field conditions by means of a tunnel constructed using bamboo sticks and plastic sheets (Zafar *et al.*, 2023). In contrast, the resistant cultivars under the effect of excessive temperatures were found to retain values of oxidative stress markers, including hydrogen peroxide and MDA, close to the control ones. In another work with a large set of cultivars, high correlation of cotton yield with activities of peroxidase and SOD under heat stress generated in the field conditions by sowing later (Yousaf *et al.*, 2022).

In experiments with cotton plant anthers, significant inverse correlation between the pollen viability and concentrations of superoxide anion-radical and hydrogen peroxide was demonstrated (Zhang *et al.*, 2023). However, Mahan and Mauget (2005) failed to register significant correlation between MDA concentrations and temperature increase in field experiments on studying responses of cotton cultivars with different genotypes. As compared to the stress-sensitive cotton cultivars, Sarwar *et al.* (2023) demonstrated higher activities of antioxidative enzymes, including SOD, catalase and peroxidase, in stress-

resistant cotton cultivars in the greenhouse. The stress-resistant cultivars had lower MDA contents, as well.

Still, there are no studies on effects of high temperatures on the wide spectrum of cotton cultivars under controlled conditions at early stages of development.

The purpose of the work is to study the possible connection between the heat resistance of cotton cultivars in the early stages of development and the state of their antioxidant system. For the work, we used varieties created by classical breeding and biotechnological methods (marker-associated selection and gene knockout).

## MATERIAL AND METHODS

### Plant material

Cotton plants of *G. hirsutum* (cultivar Bukhara-102) and *G. barbadense* (cultivar Surkhan-103) species generated using classical cotton breeding were used in experiments. Plants of gene-knockout cultivars *G. hirsutum* Porlok-1 and Porlok-4 with suppression of the *PHYA1* gene were also used, which shortens the growing season of plants (Pat. NAP 20130014, 15.11.2013 and Pat. NAP 20130017, 15.11.2013). In addition, two cultivars of *G. hirsutum* (Ravnak-1 and Ravnak-2), created by the method of marker-associated selection aimed at increasing the strength of cotton fiber, were used for experiments (Pat. NAP 201600228, 30.08.2019 and Pat. NAP 201600229, 30.08.2019).

Seeds of the gene-knockout cotton cultivars, such as Porlok-1 and Porlok-4, as well as of Ravnak-1 and Ravnak-2 generated by marker-associated selection were provided by the Center of Genomics and Bioinformatics, Uzbekistan Academy of Sciences. Seeds of the Surkhan-103 and Bukhara-102 cultivars were provided by the Cotton Breeding, Seed Production and Agrotechnologies Research Institute, Uzbekistan Ministry of Agriculture and Water Resources.

### Production of plants and exposure to heat stress

Seeds of the cotton cultivars under study were denuded in concentrated sulfuric acid, washed under cold running water for 15 minutes and kept in the distilled water for 12 hours (Babaeva *et al.* 2020). The swollen seeds wrapped in the paper rolls were germinated in a dark wet chamber for 7 days at 30°C. After the 7th day of germination, 50% of the seedlings were placed in the climatic chamber at 30°C as the controls, another half of the seedlings were exposed to heat stress. To simulate heat stress, the temperature gradually increased from 30°C to 45°C by 1°C every 10 minutes. When the desired temperature was reached, the plants were exposed to 45°C for 6 hours. The conditions were chosen based on the findings from the preliminary experiments with variations of temperature and duration of thermal effects on the plants (the findings are not presented).

After exposure to heat stress, the samples of leaves were collected to be frozen in liquid nitrogen and subsequently analyzed. Part of control and heat-exposed seedlings were used to assess either the growth response by determining

biomass of shoots and roots before exposure to heat stress and in 24 hours after stress or growth of plants under optimum conditions. In addition, biochemical parameters were measured in 24 hours after exposure to the stress. The cotyledon leaves were used for the analysis.

The inhibition of seedlings and root growth of the seedlings was determined according to the formula (Kolupaev *et al.*, 2023a):

$$I = \frac{(C_2 - C_1) - (E_2 - E_1)}{C_2 - C_1} \cdot 100\%$$

where I is growth inhibition (%); C1 and C2, E1 and E2 are, respectively, the initial and final values of seedling organ fresh weights in the control and experimental (heat stress) variants.

### **Contents of hydrogen peroxide**

The method based upon potassium iodide (PI) oxidation by hydrogen peroxide in the acidic medium was used (Junglee *et al.*, 2014). The absorption was measured spectrophotometrically at 390 nm using Shimadzu UV-1800 (Shimadzu, Japan).

### **Measurement of MDA contents**

The MDA content were measured by the reaction with 2-thiobarbituric acid (2-TBA), as described by Gür *et al.* (2010). Frozen leaf samples were homogenized in a solution containing 0.25% 2-TBA in 10% trichloroacetic acid (TCA), while the controls were homogenized in 10% TCA without 2-TBA. The samples were covered with foil caps and boiled for 30 minutes. The samples were cooled and centrifuged at 10.000 g for 10 minutes using DLAB D1524R microcentrifuge (Shandong, China). The absorbance of supernatant was measured at 532 nm. The value for absorption at 600 nm adjusted for the non-specific absorption subtracted from the basic result was measured as well.

