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INVESTIGATION OF THE EFFECT OF SALICYLIC ACID IN COMBINATION WITH SODIUM NITROPRUSSIDE ON THE DEVELOPMENT OF UPLAND COTTON (*Gossypium hirsutum* L.) GENOTYPES

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Abstract

This study investigates the combined effect of salicylic acid (SA) and sodium nitroprusside (SNP), a nitric oxide (NO) donor, on the antioxidant defense system of two upland cotton genotypes (*Gossypium hirsutum* L. Agdash-3 and AP-317) under hydroponic conditions. Seedlings were treated with 0.1 mM SA and two concentrations of SNP (0.1 and 2.5 mM), and biochemical responses were assessed after two weeks of growth. The activities of catalase, peroxidase, and polyphenol oxidase, as well as nitric oxide and proline contents were measured in the leaves. The results revealed genotype-specific responses to the treatments. In Agdash-3, antioxidant enzyme activities and proline levels increased proportionally with SNP concentration, indicating synergistic interactions between SA and SNP. In contrast, AP-317 showed higher sensitivity to SA but reduced proline accumulation in response to low SNP concentrations. The findings demonstrate that the interplay between SA and SNP modulates the antioxidant response in a genotype-dependent manner, suggesting potential applications in enhancing stress tolerance in cotton through phytohormonal and NO-based treatments.

Keywords: *upland cotton; sodium nitroprusside; salicylic acid; antioxidant system; NO content*

1. Introduction

Phytohormones, as inducers of various reactions in plant cells, play an important role in plant life, including the prooxidant-antioxidant defence system of plants. They are produced as additional stimulators for the transmission of intracellular and intercellular signals, and regulation of adaptive mechanisms, which manifests itself in the release of specific low molecular weight and protein protectors, as well as in other protective mechanisms. Salicylic acid (SA) is one of the phytohormones that plays an important role in plant defense responses to adverse environmental factors [17].

Being an additional donor of the NO radical, sodium nitroprusside (SNP) can also play a certain role in plant defense reactions. NO is a multifunctional molecule, acting also as a signal molecule. Studies involving nitric oxide donors have demonstrated their ability to regulate redox potential, thereby participating in the

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induction of the antioxidant defense system [27]. Research conducted on blueberries, wheat, and maize has revealed the influence of nitroprussides on the activity and expression of catalase, ascorbate peroxidase, and superoxide dismutase [8, 14].

The involvement of different types of activators in proline synthesis, including both phytohormones and donors of various signaling molecules, has been individually demonstrated in previous studies. In studies on different plant species, it was demonstrated that low concentration, 0.1 mM of SA, inductively affect the immune response, antioxidant enzymes activity and proline accumulation in plant cells [2, 12]. The NO radical also affects the increase in intracellular arginine, which is a minor substrate for proline synthesis. Studies on alfalfa and Arabidopsis plants confirmed that an increase in SNP concentration was directly proportional to an increase in proline synthesis [7, 25].

Cotton plays a crucial role in global agriculture and industry, serving as the most important natural fiber crop worldwide. It is a key cash crop, especially in developing countries, and significantly impacts the global economy and employment. Cotton is grown in over 80 countries, with major producers including India, China, the United States, Pakistan, and Brazil. Annually, about 25 million tons of cotton are produced globally [10, 21].

Cotton (*Gossypium hirsutum* L.) is susceptible to various environmental stress factors[19]. Despite numerous studies on antioxidant responses in cotton, the combined effect of SA and SNP on the biochemical indicators of its antioxidant defense remains underexplored.

Taking into account the fact that both types of signaling molecules, phytohormones and free NO radicals, are actively involved in antioxidant defense system and proline synthesis,this study aims to investigate the effect of SA in combination with two inducing concentrations of SNP on the antioxidant system of cotton seedlings. Specifically, the activities of catalase, peroxidase, polyphenol oxidase, proline content, and nitric oxide levels were assessed as biochemical markers of stress response.

2. Materials and Methods

Two genotypes of upland cotton, *Gossypium hirsutum* L. Agdash-3 and *G. hirsutum* L. AP-317, representing agricultural economic importance in the Azerbaijan Republic were used for investigation. Plant seeds were first surface-sterilized with 0.1% potassium permanganate solution for 3 minutes, then rinsed with distilled water and sown in a neutral substrate—perlite with a grain size of 0.6 mm. A modified Steiner nutrient solution was used as the hydroponic medium (Table 1).

Table 1. The concentration of macro- and microelements in modified Steiner nutrient solution (parts per million – ppm)[20]

Elements	Steiner Solution (ppm)
N	167
Ca	179
K	276
P	31
Mg	46
S	141
Fe	3
Mn	1.14
Cu	0.13
Zn	0.48

From the time of sowing, the plants were grown in Steiner nutrient solution throughout all growth stages, including germination, first true leaf, and up to the third true leaf stage.

