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INFLUENCE OF DINITROSALICYLIC ACID ON THE PROTECTIVE SYSTEM OF COTTON SEEDLINGS (*Gossypium hirsutum* L.)

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Abstract. Cotton growing, being an important agro-industrial object, is being improved through the introduction of new mechanisms to increase resistance to various external stress factors. Taking into account the previously studied phytohormones, the purpose of our study was to identify the effect of the salicylic acid derivative, dinitrosalicylic acid (DNSA), on the biochemical processes in the common cotton AP-317 genotype. It was established that low concentrations of DNSA-0.01 mM decreased production of NO radical, catalase and PPO activity, as well as absorption of phosphorus-anions from the nutrient medium, while higher doses of them has led to the stimulation of NO radical production, increased peroxidase activity, stabilized absorption of phosphorus anions. It was also found that at low concentrations of DNSA took place slightly activation of SOD activity, but not at relatively high concentration of DNSA. Electron paramagnetic resonance analysis of the root and leaf tissue of the plant showed an increase in the concentration of magnesium and iron ions when exposed to DNSA. It was also found that DNSA increased the concentration of lipid peroxide radicals at 0.1 mM in both tissues.

Keywords: *DNSA (dinitrosalicylic acid), Gossypium hirsutum, antioxidant enzymes, NO-content, EPR analyses.*

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1. Introduction

Cotton growing is one of the important industrial and agricultural sectors of modern crop production. Cotton of the *Gossypium hirsutum* genotype AP-317 is predominantly grown in different territorial zones of Azerbaijan (Uzundumlu *et al.*, 2023). Like other plants, cotton is also constantly exposed to various types of environmental stress factors (Han *et al.*, 2021; Guliev *et al.*, 2019; Olatunbosun *et al.*, 2023; Abdiyev *et al.*, 2019).

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These factors have a negative impact on the growth, development and productivity of plants. A sharp and prolonged stressful state of the environment can even cause the death of a plant (Hideg *et al.,* 2013; Rashid *et al.,* 2021). To alleviate and overcome the negative effects of stress and adapt to stressful conditions, plants during evolution have developed a complex and multicomponent system of protective reactions, which include both factors of stress identification and response to it. Stress-induced genes are activated, regulatory and functional proteins are synthesized and a profound change occurs in plant metabolism aimed at mitigating the influence of the stress factor (Mahajan & Tuteja, 2005; Nakashima *et al.,* 2012). The characteristics of the response of the plant organism are determined by both the species and the stress factor itself (Amrahov *et al.,* 2023). Clarification of these characteristics can be a good help for obtaining promising and tolerant varieties of various stress factors.

Data accumulated in the scientific literature indicate that phytohormones play an important role in stimulating plant defense reactions, one of which is salicylic acid (Kaltdorf & Naseem, 2013). It is a low molecular weight organic compound that acts as a signaling molecule and affects many metabolic processes in plant cells. For example, it has been found that by enhancing the activity of antioxidant enzymes, in particular catalase, ascorbate peroxidase and superoxide dismutase, salicylic acid can increase plant tolerance against various stressors (Nazar *et al.,* 2011; Agarwal *et al.,* 2005; Amrahov *et al.,* 2022). In this regard, the use of salicylic acid derivatives that can positively modulate its action for a similar purpose is of particular interest. Using the example of sulfosalicylic and acetylsalicylic acids, it has been demonstrated that they, like salicylic acid, can have a positive effect on the antioxidant system and increase the tolerance of corn and rice to relatively high concentrations of NaCl and heavy metal ions - cadmium (Tuna *et al.,* 2007; Singh & Shah, 2015). The creation of artificial regulatory molecules, including derivatives of phytohormones with high antioxidant potential that can significantly increase the anti-stress ability of plants can be considered one of the promising and priority areas in plant growth (Aguirre-Becerra *et al.,* 2021). This type of substance can also be found among salicylic acid derivatives with the prospect of their use in field conditions (Velika & Kron, 2012).

In the presented work, our studies were aimed at studying the effect of different concentrations of dinitrosalicylic acid on the antioxidant system and the formation of a paramagnetic centre in seedlings of genotype AP-317 of common cotton-*Gossypium hirsutum* L. Studying the formation of a paramagnetic centre gives some insight into changes in the metabolic processes of plants (Aliyeva *et al.,* 2023).