### **Analysis of activities of antioxidant enzymes**

To get the enzymatic extract a sample of tissue (500 mg) previously frozen in the liquid nitrogen was ground in a cold porcelain mortar with addition of appropriate extraction buffer (0.1 M Na-phosphate buffer pH 7.0 containing 20 mM EDTA, 2 mM PMSF, 1% triton X, 150 mM PVP) in 1:10 ratio. The homogenate was centrifuged at 8000 g for 15 min at temperature not higher than 4°C.

Total superoxide dismutase (SOD) activity was determined by measuring its capacity to inhibit the photochemical reduction of nitroblue tetrazolium chloride in accordance with a method by Giannopolitis and Ries (1977) with some modifications by Nikerova *et al.* (2019). To eliminate the effects of methionine- and riboflavin-like substances, two additional series of measurements were made without alternative addition of methionine and riboflavin into the incubation medium.

The peroxidase activity was determined using hydrogen peroxide as a substrate and *o*-dianisidine as a reducing agent using method described by

Christensen *et al.* (1998). The deviations in absorbance during the reaction was determined at 460 nm.

The catalase activity was measured using method described by Sinha (1972) in which dichromate in acetic acid is reduced to chromic acetate in a molar ratio of 1: 3. Lowry's method was used for protein measurement (Lowry *et al.*, 1951).

### **Proline contents**

Proline concentrations were measured using ninhydrin reagent in accordance with the method by Bates *et al.* (1973) with some modifications by Shihalyeyeva *et al.* (2014).

### **Reproducibility of experiments and statistical processing of results**

The experiments were repeated thrice, each of which was independently reproduced thrice. The data were statistically processed using Atte Stat V.10.9.6 program as a computer application to "Microsoft Excel-2007". Student's *t*-test was used to determine the significance of inter-variant differences. Different letters denote values with differences significant at  $p \leq 0.05$ .

Correlation coefficients were estimated using the R programming language version 4.1.1 (R Core Team).

## **RESULTS AND DISCUSSION**

### **Growth of cotton seedlings after heat stress**

Exposure to stress temperature caused inhibition of growth in cotton seedlings of all cultivars under study (Table 1). Growth of roots turned out to be more sensitive to heat stress, as compared to the growth of shoots. Thus, in Bukhara-102 and Porlok-1, complete termination of root growth within 24 hours after stress exposure; more than 90% root growth inhibition was seen in Ravnak-1. In other cultivars, the root growth was inhibited more than by 50-70%.

Table 1. Growth inhibition (%) in cotton shoots and roots in 24 hours after 6-hour exposure to 45°C

Cultivar	Growth inhibition, %	
	Shoots	Roots
Surkhan-103	63.7±2.4 b*	78.6±2.9 bc
Bukhara-102	68.9±2.7 b	100.0±0.0 a
Porlok-1	92.9±3.3 a	100.0±0.0 a
Porlok-4	63.5±2.5 b	53.8±2.6 d
Ravnak-1	95.5±3.2 a	90.5±2.8 b
Ravnak-2	68.0±2.8 b	71.4±3.1 c

\* The table shows:  $M \pm m$  ( $n = 3$ ), different letters denote values with differences significant at  $p \leq 0.05$  (Student's *t*-test).

Potent inhibition of shoots growth (more than by 90%) after exposure to high temperature was seen in Ravnak-1 and Porlok-1; in other cultivars the inhibition of shoots growth turned out ranging from 60 to 70%.

Generally, the highest resistance to growth after exposure to high temperature could be seen in Porlok-4; growth of both shoots and roots remained rather intensive (Table 1). Relatively high growth potential of plant organs was retained in Ravnak-2 and Surkhan-103. Roughly, the three cultivars can be considered as the relatively resistant among 6 cultivars under study. At the same time, in the cultivars Porlok-1, Ravnak-1 and Bukhara-102, the values of inhibition of organ growth were significantly greater. (Table 1).

### Parameters of oxidative stress in cotton seedlings after exposure to high temperature

Constitutive concentrations of hydrogen peroxide in various cultivars were different; thus, it was high in Porlok-1, Porlok-4 and Ravnak-1, being slightly lower in Bukhara-102 and Ravnak-2 and even lower in Surkhan-103 (Table 2). The associations between the basic concentrations of H<sub>2</sub>O<sub>2</sub> and heat resistance of seedlings could not be seen. Constitutive MDA concentrations in the seedlings were different by the cultivar. Thus, it was high in Porlok-1, Porlok-4 and Ravnak-1; while lower values were registered in other three cultivars (Table 2).

Table 2. Effect of heat stress on hydrogen peroxide and MDA contents in the cotton seedlings leaves

Cultivar	H <sub>2</sub> O <sub>2</sub> , nmol/g of fresh weight			MDA, nmol/g of fresh weight		
	Control	Heat stress		Control	Heat stress	
		After 6-hour exposure to 45°C	After 6-hour exposure to 45°C and in 24 hours of exposure to 30°C		After 6-hour exposure to 45°C	After 6-hour exposure to 45°C and in 24 hours of exposure to 30°C
Surkhan-103	117±4 e*	139±5 d	118±4 e	12.5±0.4 d	14.3±0.5 c	13.7±0.5 cd
Bukhara-102	159±6 c	195±7 bc	183±5 c	14.8±0.3 c	17.5±0.4 b	16.5±0.3 b
Porlok-1	208±8 b	285±4 a	291±5 a	17.5±0.5 b	24.0±0.8 a	26.4±0.9 a
Porlok-4	196±6 b	220±5 b	208±3 b	16.5±0.5 b	16.7±0.4 b	15.0±0.3 c
Ravnak-1	201±7 b	284±6 a	294±5 a	17.8±0.5 b	23.5±0.8 a	26.7±0.9 a
Ravnak-2	140±3 d	199±5 bc	143±5 d	13.4±0.4 cd	18.0±0.6 b	15.6±0.6 b

\* The table shows: M ± m (n = 3), different letters denote values with differences significant at p ≤ 0.05 (Student's t-test).