Preparation of SA and SNP Solutions in Hydroponics. At the early stages of plant development, SA was administered at concentration of 0.1mM from the first day of seedling growth. SA was dissolved in a portion of the nutrient solution using an ultrasonic disperser (ultrasonication method) before being added to the hydroponic medium. SNP, a known nitric oxide (NO) donor, was applied in combination with SA at varying concentrations to the cotton seedlings. Specifically, the joint effects of 0.1 and 2.5 mM SNP and SA on plants were investigated. Cotton seedlings were used for biochemical analysis after completing a two-week growth period.

Determination of Catalase Activity. Catalase (CAT, EC 1.11.1.6) activity was measured using Mosheva's gasometric method [15]. For this, 0.5 g of plant material was homogenized with 0.5 g $CaCO_3$ and 20 mL distilled water. The extract was then transferred to the neck area of a Landolt flask (Erlenmeyer flask) with an 8 mL side arm, where catalase activity was measured. To initiate the reaction, 5 mL of 3% H_2O_2 was added to the homogenate, and the mixture was stirred using a magnetic stirrer throughout the reaction. The measurement was carried out over 2 minutes. The amount of oxygen released was recorded using a burette scale. Enzyme activity was expressed in mL/sec·g (fresh sample).

Determination of Peroxidase Activity. One gram of fresh sample (leaf or root tissue, depending on the experiment) was homogenized in 5 mL of 50 mM potassium phosphate buffer (pH 7.8) containing 0.1 mM EDTA at 4°C. The homogenates were centrifuged at 9000 g for 20 minutes at 4°C. The supernatants were used for the determination of peroxidase (POX) activity.

Guaiacol peroxidase (POX, EC 1.11.1.7) activity was determined based on the method by Chance and Maehly [5]. The reaction mixture (final volume: 1 mL) contained 0.1 M sodium phosphate buffer (pH 7.2), 1 mM EDTA, 30 mM H_2O_2, 50 mM guaiacol, and 50 μL of enzyme extract. The formation of tetraguaiacol was monitored at 440 nm. The concentration of tetraguaiacol was calculated using its extinction coefficient ε = 26.6 $mM^{-1} \cdot cm^{-1}$. POX activity was expressed as the amount of tetraguaiacol formed (μkat·mg^{-1} protein).

Polyphenol oxidase activity (PPO, EC 1.10.3.2). PPO activity was determined by measuring the oxidation of 0.05 M catechol at 590 nm in 0.1 M potassium phosphate buffer with pH 7,2, according to Yermakov [28]. The activity of PPO was expressed as U/min$_*$g (FW).

Determination of NO Content in Plant Extracts. The NO content in plant tissue was determined using a modified method based on Zhou and Karpets, involving the Griess reagent [9, 29]. This method relies on the conversion of endogenous NO into nitrite, which is quantified using the Griess reaction.

One gram of fresh plant tissue was homogenized in a chilled mortar with 5 mL of 50 mM ice-cold acetic acid buffer (pH 3.6), containing 2% zinc diacetate. The homogenates were centrifuged at 8000 g for 15 minutes at 4°C. From each sample, 5 mL of the supernatant was taken and mixed with 0.250 g activated charcoal. After 3 minutes and the appearance of a white foam on the supernatant, the sample was filtered using a funnel and white filter paper (pore size 8–12 μm).

A mixture of 2 mL of the filtrate and 1 mL of Griess reagent was incubated at room temperature in the dark for 30 minutes. The absorbance of the resulting colored solution was measured at 548 nm. The NO concentration in the extract was calculated using a standard curve prepared from $NaNO_2$ solutions at various concentrations (1 μM–1 mM). These standard solutions were reacted with 1 mL of Griess reagent and their final color intensities were used to generate the calibration curve, which was then compared against the experimental variants. The NO content in cotton leaves was measured and presented as μM/g fresh weight.

Determination of Proline Content Using Paper Chromatography. Isatin (1H-indole-2,3-dione) is used as a visualization agent in paper chromatography of amino acids, producing different colors. Boctor proposed this method for the quantitative determination of proline [3]. Isatin reacts with proline to form a blue derivative, "pyrrole blue", whose intensity correlates with proline concentration. This color reaction is highly specific to proline, and interference from other amino acids is minimal. Due to its simplicity and ability to analyze multiple samples simultaneously, this method was selected for our study.

Fresh plant samples were collected and approximately 100 mg of tissue was used per sample. To the tissue, 20% ethanol was added at a ratio of 10 μL/mg and the material was homogenized. The homogenate was centrifuged at 9000×g for 5 minutes at room temperature.

Ten microliters of the resulting supernatant were applied to isatin-treated and pre-prepared chromatography paper, which was then dried for 30 minutes. The dried paper was incubated at 90°C for 20 minutes. The formation of a blue color and its shades indicated the presence of proline. The yellow background of the isatin paper was removed by rinsing in 50% ethanol for a few minutes.