2. Materials and methods

Experiments were carried out on cotton seedlings of genotype AP-317 of the Gossypium hirsutum species. Plant seeds were kindly presented by the Institute of Genetic Resources, Ministry of Education and Science of the Republic of Azerbaijan. Cotton seeds were pre-treated with a 0.2% solution of potassium permanganate for 8 minutes, washed with distilled water and then planted in plastic cups with a diameter of 7 cm containing perlite. Seedlings were grown in a phytotron (Taisite, GZX-300 E) at a temperature of 22–24°C, humidity of 65–75% and illumination of 4800 lux. The light duration was 14/10 hours day/night. Steiner's solution with the addition of DNSA at concentrations of 0.01, 0.1 and 1.0 mM was used as a nutrient medium throughout the

entire growth period, starting from the first days of seedling formation. Two-week-old embryonic cotyledons were used for analysis.

The amount of NO in cotyledons was determined using Griess reagent according to a modified method of Zhou et al. (2005) and Karpets et al. (2015). Leaves (1 g) were ground with a pestle and mortar in 5 ml of 50 mM cold acetic acid buffer with pH 3.6 containing 2% zinc diacetate. The homogenate was centrifuged at 8000 g for 15 min at 4^0C. After centrifugation, 0.25 g of activated carbon was added to the supernatant. After shaking and filtering with white filter paper (pore size 8–12 μm), the filtrate was collected. A mixture of 2 ml of filtrate and 1 ml of Greiss reagent was incubated at room temperature in the dark for 30 min. Absorbance was determined at 548 nm. NO content was calculated by comparison with the $NaNO_2$ standard curve. This method is based on the conversion of endogenous NO to nitrite and the determination of the amount of nitrite using the Griess reaction.

Catalase activity was determined by Mosheva gasometric method (1982). First, 0.5 g of plant material was measured and homogenized with the gradual addition of 0.5 g of $CaCO_3$ and 20 ml of distilled water. The extract was then transferred to a Landolt flask (Erlenmeyer flask) with the neck on the side and the catalase activity was measured. To start the reaction, 5 ml of 3% H_2O_2 was added to the extract and stirred on a magnetic stirrer throughout the reaction. The measurement was carried out after 3 minutes. After this time, the amount of oxygen released could be determined on the burette scale. Enzyme activity was expressed in ml O_2/sec* g^{-1} (fresh weight).

Peroxidase activity (POX, EC 1.11.1.7) was determined according to the method of Chance and Maehly (1955). The reaction mixture contained 0.1 M Na-phosphate buffer, pH 7.2, containing 1 mM EDTA, 30 mM H_2O_2, 50 mM guaiacol and 500 μl of enzyme extract in a final volume of 4 ml. The formed tetraguaiacol was measured at 440 nm. The concentration of tetraguaiacol was calculated using the extinction coefficient of tetraguaiacol (26.6 mM^{-1} cm^{-1}). POX activity was expressed as $\Delta A590^* g^{-1} * min^{-1}$.

Superoxide dismutase (EC 1.15.1.1) **activity** was determined spectrophotometrically by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) according to Beauchamp and Fridovich (1971). The reaction mixture contained 50 mM of K phosphate buffer (pH 7.8), 0.1 mM of EDTA, 150 μM of NBT, 26 mM of methionine, 8 μM of riboflavin and 100 μl of enzyme extract in the final volume of 4.1 ml. Reaction mixtures were incubated for 8 min under light conditions. One unit of SOD activity was defined as the amount of enzyme that inhibits 50% of NBT photoreduction.

Polyphenol oxidase (PPO, EC 1.10.3.2) **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 et al. (1987). The activity of polyphenol oxidase was expressed as U/min/g (FW).

Inorganic phosphate determination in nutrient solution. The quantification of inorganic phosphate was conducted by Pradhan and Pokhrel (2013). Steiner solution, containing various concentrations (0.01mM, 0.1mM and 1 mM) of DNSA, obtained from the plant growing medium, were utilized for phosphate analysis. Steiner solution served as the standard in this analysis. To eliminate soluble particles such as perlite and root parts, the samples were passed through Whatman-41 filter paper. The resulting filtrate contained orthophosphate, as well as condensed phosphate in its pyro, meta and poly forms, along with organically bound phosphorus. Phosphate levels were determined using the molybdenum blue phosphorus method in conjunction with a UV-visible

spectrophotometer, specifically the WPAS 104 UK model equipped with 1 cm matched quartz cells. This method relies on the formation of a phosphomolybdate complex upon the addition of molybdate, followed by the reduction of the complex using hydrazine hydrate in an aqueous sulfuric acid medium. The system follows Lambert-Beer's law at 840 nm within a concentration range of 0.1-11 ppm.