The 6-hour exposure to high temperature caused increase in the concentrations of hydrogen peroxide and MDA in the leaves of all plants under study, excluding those of Porlok-4 cultivar. Ravnak-1, Ravnak-2 and Porlok-1 cultivars demonstrated the most significant increase of the heat stress parameters

(Table 2). The less significant effect of increase in concentrations of H<sub>2</sub>O<sub>2</sub> and MDA under heat stress was observed in Bukhara-102 and Surkhan-103.

Within 24 hours after stress, the concentrations of H<sub>2</sub>O<sub>2</sub> and MDA tended to decrease in Surkhan-102, Ravnak-2 and Bukhara-102 cultivars; that is, the values reached the control ones (Table 2). At the same time, the parameters in Porlok-1 and Ravnak-1 remained increased, as compared to the control values. In Porlok-4 cultivar, as it was earlier pointed out, concentrations of H<sub>2</sub>O<sub>2</sub> and MDA remained unchanged in 6 hours of stress exposure; no changes in the parameters could be seen in 24 hours of incubation at optimum temperature, as well.

Generally, no signs of oxidative stress after exposure to 45°C could be seen in Porlok-4 cultivar only. On the contrary, significant increase of hydrogen peroxide and MDA in the leaves after heat stress could be seen in Porlok-1 and Ravnak-1 cultivars; the rest cultivars kept intermediate position by the parameters in question.

### **Activities of antioxidant enzymes in leaves of cotton seedlings after heat stress**

Basic SOD activity in the leaves of different cultivars varied a lot (Table 3). The highest values were registered in Surkhan-103, the only representative of *G. barbadense*. Alterations in activity of the enzyme under heat stress and in post-stress period manifested differently depending on peculiarities of the cultivars. A significant increase in the SOD activity after 6-hour exposure to stress temperature was found in Surkhan-103 cultivar only. In Porlok-1, Ravnak-1 and Ravnak-2 cultivars, activity of the enzyme under the stress of the kind did not significantly change; in Bukhara-102 and Porlok-4 cultivars the SOD activity was found to be reducing (Table 3). In post-stress period, a significant increase in the SOD activity could be seen in Bukhara-102 and Porlok-4. In other cultivars, no alterations in activity of the enzyme significant at  $P \leq 0.05$  could be seen.

Basic activity of catalase in various cotton cultivars under study differed significantly (Table 3). After 6-hour exposure to 45°C, activity of the enzyme decreased in all cultivars excluding Surkhan-103 cultivar. In post-stress period activity of the enzyme remained steadily high in Surkahn-103 cultivar (*G. barbadense*). A significant increase in the activity up to the values, exceeding the control ones, was registered in Bukhara-102, Porlok-4 and Ravnak-2 cultivars. At the same time activity of CAT in Porlok-1 cultivar remained significantly decreased, while in Ravnak-1 it slightly increased not exceeding the control values (Table 3).

As compared to the activities of the other two enzymes, activity of POX in various cultivars in the control varied less significantly. In response to a 6-hour stress exposure, most cultivars experienced an increase in peroxidase activity. However, in Ravnak-1 and Ravnak-2 cultivars, activity of the enzyme decreased. In 24 hours after stress exposure, the activity in most cultivars remained on the level registered in 6 hours after stress exposure, respectively did not change significantly. Ravnak-1 was the exclusion with significant increase in activity of the enzyme.

Table 3. Effect of heat stress on activity of antioxidant enzymes in the cotton seedlings leaves

Cultivar	Control	Heat stress	
		After 6-hour exposure to 45°C	After 6-hour exposure to 45°C and in 24 hours of exposure to 30°C
SOD activity (U/mg protein)			
Surkhan-103	20.1±0.7 c*	25.8±0.5 ab	28.0±0.9 a
Bukhara-102	15.8±0.3 d	13.1±0.4 e	20.2±0.6 c
Porlok-1	15.0±0.6 de	15.9±0.5 d	17.0±0.6 cd
Porlok-4	17.4±0.5 cd	11.5±0.3 f	24.0±0.5 b
Ravnak-1	12.8±0.4 e	12.5±0.4 e	14.0±0.5 de
Ravnak-2	14.4±0.6 de	15.6±0.6 de	15.0±0.6 de
CAT activity (U/mg protein)			
Surkhan-103	150.0±4.8 b	160.0±2.5 b	170.0±6.5 ab
Bukhara-102	130.0±5.9 bc	90.0±4.5 d	190.3±4.5 a
Porlok-1	92.5±3.6 d	54.4±1.5 ef	52.5±1.4 ef
Porlok-4	110.0±3.2 c	78.0±2.7 d	175.0±6.5 ab
Ravnak-1	55.8±1.4 ef	30.3±0.9 h	50.0±1.2 f
Ravnak-2	60.4±1.8 e	42.0±1.0 g	98.0±3.7 cd
POX activity (U/mg protein)			
Surkhan-103	18.0±0.6 cd	23.0±0.9 b	22.0±0.8 b
Bukhara-102	15.8±0.5 d	28.0±0.7 a	25.0±0.8 ab
Porlok-1	17.4±0.3 cd	19.3±0.8 b	25.0±0.7 ab
Porlok-4	18.0±0.5 cd	25.0±0.9 ab	25.4±0.6 ab
Ravnak-1	18.8±0.5 c	11.2±0.3 e	28.0±0.5 a
Ravnak-2	14.4±0.4 de	10.5±0.5 e	12.6±0.5 e

\* The table shows:  $M \pm m$  ( $n = 3$ ), different letters denote values with differences significant at  $p \leq 0.05$  (Student's t-test).