The dried paper was placed on a flat surface in the dark and photographed. The intensity of the proline-isatin complex was quantified using ImageJ software. To create a standard curve, a concentration series of proline (0–5 mg/mL in 20% ethanol) was prepared and reacted with isatin as described above. The resulting densitometric values were plotted in Excel and subjected to statistical analysis.

3. Results and disscussion

The data obtained from studying the effect of the inductive concentration (0.1 mM) of SA in combination with SNP on catalase activity indicated a synergistic interaction between SNP and SA (Fig. 1).

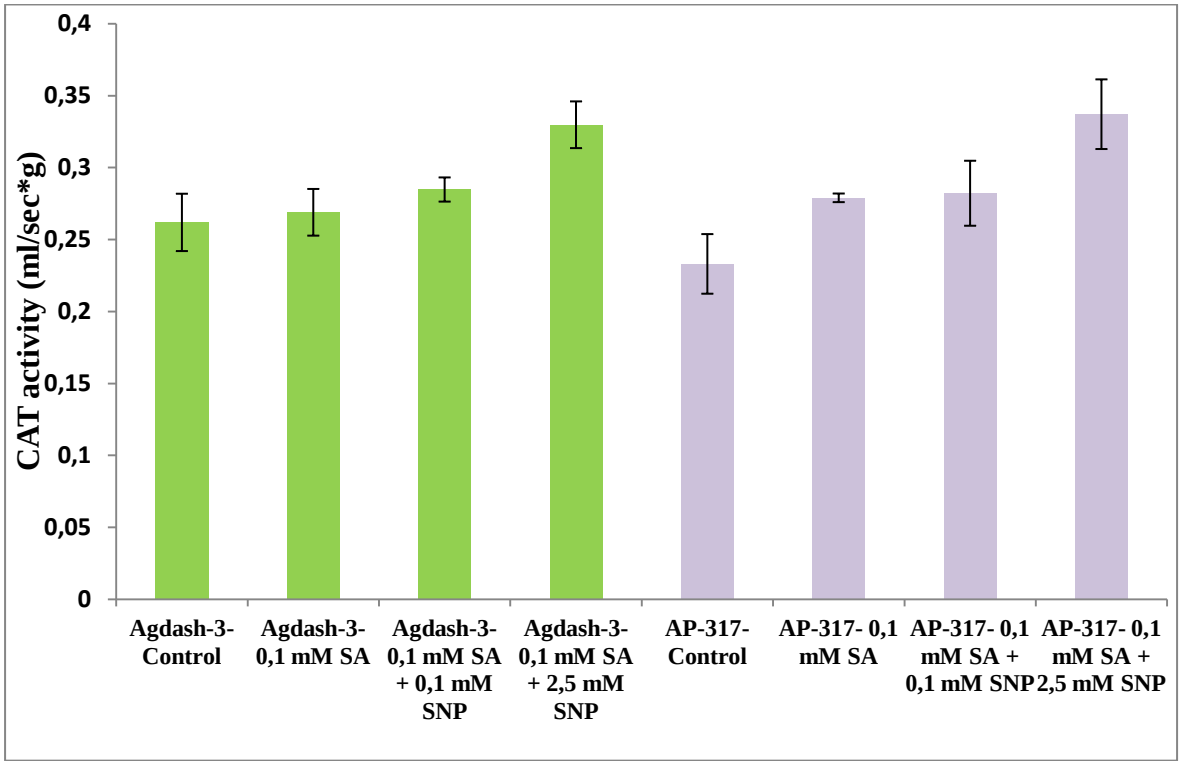

Fig. 1. Effect of 0.1 mM salicylic acid in combination with 0.1 and 2.5 mM NNP on catalase (CAT) activity in the leaves of upland cotton genotypes Agdash-3 and AP-317

In the Agdash-3 genotype, CAT activity remained almost unchanged after treatment with 0.1 mM SA. However, the application of 0.1 mM and 2.5 mM sodium SNP increased CAT activity, with the highest level observed at the 2.5 mM SNP concentration. In contrast, when a mixture of 0.1 mM SNP and SA was applied, the increase was not significantly different from the control group or the 0.1 mM SA treatment.

In the AP-317 genotype, catalase activity exhibited more pronounced changes. At 0.1 mM SA concentration, catalase activity increased by 20% compared to the control group. Furthermore, the mixture of 0.1 mM SNP and 0.1 mM SA did not significantly enhance catalase activity. However, the combination of 2.5 mM SNPand 0.1 mM SA resulted in a significant increase in catalase activity.

Overall, the response to SA was different in both genotypes. The AP-317 genotype, which is more sensitive to external changes, exhibited an increase in CAT activity even at low SA concentrations. In contrast, the more stable Agdash-3 genotype did not show a high response to SA. Additionally, CAT activity increased in both genotypes when treated with the combination of 2.5 mM SNP and SA, indicating their synergistic effect. This was associated with both the individual effect of SNP and the induction of CAT activity by SA, which activates various antioxidant enzyme mechanisms.

