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Effect of exogenous hydrogen peroxide on biochemical parameters of cotton cultivars exposed to high temperature

Munavvar Nurmatova[1*], Ali Akhunov[1], Nigora Khashimova[1], Karomat Kuldoshova[1,2] & Sanjar Navruzov[1]

[1]Laboratory of Chemistry of enzymes, A.S. Sadykov Institute of Bioorganic Chemistry, Uzbekistan Academy of Sciences, 100 125, Tashkent, Republic of Uzbekistan

[2]National University of Uzbekistan named after Mirzo Ulugbek, Uzbekistan Ministry of Higher Education, Science and Innovations, Almazor 100 174, Tashkent, Republic of Uzbekistan

*Email - munavvarnurmatova90@gmail.com

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Abstract

The increased production of reactive oxygen species (ROS), possibly leading to damage to intracellular homeostasis, is a response to temperature stresses. Hydrogen peroxide in high concentrations results in oxidative stress and even causes cell death; it acts as a signalling molecule and increases the activities of antioxidant enzymes due to gene expression in low concentrations. At the same time, H_2O_2 is the most stable ROS and acts as a key regulator of some physiological and biochemical processes. Consequently, the work was performed to study the stimulating effect of pretreatment with H_2O_2 (10mM) in 6 cotton cultivars exposed to high temperatures (45°C). The findings from the study clearly showed an increase in the activities of antioxidant enzymes, including superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD), as well as of proline concentrations in the cotton sprouts exposed to heat stress, as compared to not pretreated ones. Pretreatment with 10mM H_2O_2 improved tolerance to oxidative stress arising under heat stress. The extent of lipid membrane damage was low in the heat-resistant and heat-tolerant cotton cultivars. Spraying with 10mM H_2O_2 is thought to cause accumulation of intracellular hydrogen peroxide before exposure to stress; it's further increased quantity as a signalling molecule led to the expression of genes of protective proteins of the antioxidant system and osmolytes (proline), providing the balance between the accumulation and removal of free oxygen radicals.

Keywords

antioxidant system; catalase; heat stress; malondialdehyde; peroxidase; proline; reactive oxygen species; superoxide dismutase

Introduction

Accumulation of reactive oxygen species (ROS) is an early cell response to effect of high temperature. In higher plants, the ROS neutralization is under enzyme control to be removed separately or conservatively by the antioxidant enzymes including superoxide dismutase (SOD), catalase (CAT), peroxidase (POD) and others (1). SOD is brought front of the antioxidant protection, as transfer of an aggressive form of superoxide anion radical (O_2^-) generating in various physiological processes into H_2O_2 as more stable compound is the essential function of this enzyme (2). Further neutralization of hydrogen peroxide (H_2O_2) takes place with the participation of CAT, effectively acting under high concentrations of H_2O_2 in contrast to POD. One molecule of CAT is known to transform 6 million molecules of H_2O_2 into water and molecular oxygen (3, 4). That means that under physiological conditions, the activity of CAT is 10,000

times higher than that of POD (5). However, this activity in plants can be inconsistent, probably depending on the concentration of H_2O_2 that isformed. Along with this, proline protects membranes and proteins from the adverse effects of high concentrations of hydroxyl radicals under stress. Its structural peculiarities give grounds for the direct inactivation of ROS. Proline is known to be able to form a stable radical as it contains a tertiary carbon atom. Forming a radical like this results in suppression or breakage of a cascade of free radical reactions triggered by superoxide radicals, such as peroxide radicals or hydroxide radical (6). The association between accumulation of proline and plant stability to stressors was demonstrated in many works. In some plants, more than a hundredfold increase in proline concentrations in response to effects of adverse stressors (7). As a compatible soluble compound, proline takes part in the stabilization of proteins and protein components in chloroplasts and cytosol, in the protection of photosynthetic apparatus and enzymes participating in the detoxification of ROS, as well as in the stabilization of redox balance (8).