EPR analyses were carried out on an EMX plus radio spectrometer manufactured by the Bruker company, which operates in the X-region of microwaves, has a frequency of ν~9.8 gigahertz (GHs) (a wavelength of λ~3cm), a magnetic field range of 0-6000 Gauss (G), modulation frequency 100 kilohertz (kHz). All spectra were recorded at room temperature in ultrapure glass tubes manufactured by Wilmad and the modulation amplitude was selected considering the width of the spectral lines to receive appropriate signals (Ismayilova *et al*., 2021). The test samples were dried at room temperature and crushed into small pieces for filling into EPR tubes with an internal diameter of 3 mm.

3. Results

One of the features of the indicators of plant defense reactions is the formation of NO, which behaves as a multifunctional molecule and has not only bactericidal and fungicidal properties, but also plays the role of a regulatory and signaling molecule (Porrini *et al.*, 2020; Kohli *el al.*, 2019). Our studies showed that low concentrations (0,01 and 0,1 mM) of DNSA compared to the control caused a decrease and its relatively high concentration caused a noticeable increase in the formation of NO in the leaves of AP-317 cotton seedlings (Figure 1).

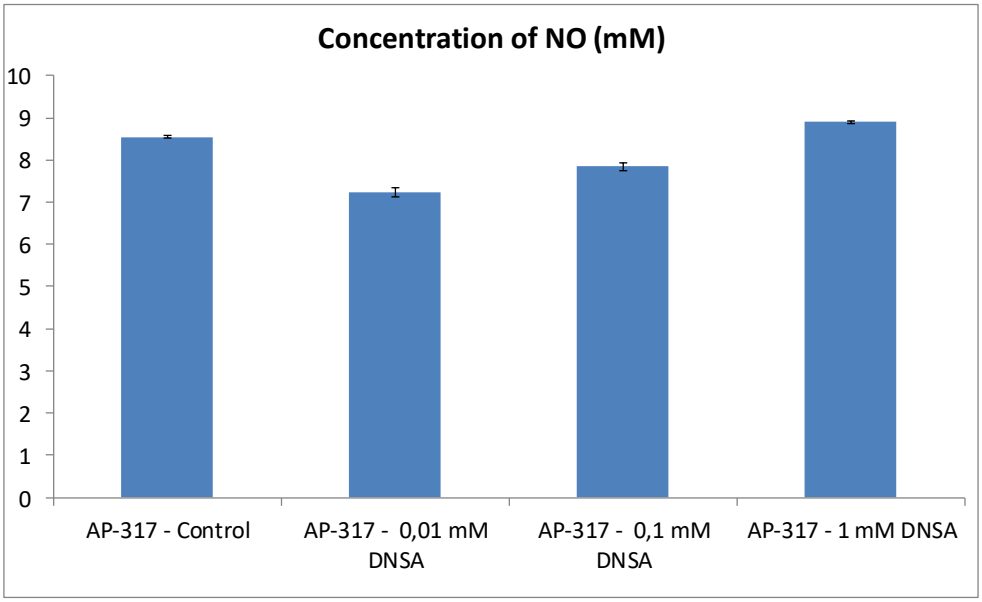

Figure 1. Effect of DNSA on the formation of NO in leaves of common cotton genotype AP-317

Catalase is one of the widespread antioxidant enzymes in nature and plays an important role in neutralizing H_2O_2. H_2O_2 is normally formed in a certain amount in plants and like NO, is a multifunctional molecule. However, in stressful situations, it can be produced in excess quantities and its excess can hurt plants (Corpas *et al.*, 2017).