To investigate the effect of 0.1 mM SA in combination with SNP on POX activity, the study revealed distinct differences between genotypes in response to the interaction with SNP (Fig. 2).

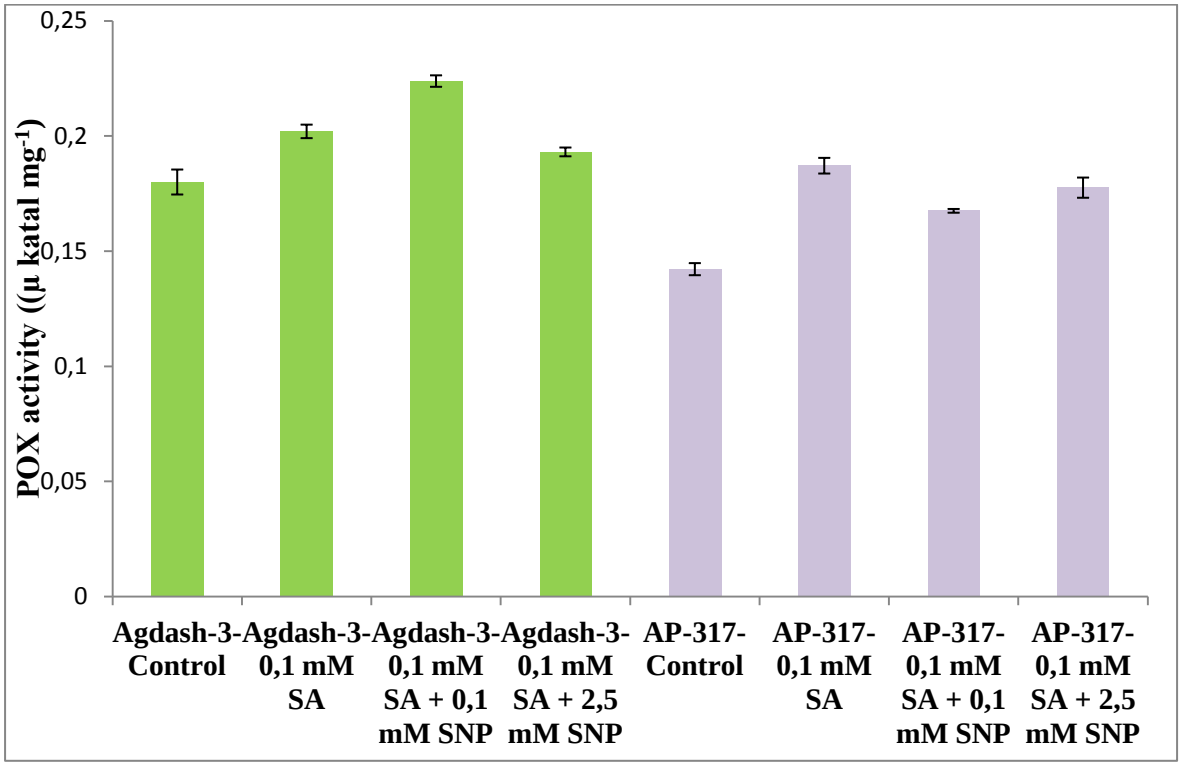

Figure 2. Effect of 0.1 mM SA in combination with 0.1 and 2.5 mM SNPon POX activity in true leaves of the upland cotton genotypes – Agdash-3 and AP-317

0.1 mM SA increased POX activity in both Agdash-3 and AP-317 genotypes by 12% and 28%, respectively. Additionally, the combination of 0.1 mM SNP and 0.1 mM SA in the Agdash-3 genotype resulted in a 28% increase in activity. Increasing the SNP concentration to 2.5 mM negatively affected the enzyme activity compared to the 0.1 mM SNP treatment. For the AP-317 genotype, the effect of the combination of 0.1 mM SNP and 0.1 mM SA on peroxidase activity was negative compared to 0.1 mM SA alone. However, the combination of 2.5 mM SNP and 0.1 mM SA increased POX activity, bringing it in line with the effect of 0.1 mM SA.

Overall, the Agdash-3 genotype maintained its stability, but the increased concentration of SNP had a negative effect. This may have disrupted its metabolic systems responsible for the synthesis and breakdown of free oxygen radicals.

For the AP-317 genotype, POX enzyme activity was more sensitive to the varying concentrations of SNP at the biochemical or molecular level. This may have simulated its energy balance, causing uncertainty in POX activity due to a lack of resources or direct effects of SNP on enzyme biosynthesis, potentially leading to an imbalance between enzyme concentration and activity.