ROS were thought to play a negative role in cells causing oxidative stress for quite a long time. However, recent studies demonstrated ROS's high physiological value in plant organisms' ontogenesis (9). Exogenously applied eco-friendly chemicals like proline, hydrogen peroxide and salicylic acid may improve the tolerance of plants by fine-regulating the activities of SOD, CAT, POX and key enzymes of the ascorbate–glutathione pathway (10). $H_2O_2$is a potent regulator in signalling pathways when the plant is under stress. The oxidative damage triggered by the various ROS generated in response to multiple stresses continuously decreases the productivity of plants. Abiotic stress tolerance could be attained by fine regulation of the antioxidant responses in plants. H_2O_2 treatment also protected the sensitive cultivars from drought stress by increasing CAT, POD, ascorbate peroxidase (APX), monodehydroasorbate reductase (MDHAR) and glutathione reductase (GR) enzymes (11). H_2O_2 plays an essential role in cell signalling at low concentrations. Drought stress reduced H_2O_2in sensitive cultivars (12). Many other reports also depicted that exogenous application of various biomolecules resulted in an increase in SOD and CAT activities and membrane integrity which may further be involved in the maintenance of photosynthetic pigments under drought-affected conditions (13). Despite the importance of the exogenous application of H_2O_2 in different crops, there is limited information about the protective effects of seed pretreatment with H_2O_2. In our earlier studies, concentrations of H_2O_2 ranging from 10 to 100 mM were shown to cause a linear increase in intracellular H2O2 and malondialdehyde (MDA) concentrations by the concentrations of H_2O_2 (14). Experiments demonstrated that 10mM H_2O_2 did not stimulate oxidative stress, stimulating the activity of CAT and POD. The work was initiated to study the effects of pretreatment cotton cultivars with 10mM H_2O_2 and the ability to generate tolerance to oxidative stress caused by the 6-hour exposure to high temperature (45°C).

Materials and Methods

The study used biotechnological cotton cultivars generated by the gene knockout method (Porlok-1 and Porlok-4), those generated using marker-associated selection (Ravnak-1 and Ravnak-4) provided by the Center of Genomics and Bioinformatics, Uzbekistan Academy of Sciences, as well as medium-fiber cotton cultivar Bukhara-102 (*G. Hirsutum*) and fine-fiber cotton cultivar Surkhan-103 (*G. barbadense*) generated using classical cotton breeding provided by the Cotton Breeding, Seed Production and Agrotechnologies Research Institute, Uzbekistan Ministry of Agriculture and Water Resources.

Germination of sprouts

Seeds of the cotton cultivars under study were denuded in concentrated sulfuric acid, washed under cold running water for 15 min and kept in the distilled water for 12 hrs (15). We used 40 seeds per group. Each group contains three samples (Table 1). Several experiments were performed in the climatic chamber with a temperature of 30 and 45 °C and a relative humidity of 60 % as climatic conditions desired.

After the 7th day of germination, the leaves of the sprouts were sprayed with 10mM H_2O_2 and stored in the chamber for 24 hrs for complete absorption (16). The seeds were exposed to high temperatures (45 °C) for 6 hrs. After the exposure, the leaves were collected for analysis. The control set of leaves was sprayed with distilled water.

Measurement of activities of antioxidant enzymes

To get the enzymatic extract, a 500-mg sample of tissue previously frozen in the liquid nitrogen was ground in a cold porcelain mortar with the addition of appropriate extraction buffer (0.1 M Na-phosphate buffer, pH 7.0, 20 mM EDTA, 2 mM PMSF, 1 % triton X, 150 mM PVP) in 1: 10 plant tissue: water ratio.

Measurement of SOD activity:

Total SOD activity was determined by measuring its capacity to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) chloride following a method by Giannopolitis and Ries (17) with some modifications (18).