### Contents of proline in the cotton seed leaves under heat stress

Constitutive contents of proline in the cotton leaves were found to vary from 0.28  $\mu\text{mol/g}$  in Porlok-4 to 0.73  $\mu\text{mol/g}$  in Ravnak-2 cultivar (Table 4). The 6-hour exposure of seedlings at 45°C caused various effects depending on the cultivar. A significant increase of the proline contents could be seen in Ravnak-2 cultivar only. In Porlok-1 and Porlok-4 cultivars, after the 6-hour stress exposure proline did not change significantly. Subsequent 24-hour exposure of seedlings

exposed to optimum temperature (30°C) caused significant increase in concentrations of proline in Surkhan-103, Bukhara-102 and Porlok-4 cultivars.

In other cultivars, after transfer of seedlings to the optimum conditions proline either unchanged significantly, or tended to slight decrease (Porlok-1, Ravnak-1 and Ravnak-2 cultivars).

Table 4. Effect of heat stress on concentrations of proline ( $\mu\text{mol/g}$  of fresh weight) in the cotton seedlings leaves

Cultivar	Control	Heat stress	
		After 6-hour exposure to 45°C	After 6-hour exposure to 45°C and in 24 hours of exposure to 30°C
Surkhan-103	0.34±0.01 d	0.24±0.01 ef	0.45±0.01 c
Bukhara-102	0.67±0.02 b	0.40±0.01 c	0.83±0.03 ab
Porlok-1	0.66±0.02 b	0.59±0.03 bc	0.44±0.01 c
Porlok-4	0.28±0.01 e	0.25±0.01 ef	0.48±0.02 c
Ravnak-1	0.34±0.01 d	0.26±0.01 ef	0.22±0.01 f
Ravnak-2	0.73±0.03 b	1.00±0.05 a	0.92±0.01 a

\* The table shows:  $M \pm m$  ( $n = 3$ ), different letters denote values with differences significant at  $p \leq 0.05$  (Student's t-test).

### Correlations between growth response of plants to heat stress and biochemical parameters

Absolute values of contents of hydrogen peroxide and MDA in leaves at the moment of elimination of the 6-hour exposure to 45°C were found to closely correlate with the growth inhibition (Figure 1). Activities of antioxidant enzymes were in inverse correlation with the growth inhibition of seedlings, though their values were not significant at  $P \leq 0.05$ . Attention should be paid to high coefficient of inverse correlation between catalase activity and concentrations of hydrogen peroxide, being the evidence for the contribution of the enzyme in the control of  $\text{H}_2\text{O}_2$  contents in the cotton leaves.

Correlation between growth inhibition of seedlings and the AOS parameters was found closer not immediately after stress, but after subsequent 24- hour very high correlations between growth inhibition and oxidative stress parameters (contents of  $\text{H}_2\text{O}_2$  and MDA) could be seen. Inverse correlation between growth inhibition and catalase activity was registered, as well (Figure 2).

Thus, absolute values of some parameters characterizing the status of pro-/antioxidant balance, including of hydrogen peroxide and MDA contents and CAT activity, significantly correlated with the seedlings growth potential in post-stress period. Still, the data obtained for other plant species demonstrate that in some cases not absolute values of biochemical parameters but the relative ones

(in % to the controls) reflecting the character of changes in these parameters under stress and in the post-stress period (Kolupaev *et al.*, 2023a).