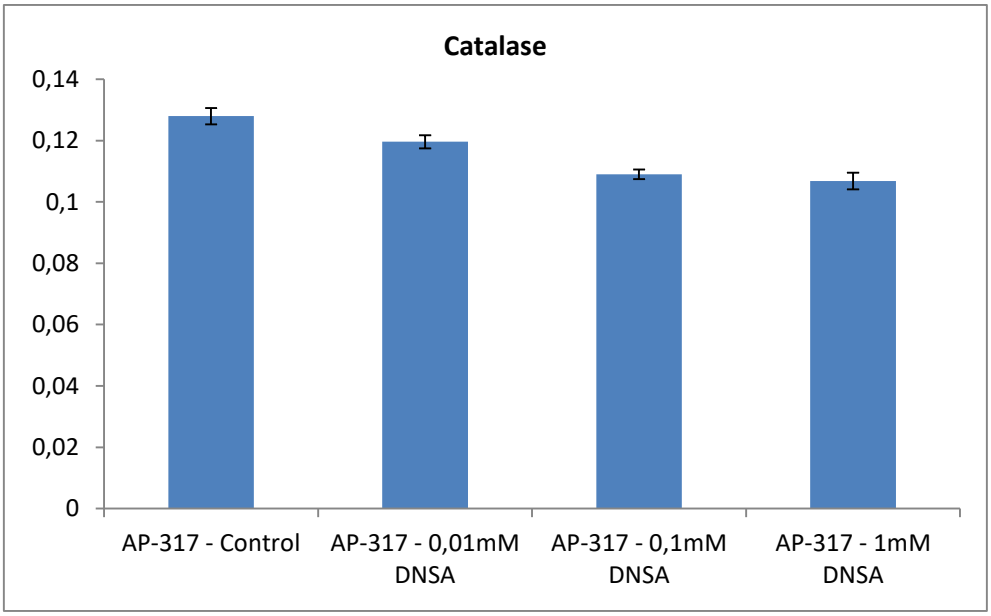

Figure 2. Effect of DNSA on catalase activity in leaves of common cotton genotype AP-317

Our studies showed that DNSA at all tested concentrations suppressed the activity of catalase and a directly proportional relationship was observed between its concentrations and the degree of inhibition. As it appears, the participation of DNSA in the defense reaction of this cotton plant is not associated with the involvement of catalase in this process (Figure 2).

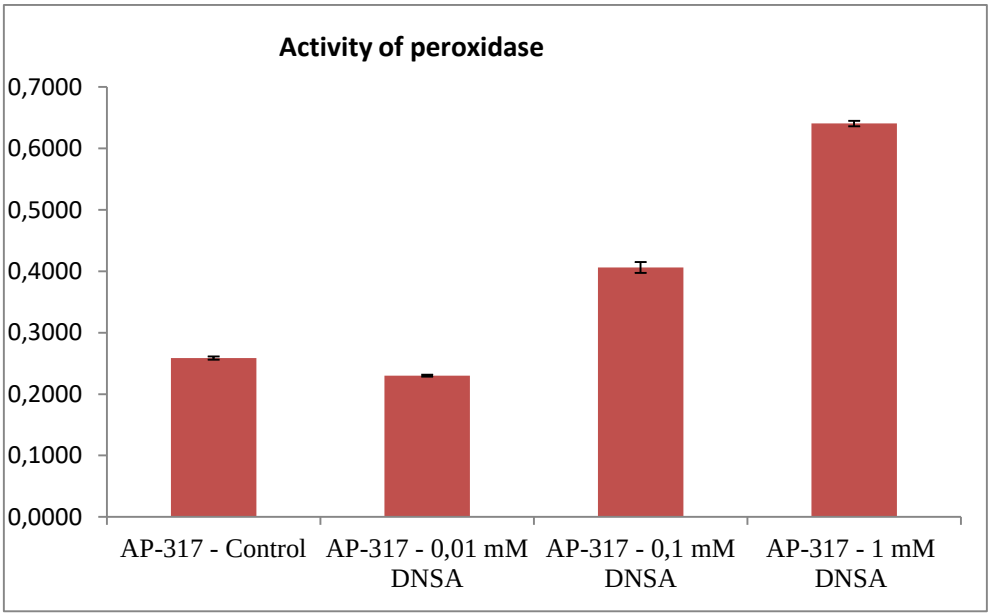

Figure 3. Effect of DNSA on the activity of peroxidase in leaves of cotton seedlings of the common genotype AP-317

Figure 3 shows the effect of different concentrations of DNSA on the activity of peroxidase in the leaves of cotton seedlings of the AP-317 genotype. As can be seen from the figure, the activity of this important antioxidant enzyme involved in the neutralization

of excess H_2O_2 decreased only slightly at a low concentration (0.01 mM) of DNSA and increased significantly as its concentration increased. For example, at a concentration of 1 mM DNSA, the enzyme activity increased approximately 2.5 times.