Measurement of POD activity

For POD activity, Hydrogen peroxide was used as a substrate; o-Dianisidine (another name, 3,3'-dimethoxy-4,4'-benzidine) was used as the reducing agent. Changes in the absorption in the reaction were assessed at 460 nm. The reaction was initiated by adding 50 µL of H_2O_2 for 1 min. POD activity was expressed in U/mg of protein (19). Lowry's method was used for protein measurement (20).

Table 1. Groups used for establishing heat stress

	Control	Heat stress	H_2O_2 and heat stress
Temperature	30 °C	45 °C	45 °C
Duration	7 days-old seedlings	6 days-old seedlings were subjected to heat stress for 6 h on 7th day	6 days-old seedlings were subjected to H_2O_2 and heat stress for 6 h on 7th day

*We used three replicates in each group. Depending on the seasons, we provided 20 % humidity in summer and 60 % humidity in winter.

Measurement of CAT activity

The CAT activity was measured with the modified method in which dichromate in acetic acid is reduced to chromic acetate in a molar ratio of 1: 3 with some modifications (21, 22).

Measurement of H_2O_2 concentrations

To measure concentrations of H_2O_2, the method based upon potassium iodide (PI) oxidation by H_2O_2 in the acidic medium was used (23).

Measurement of MDA concentration

The MDA content was measured by the reaction with 2-thiobarbituric acid (2-TBA) (24). 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 min using DLAB D1524R microcentrifuge (Shandong, China). The absorbance of the supernatant was measured at 532 nm. The value for absorption at 600 nm adjusted for the nonspecific absorption subtracted from the basic result was also measured.

Statistical processing of the data

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 significant differences at $p \leq 0.05$. Correlation coefficients were estimated using the R programming language version 4.1.1 (R Core Team) as a computer application to "Microsoft Excel-2007".

Results and Discussion

The heat shock protein gene is essential in plants, as it helps the plant's cellular machinery defend against various abiotic stresses such as heat. It also performs an additional role by interacting with extended sections of protein peptides and with proteins that are folding. The interaction of HSP70 with other proteins prevents them from aggregating together, facilitates changes in their folding to reach their final shape and controls how the proteins perform (25). Molecular signalling is triggered when newly synthesized proteins are released from the ribosome and substrate binding domains of Heat Shock Protein (HSP70) help to identify and bind to the hydrophobic amino acid residue sequence (26). Similarly, research identified long non-coding RNAs (lncRNAs) targeting essential candidate genes associated with high-temperature tolerance in cotton. These candidate genes encompass chlorophyll a-b binding proteins, ribosomal proteins and heat shock proteins. The roles of circular RNAs (circRNAs) in cotton's growth, development and stress response were investigated (28, 29).

Under stress situations, heat shock factor A2 (HSF A2) makes a super activator hetero oligomer structure with HSFA1, which is more efficient than the individual HSFs, which not only regulate the down-stream stress related HSP genes but also the protective enzyme genes such as GST, GR, POX and APX (30). Some studies also report that HSP gene expression positively regulates the protective enzyme activities. As observed in Arabidopsis, the overexpression of HSP17.8 enhanced the SOD activity and in tobacco, HSP16.9 increased the activities of POD, CAT and SOD (31). Our findings demonstrated that the pretreatment of cotton sprouts with 10 mM H_2O_2 under the 6-hour exposure to high temperature (45 °C) caused an increase in the SOD activity in Bukhara-102 (91 %) and Porlok-1 (89 %). Along with this, as compared to the samples not pretreated with H_2O_2 (74.3 ± 2.8 µmol of H_2O_2 mg of protein min^{-1}), the activity of CAT, an essential enzyme decomposing H_2O_2, in the pretreated ones increased by 66 % in Bukhara-102 (119.6±3.41 µmol of H_2O_2 mg of protein min-1) and by 58 % in Porlok-1 (118.0 ± 4.5 µmol of H_2O_2 mg of protein min^{-1}) (Fig. 1).