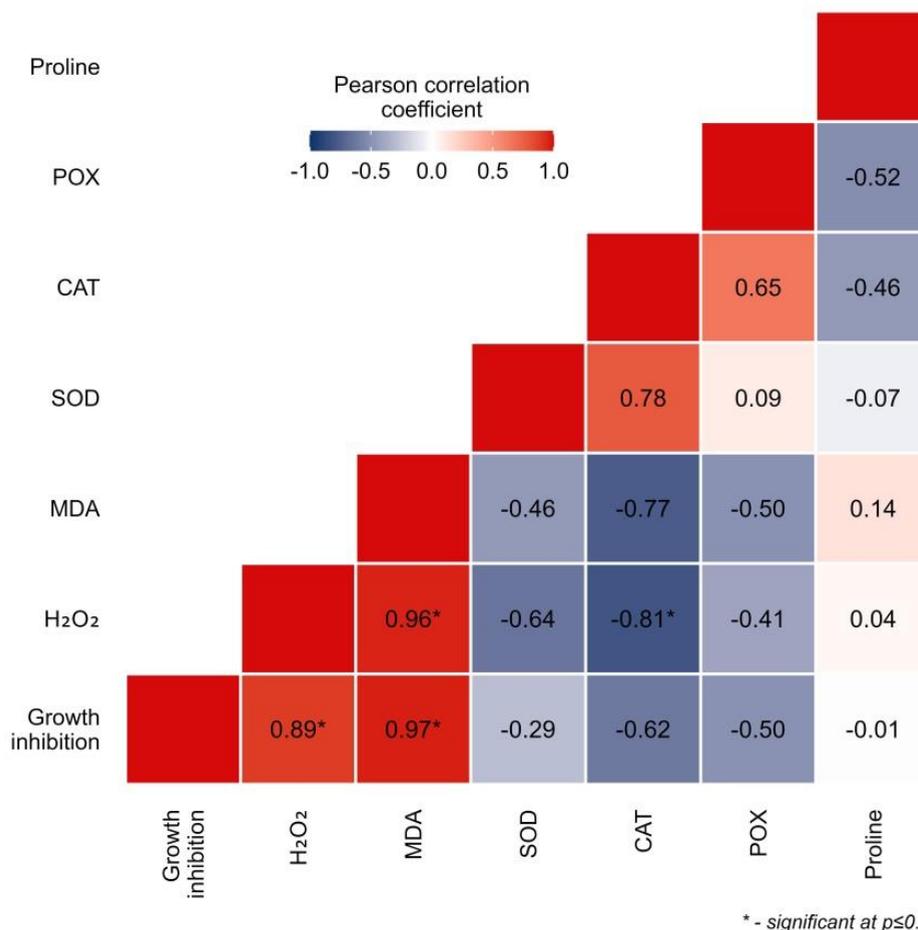


Figure 1. Correlation between growth inhibition and absolute values of biochemical parameters in cotton cultivars after 6-hour exposure to 45°C, CAT – catalase, MDA – malondialdehyde, POX – peroxidase, SOD – superoxide dismutase.

That was the reason for us to present the results as the heat map of values calculated in % to respective control values (Figure 3). This helped see some patterns less noticeable in analysis of absolute values. Thus, in Surkhan-103 and Porlok-4 cultivars with the lowest growth inhibition of the above ground part of plant (Table 1) only insignificant increase in H<sub>2</sub>O<sub>2</sub> and MDA contents could be seen after the 6-hour stress exposure; while subsequently after transfer of plants to optimum conditions the parameters decreased to the control levels.

It is of note, that in the stress resistant Surkhan-103 and Porlok-4, the SOD activity was increased more significantly than the one in other cultivars in 24

hours after elimination of stress exposure (Figure 3). Significant increase in catalase activity in post-stress period took place in the stress resistant cultivars – Porlok-4 and Ravnak-2. At the same time, dynamics of changes in the peroxidase activity was found to slightly depend on the resistance of cultivars.

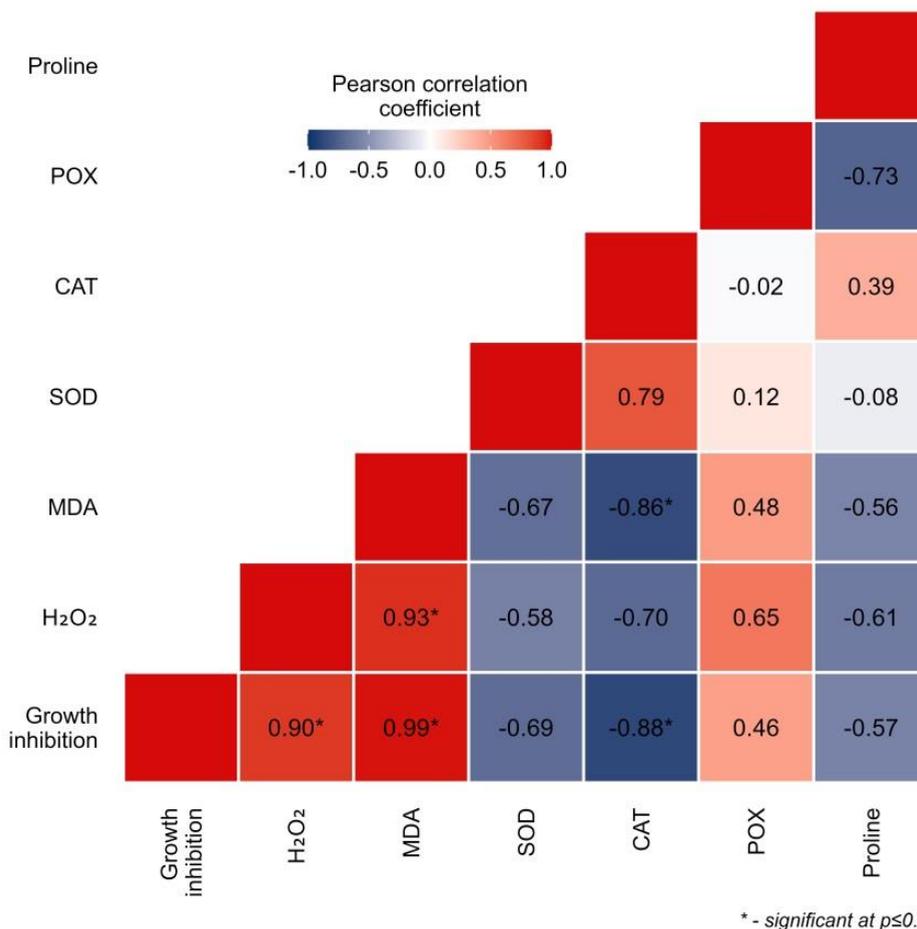


Figure 2. Correlation between growth inhibition and absolute values of biochemical parameters in cotton cultivars after 6-hour exposure to 45°C and subsequent 24-hour exposure to 30°C, CAT – catalase, MDA -malondialdehyde, POX – peroxidase, SOD – superoxide dismutase

Contents of proline immediately after stress exposure slightly depended on the growth parameters. However, the contents in post-stress period significantly increased in two cultivars maintaining high growth potential, to name Porlok-4 and Surkhan-103.