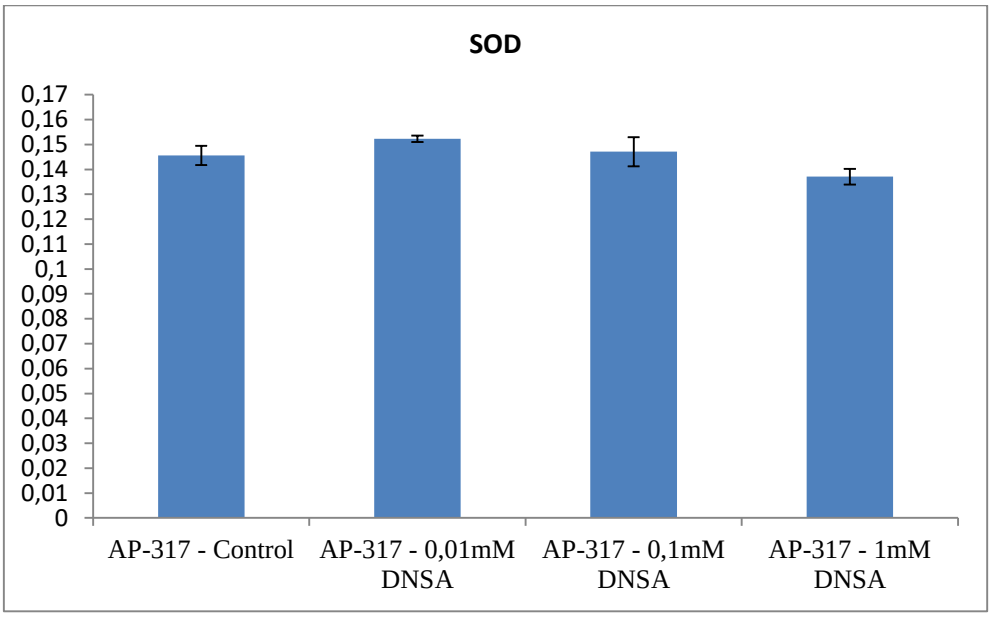

Figure 4. Effect of DNSA on the SOD activity of leaves of upland cotton seedlings of genotype AP-317

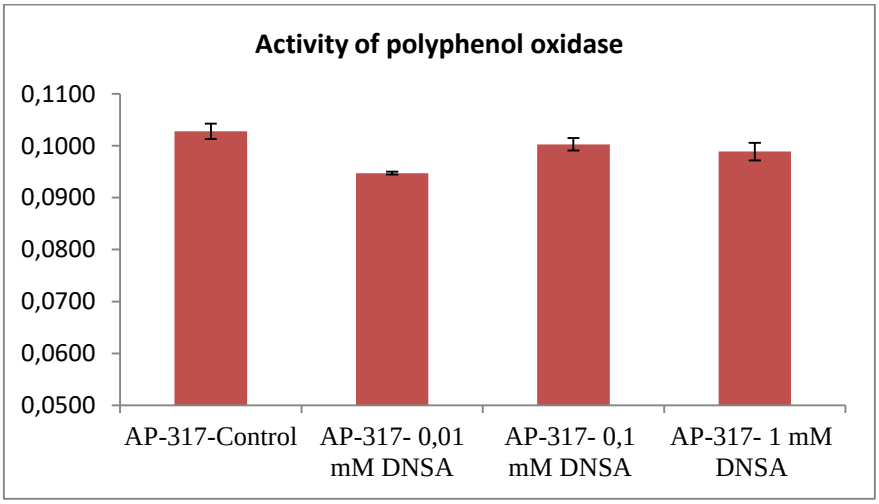

Figure 5. Effect of DNSA on the activity of polyphenol oxidase in leaves of upland cotton genotype AP-317

DNSA did not have a significant effect on the activity of SOD, which is considered one of the key antioxidant enzymes in plants (Figure 4). A low concentration slightly stimulated it and a high concentration slightly inhibited the activity of this electron-distributing enzyme.