The increase in catalase activity as a response to pretreatment with hydrogen peroxide suggests potential role in regulating H_2O_2 in a plant cell, probably associated with the increase in the endogenous hydrogen peroxide caused by the treatment with 10 mM H_2O_2. Multiple data demonstrate that exogenous administration of H_2O_2 causes an increase in quantities of SOD and CAT isoenzymes under stress conditions. Similar results were obtained in studies of wheat, explaining the increase by the fact that the exogenous administration of H_2O_2 caused the accumulation of the intracellular H_2O_2 further, resulting in a rise in activity of SOD and CAT under stress conditions (32). Findings from many studies demonstrated the effect of H_2O_2 as a key regulator of essential physiological and biochemical processes (33). Research showed the report on the participation of H_2O_2 in the adaptation of plants under salt stress conditions by increasing the activity of antioxidant enzymes (34). Research indicated the significance of CAT activation in neutralizing H_2O_2 generated under stress by the example of transgenic tobacco deficient in the CAT gene with greater damage of leaves due to oxidative stress, compared to leaves of common tobacco (35).

In contrast to other cultivars, an increase in the POD activity was found as a response to the pretreatment with 10mM H_2O_2 in Ravnak-1, as compared to the cotton sprouts not pretreated with hydrogen peroxide under effects of high temperature. Still, a significant increase in the POD activity was demonstrated in Ravnak-2 (97 %), while no substantial changes were found in the SOD and CAT activities. The key role of the essential enzyme in determining resistance to oxidative stress is suggested to depend on the plant species (36). The CAT activity was reduced by 23% and 14 % in Surkhan-103 and Porlok-4, respectively, after pretreatment with hydrogen peroxide under high temperatures.

In these cultivars, the activity of POD increased basically. Reduction in CAT activity can be seen under some conditions, while SOD, POD and APX, as a rule, are induced for removal of ROS (37). The findings are consistent with data from other studies confirming that there is no clear boundary between activities of antioxidant enzymes; under heat stress, activating the enzymes is compensatory. Activation of plant antioxidant system in response to exogenous pro-oxidant effects seems quite logical. Pretreatment of sprouts of arabidopsis with hydrogen peroxide was demonstrated to trigger the two-phase increase of Ca^{2+} concentrations in