Based on the values expressed in percent to the control, we calculated coefficients of correlation between growth inhibition and parameters characterizing the AOS functioning (Figure 4). It turned out that immediately after stress exposure rather high direct correlation was registered between the

values of growth inhibition of seedlings and increase in contents of oxidative stress markers – hydrogen peroxide and MDA, as well as the inverse correlation between growth inhibition and increase in catalase activity. However, the correlations were not significant at  $P \leq 0.05$ . Correlations of growth inhibition with other biochemical parameters turned out relatively low.

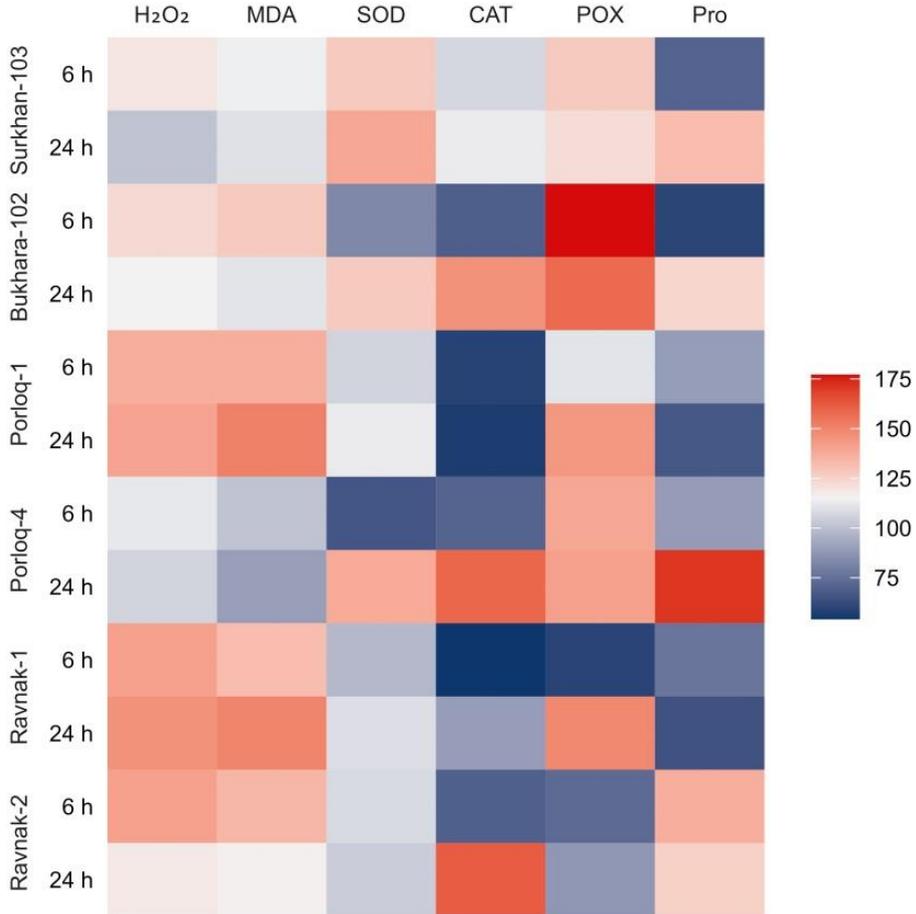
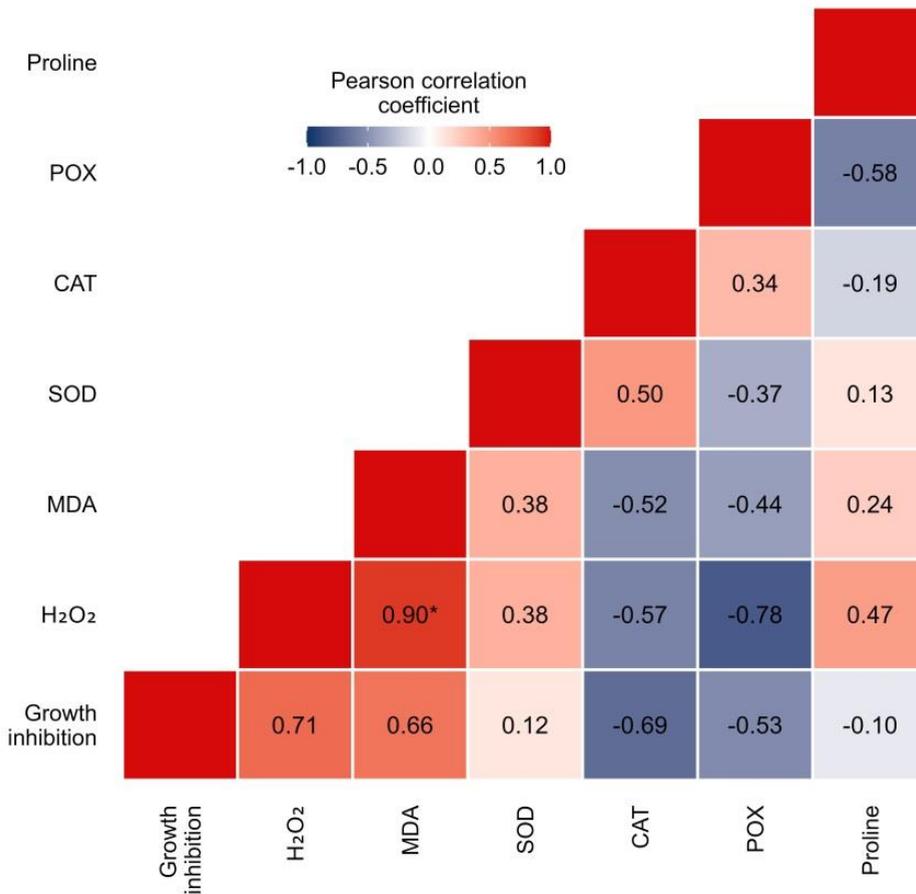