Impact of SNP and of SA on PPO activity in the true leaves of the upland cotton genotypes, Agdash-3 and AP-317 is presented in Fig.3. Effect of 0.1 mM SA on the Agdash-3 genotype led to a 10% decrease in PPO activity. The combined effect of 0.1 mM SA and 0.1 mM SNP did not have a significant effect on PPO activity. However, the application of 0.1 mM SA with 2.5 mM SNP resulted in the restoration of PPO activity to the initial level observed in the control group, which demonstrated the restorative effect of higher SNP concentrations on activity.

In the AP-317 genotype, no significant changes in PPO activity were observed under the influence of 0.1 mM SA. The addition of 0.1 mM SNP decreased PPO activity, negatively affecting enzyme function. The 2.5 mM SNP concentration did not cause significant changes in PPO activity; however, in contrast to 0.1 mM SNP, it showed a slight amount of activity.

The results clearly indicate that the combined use of low SNP concentrations with SA did not significantly affect the increase in PPO activity. This suggests that at low concentrations, it may not be sufficiently effective to stimulate enzymatic activity. High SNP concentrations (2.5 mM) had a more pronounced effect on PPO activity, especially in the Agdash-3 genotype, where activity returned to the control level. This demonstrates the role of SNP in modulating PPO activity and its interaction with SA.

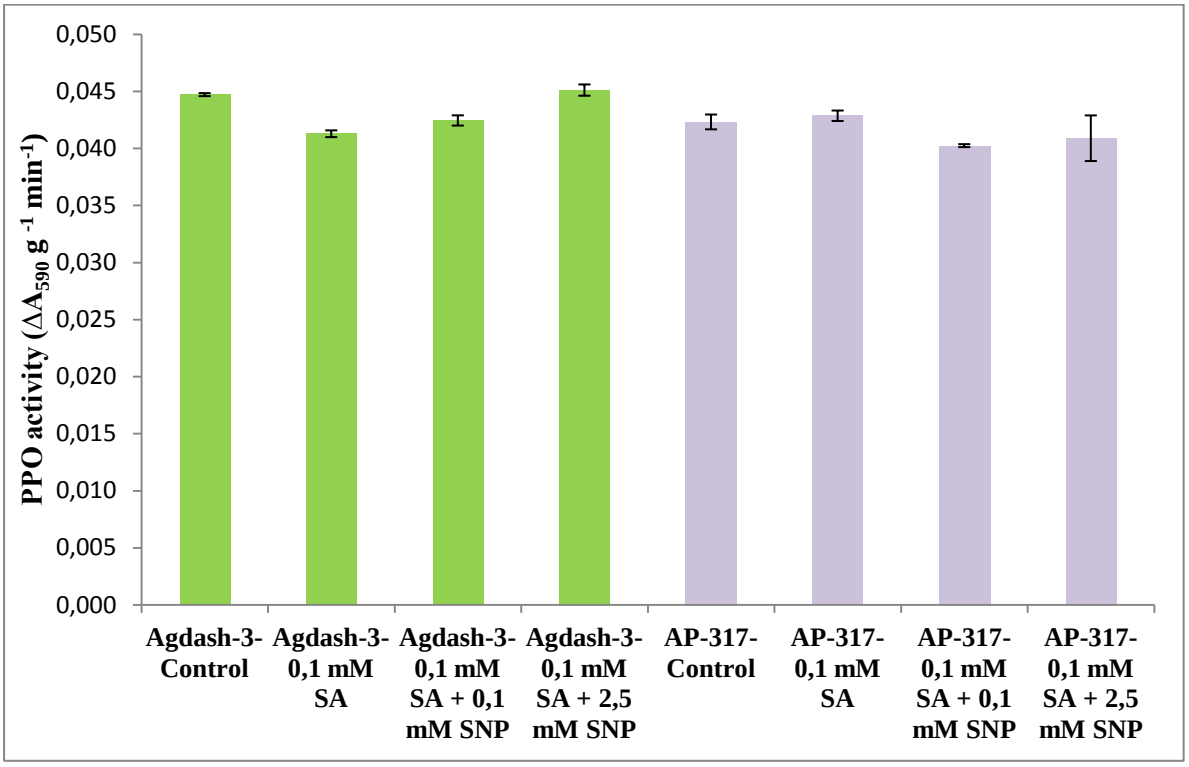

Fig. 3. Effect of 0.1 and 2.5 mM SNP in the presence of 0.1 mM salicylic acid on PPO activity in the true leaves of the upland cotton genotypes, Agdash-3 and AP-317

Further analysis in the next steps of the study revealed unexpected results regarding the effect of SA, both individually and in combination with varying SNP concentrations, on nitric oxide levels in the leaf extracts of different cotton genotypes (Fig. 4).