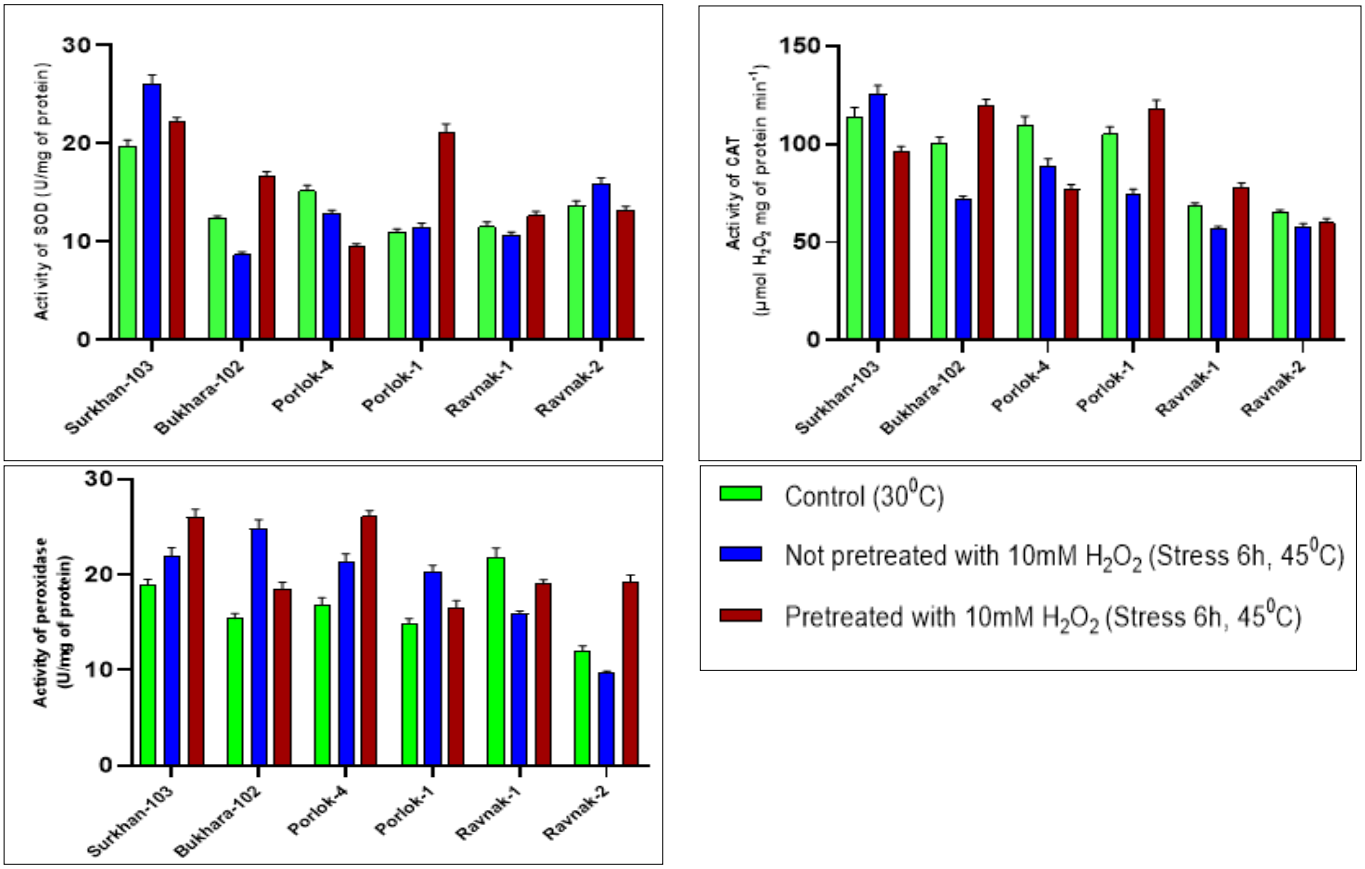

Fig. 1. Activity of superoxide dismutase, CAT and POD in leaves of cotton sprouts pretreated with 10mM H_2O_2 under the 6-hour exposure to 45°C. * The table shows: M ± m (n = 3), different letters denote values with differences significant at $p \leq 0.05$ (Student's t-test).

cytosol and subsequent expression of the gene of glutathione -S-transferase (38).

Key ROS scavenging enzymes reported are SOD, CAT, POX, APX and GR. In contrast, plants use glutathione, ascorbic acid (vitamin C) and tocopherols as vital non-enzyme antioxidants under stressful conditions to alleviate the adverse effects of ROS. Pretreatment of seeds with 60 mM H_2O_2 alleviated water stress-induced growth inhibition in all three wheat cultivars. Further, it enhanced the drought tolerance of PBW 644 by upregulating SOD, POX, APX and GR enzymes, accompanied by an increase in total phenols and ascorbate content. Compared to stress, H_2O_2 pretreatment caused a rise in the POX activity of roots of PBW 644 and shoots of PBW 621 in wheat cultivars (11). Thus, oxidative stress caused by the effect of exogenous H_2O_2 can be assumed to serve as the signal for activation of antioxidant enzymes. Despite the various levels of activity of the antioxidant enzymes, concentrations of intracellular hydrogen peroxide and malondialdehyde in all-cotton cultivars under study decreased in response to the combined effects of 10mM H_2O_2 and high temperature (45°C) (Fig. 2).