Figure 3. The heat map of alterations in biochemical parameters in cotton cultivars after 6-hour exposure to 45°C and after subsequent 24-hour exposure to 30°C. All values are presented in % to values in the controls, CAT – catalase, MDA -malondialdehyde, POX – peroxidase, SOD – superoxide dismutase

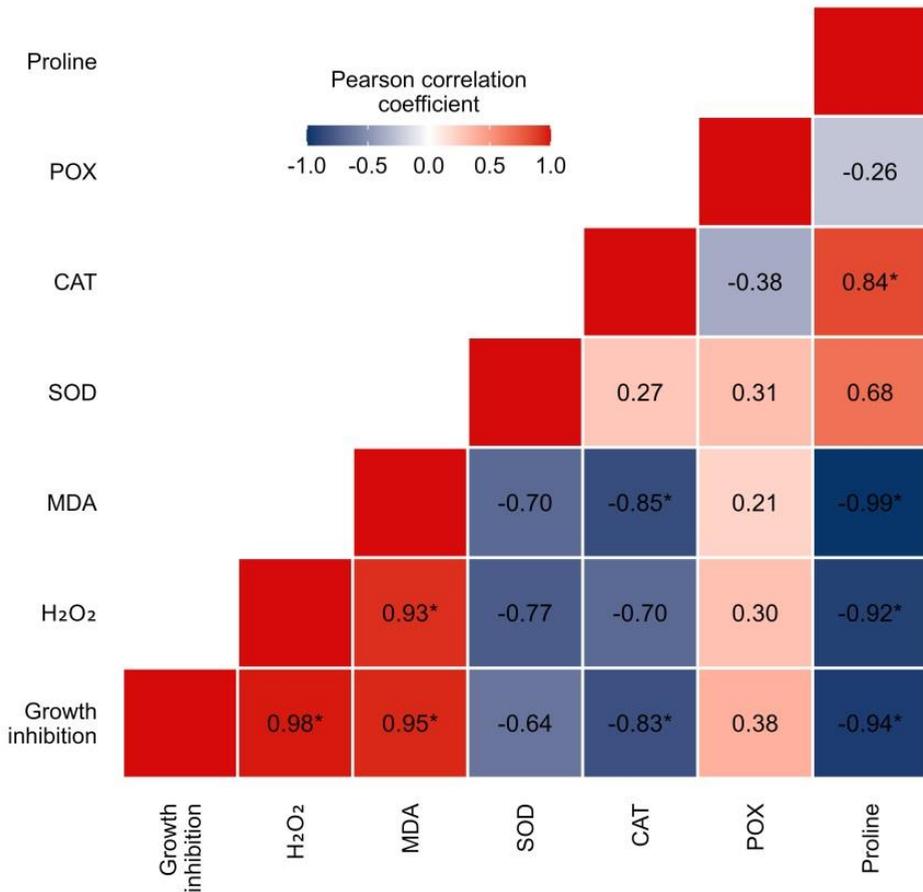
More explicit picture of correlations between growth and relative biochemical parameters could be seen in 24 hours after of stress effect. In this case, the correlation between growth inhibition and alterations in contents of H<sub>2</sub>O<sub>2</sub> and MDA (0.98 and 0.95, respectively) turned out the highest one (Figure 5). Significant ( $P \leq 0.05$ ) inverse correlations between growth inhibition and alterations in activity of catalase and contents of proline were found, as well. In

addition, attention should be paid to the high inverse correlation between alterations in the contents of MDA and proline, as well as to the one between alteration in activity of catalase and MDA contents. Alterations in contents of proline were found closely inversely correlated with alterations in contents of hydrogen peroxide in leaves. It is reasonable to think that the activity of catalase and contents of proline might be essential elements in the strategy of prevention for irreversible progress of oxidative stress and excessive inhibition of growth. It is noteworthy that a high direct correlation between alterations in proline contents and catalase activity could be seen as well. Rather high but insignificant ( $P \leq 0.05$ ) inverse correlation between growth inhibition and alteration in the SOD activity was registered to be indicative of considerable contribution of the enzyme to the maintenance of post-stress pro-/antioxidant balance.