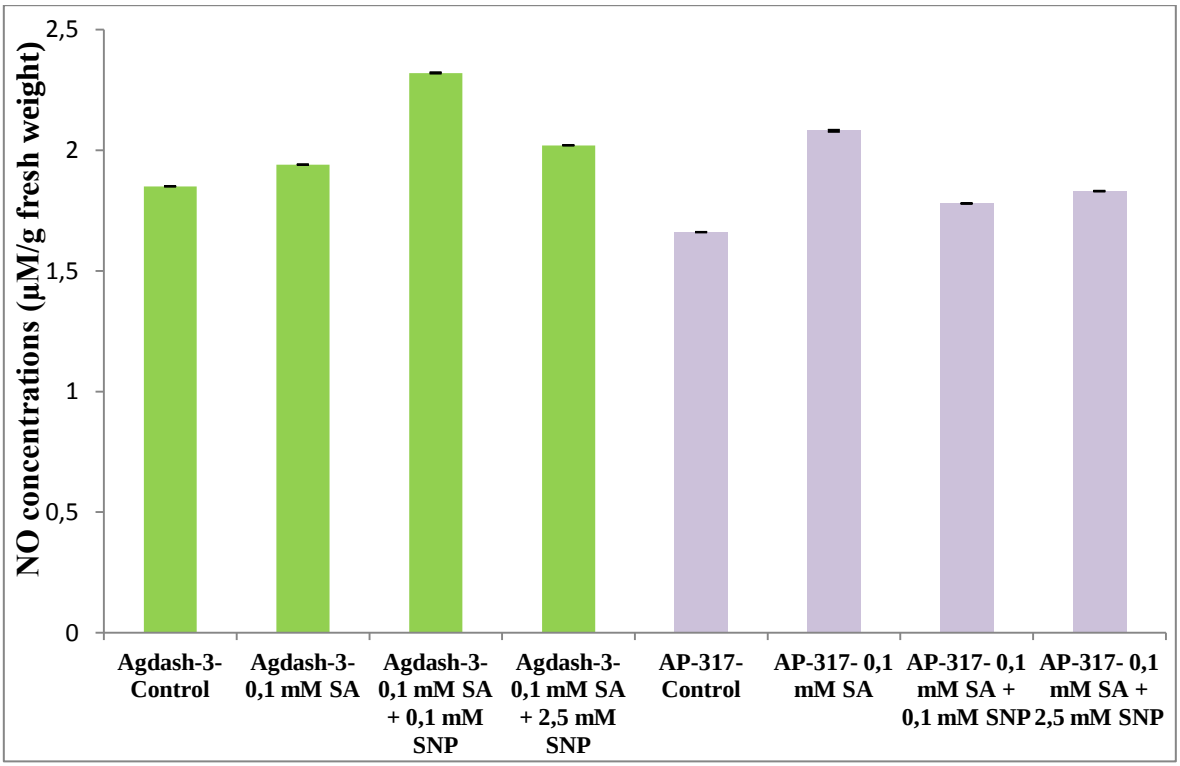

Fig.4. The effect of 0.1 mM salicylic acid in combination with 0.1 and 2.5 mM SNP on the nitric oxide (NO) content in the leaf extracts of the cotton genotypes Agdash-3 and AP-317

First, it has been determined that in the Agdash-3 genotype, 0.1 mM SA slightly increased the nitric oxide (NO) content (by 5%), which falls within the statistical error range considering the standard error. The addition of 0.1 mM SNP to the nutrient medium led to a 25% increase in the NO content. In contrast, the combination of high concentrations of SNP and SA resulted in a less pronounced increase in the NO level, with a total increase of only 10%.

For the AP-317 genotype, changes in the NO content were observed only under the effect of SA, where the NO level increased by 25% compared to the control group. It is noteworthy that both low and high concentrations of SNP in the presence of SA did not induce significant changes in the NO content.

Regarding proline content, it has been found that (Fig. 5, 6), in all variants of *Gossypium hirsutum* L. Agdash-3, 0.1 mM SA, as well as the combinations of 0.1 mM SA + 0.1 mM SNP and 0.1 mM SA + 2.5 mM SNP led to an increase in proline content in the leaf extracts. It was also determined that in the Agdash-3 genotype, the endogenous proline content was directly proportional to the concentrations of the additional inducers, SA and SNP, whereas in the AP-317 genotype, the proline content reached its maximum only at high SNP + SA concentrations. In contrast to *Gossypium hirsutum* Agdash-3 variants, the proline content in the analogous variants of *Gossypium hirsutum* AP-317 did not increase but rather decreased compared to control. Only at the highest SNP concentration (2.5 mM) with 0.1 mM SA, it reached the control level. However, the initial proline level in *Gossypium hirsutum* AP-317 extract was significantly higher compared to the extract of *Gossypium hirsutum* Agdash-3 when compared to the control.

Fig. 5. Analysis of proline in the true leaf samples of plants treated with ST and NNP using the Thin Layer Chromatography (TLC) method

Top row (from left to right): *Gossypium hirsutum* Agdash-3: Control; 0.1 mM SA; 0.1 mM SA + 0.1 mM SNP; 0.1 mM SA + 2.5 mM SNP; *Gossypium hirsutum* AP-317: Control; 0.1 mM SA; 0.1 mM SA + 0.1 mM SNP; 0.1 mM SA + 2.5 mM SNP. Bottom row (from left to right): A, B, C, D, E, F – control variants of proline at different concentrations (10 mM (A), 2 mM (B), 1 mM (C), 0.2 mM (D), 0.1 mM (E), 0.02 mM (F)).