Sprouts of Porlok-1, a biotechnological cotton cultivar sensitive to high temperature, demonstrated the most significant increase in concentrations of H_2O_2 and MDA under the effect of high temperature. Pretreatment of the sprouts with H_2O_2 significantly decreased concentrations of intracellular hydrogen peroxide and level of lipid peroxidation under heat stress. Improvement in heat stress tolerability can be associated with the increase in the activities of SOD and CAT after pretreatment of plants with hydrogen peroxide. Research indicates that hydrogen peroxide and MDA can increase with the increase in concentrations of H_2O_2, but low concentrations of hydrogen peroxide cause the reverse effect (39). Elevated MDA content indicates oxidative stress in plants subjected to various stresses, including drought stress. The levels of MDA content were reduced in the treated seedlings of all the cultivars, which further revealed the role of H_2O_2 pretreatment in alleviating membrane damage (11). Compared to drought stress, H_2O_2 pretreatment led to a significant decrease in MDA levels (13). Therefore, the above-discussed data revealed that H_2O_2 pretreatment played a role in maintaining MDA levels by activating defense responses and assisting them in coping with the adverse effects of high temperatures.

Under the effect of H_2O_2 , an increase in the stability of cell membranes can be associated with an increase in proline, a free amino acid. Proline protects membranes and proteins from adverse environmental effects, acting as an absorber of hydroxyl radicals. Our findings demonstrated that the 6-hour exposure to high temperature caused a decline in proline levels. Similar results were obtained in the wheat after exposure to various temperature regimes (30, 35 and 40 °C) (25). Our findings demonstrated an increase in the levels of proline in Surkhan-103 (100 %), Bukhara-102 (37.2 %), Porlok-4 (50 %) and Ravnak-1 (107 %) under the effect of high temperature in the pretreated sprouts. Kumar *et al.* explain the similarity of findings by the fact that the exogenous administration of H_2O_2 causes intracellular accumulation of proline, making possible the conclusion that the intracellular concentrations of hydrogen peroxide somehow correlate with the genes participating in the proline biosynthesis (32). Accumulation of proline under heat stress increases heat tolerance, probably due to increased ROS production via the