\* - significant at  $p \leq 0.05$

Figure 4. Correlation between growth inhibition and alterations in biochemical parameters (in % to control) in cotton cultivars after 6-hour exposure to 45°C, CAT – catalase, MDA -malondialdehyde, POX – peroxidase, SOD – superoxide dismutase



\* - significant at  $p \leq 0.05$

Figure 5. Correlation between growth inhibition and alterations in biochemical parameters (in % to control) in cotton cultivars after 6-hour exposure to 45°C and subsequent 24-hour exposure to 30°C, CAT – catalase, MDA - malondialdehyde, POX – peroxidase, SOD – superoxide dismutase

Generally, our findings are consistent with those of Hasan et al. (2018). This study compares the AOC status of three cotton cultivars having different drought tolerance and belonging to three different species including *Gossypium hirsutum*, *Gossypium arboreum* and *Gossypium barbadense* under drought conditions. According to the authors, the most frequently used species in breeding, *Gossypium hirsutum*, exceeds the other two in terms of resistance (Hasan et al., 2018). The experimental data obtained by the authors indicated the presence of correlations between resistance and concentrations of proline, soluble proteins and carbohydrates, as well as the activities of SOD, POX, ascorbate peroxidase and oxidative stress parameters. In study on correlations between antioxidant activity and drought resistance of the cotton species belonging to

*Gossypium hirsutum*, the reduction in the activities of catalase and peroxidase was demonstrated in non-resistant genotypes and the increase in the parameters of the resistant ones (Singh *et al.*, 2021). Upon comparison responses of tolerant and resistant cotton cultivars to the combined effect of drought and high temperature, Sekmen *et al.* (2014) established that the resistance to the stresses was associated with the capability of the cultivars to preserve constitutive activity of SOD and ascorbate, as well as to increase activity of catalase and non-specific peroxidase. The cultivar most resistant to the combined effects of stressors had the capacity of accumulating higher concentrations of proline.

As it was pointed out, in the field experiments on the stage of cotton flowering activities of catalase, SOD, POX as well as contents of proline under heat stress were found increased in the resistant cultivars, while contents of MDA and hydrogen peroxide were typically lower in them (Zafar *et al.*, 2023).

Yousaf *et al.* (2022) demonstrated significant positive correlation of seed cotton yield with activity of POX ( $r = 0.974$ ) and SOD ( $r = 0.868$ ) under heat stress field conditions by shift in sowing period. In several works, attempts to assess the association between heat resistance of cotton cultivars to high temperatures and accumulation of MDA as a main marker of oxidative stress have been made. Sarwar *et al.* (2023) demonstrated close correlation of MDA contents with the cell damage in plants grown in the hothouse under local terminal heat stress. At the same time, no inverse correlation between heat resistance and MDA accumulation could be seen in the control plants and those exposed to heat stress due to late sowing in the field (James *et al.*, 2005).

In our findings, cotton cultivars in the post-stress period showed high correlation between growth inhibition and accumulation of markers of oxidative damages, to name hydrogen peroxide and MDA. High negative correlation has been found between growth inhibition and increased catalase activity and proline contents in post-stress period (Figure 5), as well. Close inverse correlation between alterations in MDA concentrations and those in proline contents was registered. This indicates considerable contribution of the AOS components above to the protection of cotton seedlings from oxidative stress. It is of note, that the inverse correlation between contents of MDA and proline under stress can be seen not in all plant species. Thus, the wheat cultivars under heat stress demonstrated high direct correlation between the increased contents of MDA and proline (Kolupaev *et al.*, 2023b). Close direct correlation was demonstrated in the rapeseed plants exposed to toxic effect of copper (Kholodova *et al.*, 2018). The accumulation of proline in these plant species is possible to take place when there are the oxidative damage occurrences; this can be the basis for argument about contribution of proline to the antioxidant protection. Participation of proline in response to heat stress and, possibly, to other ones can be suggested to depend on the plant species peculiarities. This seems to increase the relevance of special study on the AOS behavior in plants of definite species under definite types of stress. Generally, status of the AOS components in plants of different genotypes can be a significant marker for their resistance to stresses of various natures. In

particular, the association found between resistance of the wheat cultivars to the drought and heat stress and their capability to withstand damages under direct effect of oxidative stress agents, including hydrogen peroxide and iron sulfate (III) (Yastreb *et al.*, 2023). The association between the drought resistance of the rice cultivars and their resistance to methyl viologen, an active ingredient of herbicides causing oxidative damages was demonstrated, as well (Iseki *et al.*, 2014).

Our work presents the comparative estimation of the thermal stability in cotton seedlings of two botanical species and 6 cultivars obtained by various methods. Of note, under extreme conditions, Surkhan-103 cultivar (*G. barbadense* species) demonstrated rather high resistance to high temperature; low parameters of growth inhibition and accumulation of oxidative damage markers being the evidence for that (Tables 1, 2). As compared to others, Porlok-4, a cultivar with a phytochrome knocked out gene, demonstrated high resistance. Ravnak-2, a cultivar generated using marker-associated selection, was found to be rather resistant to high temperatures. It should be noted, two other cultivars generated using biotechnological methods, to name Porlok-1 and Ravnak-1 turned out less resistant to high temperatures. By this parameter, they conceded to the moderately resistant Bukhara-102 generated using classical selection.

## CONCLUSIONS

The cotton seedlings can serve as a model for estimation of heat resistance of cultivars and study on contribution of components of stress-protective systems in manifestation of heat resistance as a feature. A direct correlation has been established between the accumulation of hydrogen peroxide, the LPO product of MDA, and the amount of growth inhibition of cotton cultivars after heat stress. An inverse relationship has also been established between the inhibition of plant growth caused by heat stress and the accumulation of proline, an increase in the activity of CAT and, partly, SOD in the period after stress. These parameters can be used in assessment of the selection cotton material by heat resistance.

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