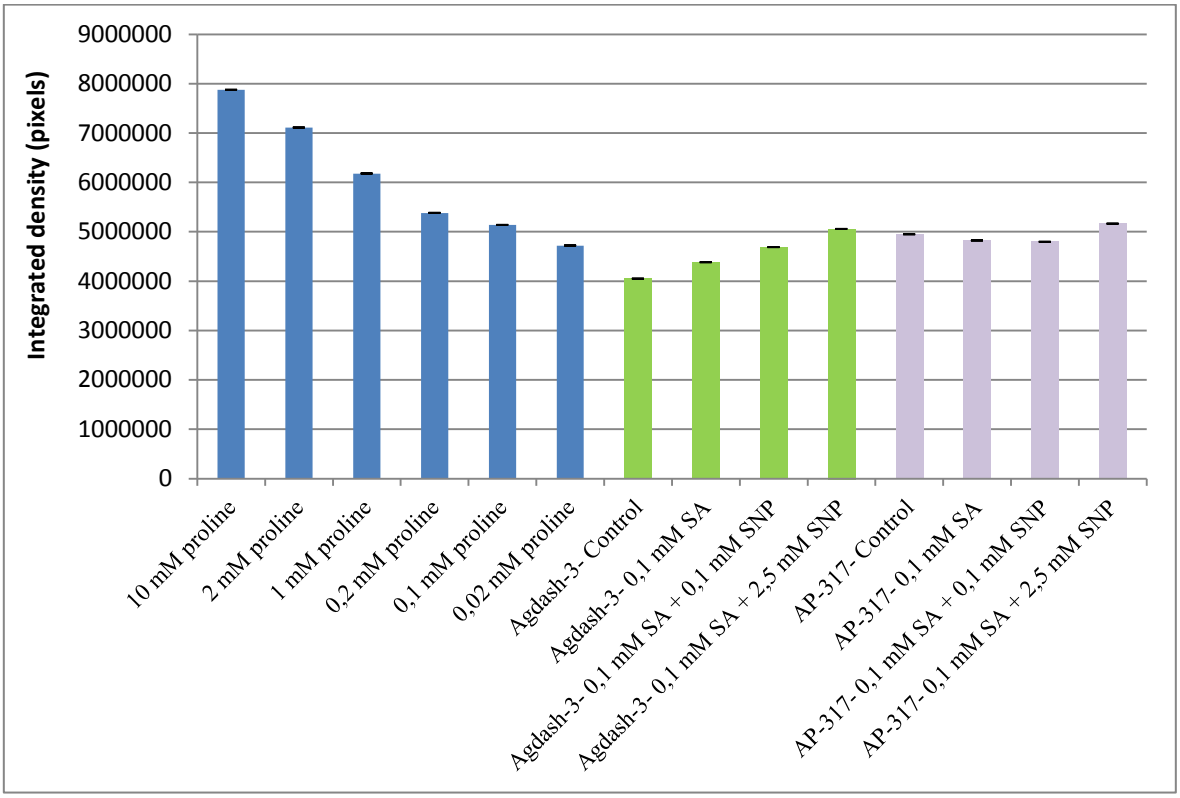

Fig. 6. Results of densitometric analysis of proline determination in the leaves of plants treated with ST and NNP using ImageJ software

Overall, SA is an important molecule with plant hormonal activity, participating in the regulation of critical defense mechanisms. However, SNP, with its chemical formula Na2[Fe(CN)5NO], is the most commonly encountered compound, acting as a NO donor and playing a pivotal role in the regulation of NO signaling pathways in plants [6, 16)]. The released NO plays a vital role in various biochemical processes, actively influencing plant growth, development, and stress responses. However, it is important to note that excessive levels of SNP lead to undesirable outcomes, including osmotic stress [7]. Previous studies have shown that the negative role of nitrosative stress, associated with excessive synthesis of NO, leads to the formation of peroxynitrites, which participate in the oxidative modification of proteins and nucleic acids, potentially causing cell wall degradation [1, 26].

Understanding the interaction between SA and SNP is crucial for identifying complex synergistic or antagonistic intracellular interactions resulting from normal and excessive synthesis of NO, as well as determining how hormonal regulation through SA integrates into the toxic processes induced by nitroprusside.

Analysis of antioxidant enzyme activities showed a clear increase in CAT and POX activities, which may be linked to the elevation of free radical levels in both genotypes under nitroprusside treatment. It is also important to note that the Agdash-3 genotype maintained its stability, but increasing SNP concentrations had a negative impact, potentially disrupting metabolic systems responsible for the synthesis and degradation of reactive oxygen species. This process, which had a more gradual effect on this genotype, suggests that it may have a defense potential against oxidative damage caused by nitroprusside in the presence of SA.