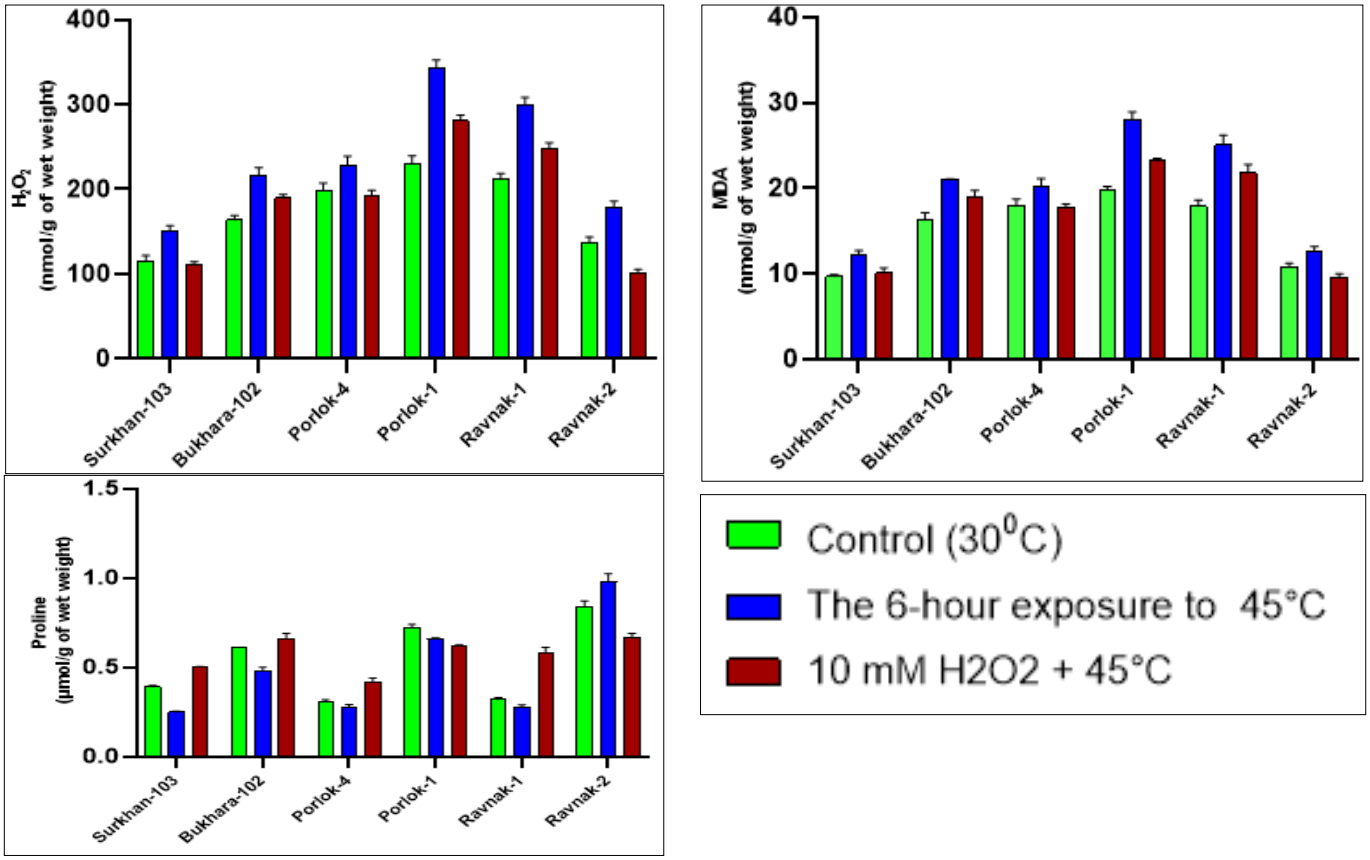

Fig. 2. Concentrations of hydrogen peroxide, malondialdehyde and proline in leaves of cotton sprouts pretreated with 10mM H_2O_2 under the 6-hour exposure to 45°C. * The table shows: M ± m (n = 3), different letters denote values with differences significant at $p \leq 0.05$ (Student's t-test).

Pro/P5C cycle of the redox control system, triggering the expression of the protective gene (40). In addition, Orabi and Sadak found that soaking the wheat seeds in 50 and 100 mM H_2O_2 significantly increased free amino acids, including proline, total quantities of the soluble carbohydrates and activities of some enzymes (41).

Our findings demonstrated that pretreatment with 10mM H_2O_2 generated heat tolerance in cotton cultivars. Early increase of endogenous H_2O_2 causes the effect of elicitor, the compounds that mitigate abiotic stress effects by enhancing the antioxidant profile and accumulation of bioactive compounds (42). Our findings demonstrated the double role of H_2O_2 in cotton; hydrogen peroxide in high concentrations results in oxidative stress and causes the death of plant cells, while in low concentrations, it acts as a signalling molecule. This is consistent with the data that hydrogen peroxide possesses a regulating ability to activate antioxidant enzymes under heat stress. Thus, an increase in plant tolerance to high temperatures is possible by inducing the expression of one's genes with the participation of ROS, which provides protective responses.

Conclusion

Based on the above results, it was concluded that high temperature causes an increase in ROS, leading to oxidative stress and lipid peroxidation. The results clearly showed that H_2O_2 at low concentrations (10 mM) plays a regulatory role in the activation of antioxidant enzymes such as SOD, CAT and POD, reducing oxidative stress against the background of MDA and also causing the accumulation of proline. Thus, it was concluded that pretreatment of cotton varieties with low concentrations of H_2O_2 helps to resist oxidative stress caused by heat stress.

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Authors' contributions

AA wrote the manuscript and supervised the project. KN performed the statistical processing of the data. NM determined the activities of SOD and CAT. KK determined concentrations of proline and hydrogen peroxide. NS measured concentrations of MDA and activities peroxidase activity. All authors read and approved the final manuscript.

Compliance with ethical standards

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

Ethical issues: None

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