Regarding POX, a clear decrease in POX activity was observed at a concentration of 2.5 mM nitroprusside, which may be related to both the regulatory mechanisms of SA and the cell's own compensatory mechanisms. In the AP-317 genotype, the POX enzyme appeared to be more sensitive to changing concentrations of SNP at the biochemical or molecular level. This could simulate its energetic balance, resulting in a direct effect of SNP on enzyme synthesis, thus creating an imbalance between enzyme concentration and activity [8].

Increased synthesis or accumulation of NO in the cell, induced by the administration of nitro-oxidative stimulants such as nitroprusside, provokes iron and ferritin ions, which in turn induce the additional release of reactive oxygen species [22, 23].

PPO acts as a key oxidative enzyme in the breakdown of phenolic compounds [24]. Previous studies have shown that, although PPO is not considered a component of the antioxidant system, its activity increases under stress conditions (radiation, mechanical damage, changes in environmental chemical composition, etc.), leading to the formation of chemical barriers such as lignin precipitates, which prevent the spread of active oxygen species and strengthen the cell wall [4, 18].

Results showed that low concentrations of SNP did not significantly affect PPO activity when used in combination with SA. This suggests that low concentrations might not be sufficient to stimulate enzymatic activity. However, high SNP concentrations (2.5 mM) had a stronger effect on PPO activity, especially in the Agdash-3 genotype, where the activity reached the control level. This demonstrates the role of SNP in modulating PPO activity and its interaction with SA.

These results also suggest that chemical defense mechanisms against free radicals are activated, which is directly related to previous data on antioxidant enzymes, indicating the important role of this enzyme in neutralizing the negative effects of high SNP concentrations.

SA's activity is not only evident in activating antioxidant systems but also in the production of NO, a secondary signaling molecule [30]. Given that nitroprusside participates in the production of NO and enhances the expression of antioxidant enzymes, it was important to determine how SA influences the intracellular NO levels in different variants. This analysis showed that SA increases the amount of NO in leaf extracts, particularly in the AP-317 genotype, which suggests that this genotype may have a higher sensitivity to SA and generally to changing external factors.

The Agdash-3 genotype showed less response to SA and nitroprusside, with a gradual increase in NO levels, peaking at a specific concentration, and then decreasing at the maximal concentration of 2.5 mM sodium nitroprusside. This effect may be related to the process of mobilizing additional resources for neutralizing NO or the involvement of enzymatic systems in the clearance of excess NO. The effect of SNP on NO synthesis was more pronounced in the Agdash-3 genotype, while the effect in the AP-317 genotype was minimal. This result likely points to interspecies differences at the level of interaction with different chemical compounds.

Previous studies have shown that SNP, as an NO-radical activator, directly participates in osmoregulation through the involvement of NO radicals in proline synthesis. Proline concentration was directly proportional to SNP concentration [13]. It was also determined that high SNP concentrations lead to a decrease in photosynthesis rate and stroma permeability, which is accompanied by the extracellular accumulation of proline [7]. SA, as an inducer of many metabolic processes, also participates in proline accumulation due to an increase in γ-glutamyl kinase (GK) activity and a decrease in proline oxidase (PROX) activity [11]. The results of the analysis showed that the Agdash-3 genotype exhibited an efficient interaction with SNP and SA, resulting in an increase in proline concentration. In contrast, the AP-317 genotype showed a decrease in proline synthesis activity with low concentrations of SA and 0.1 mM SNP + ST, suggesting the presence of additional pathways for regulating intracellular proline levels.

The data obtained showed that SNP increased proline production in the Gossypium hirsutum Agdash-3 genotype in direct proportion to the applied concentration, thus participating in the osmoprotective mechanisms of this genotype. In the Gossypium hirsutum AP-317 genotype, the initially high proline levels allowed the synthesis of proline to proceed via the arginine pathway without specific induction or participation in this process.

4. Conclusion

The results of this study highlight the significant influence of SA and SNP on the regulation of antioxidant defense mechanisms in upland cotton. Both genotypes, Agdash-3 and AP-317, exhibited distinct biochemical responses to the combined treatment, reflecting their genetic variability in stress response. Agdash-3 demonstrated a more stable and proportional response to increasing SNP concentrations, including enhanced CAT and POX activities, increased NO levels, and elevated proline content, indicating its capacity for controlled oxidative stress management and osmotic adjustment. In contrast, AP-317 was more sensitive to SA alone and displayed a less consistent response to SNP, particularly in PPO activity and proline synthesis. These findings suggest that the combined application of SA and SNP can be optimized based on genotype-specific traits to modulate antioxidant defense and improve stress resilience in cotton. Further studies are recommended to explore the molecular pathways involved and to evaluate the long-term impact under field conditions.

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