The inhibitory effect of DNSA is also evident in the activity of PPO, an enzyme believed to play an important role in plant defense, particularly in protection against biotic stress (Taranto *et al.*, 2017; Ngadze *et al.*, 2012). Moreover, the most effective concentration inhibiting it in this process turned out to be 0.01 mM (Figure 5).

One of the essential points in the growth, development and productivity, as well as immunological tolerance to unfavorable environmental conditions of plants, is the provision of phosphorus ions (Hawkesford *et al*., 2023, Lambers, 2022). Therefore, the influence of plant growth regulators on the course of this process is of particular interest. Based on the decrease in the content of dihydrophosphorus anion from the nutrient medium in the hydroponic medium, it was revealed that DNSA not only does not contribute but, on the contrary, impedes the absorption of phosphorus ions by the roots of cotton seedlings. This occurs especially severely at low concentrations of DNSA (Figure 6). At relatively higher concentrations of DNSA, this effect manifested itself to a much lesser extent than at a concentration of 0.01 mM.

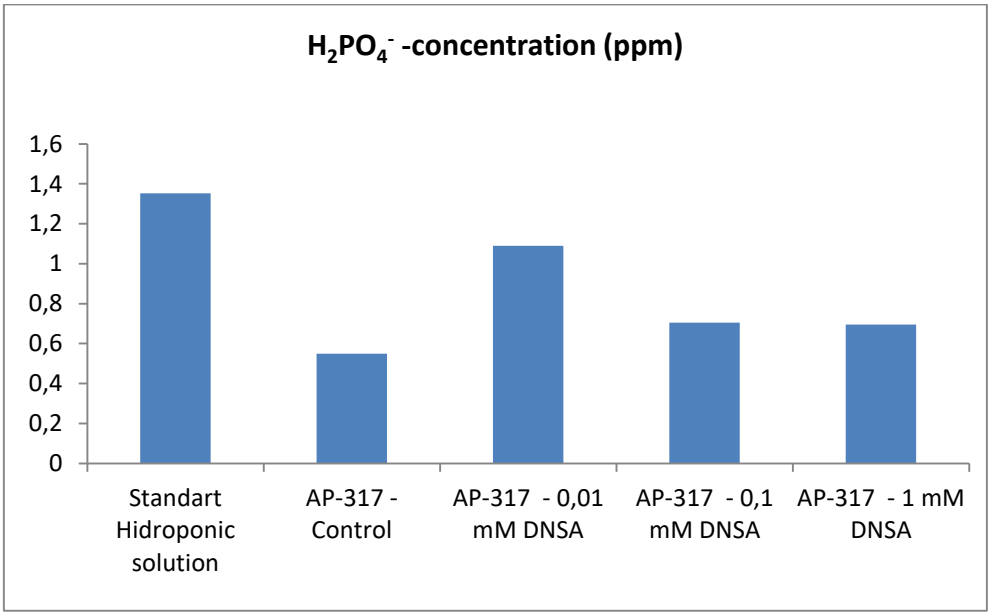

Figure 6. Effect of DNSA on the intensity of phosphate ion absorption by cotton seedlings of the upland genotype AP-317

Thus, from the results obtained, it becomes clear that DNSA had different effects on the activity of enzymes involved in the plant defense response. It had virtually no effect on the activity of SOD, stimulated the activity of POX and on the contrary, suppressed the activity of CAT and PPO in the leaves of cotton seedlings. It also suppressed the formation of NO and the absorption of phosphorus ions from the nutrient medium.

EPR spectra obtained in a wide magnetic field range of root and leaf samples of plants exposed to different concentrations of DNSA are shown in Figure 7.

In the spectrum of leaf samples, the singlet line observed at low magnetic field in roots (Figure 7) (g=4.25) disappears in leaves samples (Figure 8), but the central part is visible. In leaf specimens, the sextet and singlet lines in the center are more clearly distinguished. Although the distance between the individual lines of the sextet line lays on average 90 G, the distances between the individual lines increase as the magnetic field increases.

It was found that the concentration of lipid peroxidase radicals increased in root tissues with the addition of DNSA. They obtained their maximum value at DNSA 0.1 mM. The same was observed in leaf tissue. The data correlates with the result of peroxidase activity.

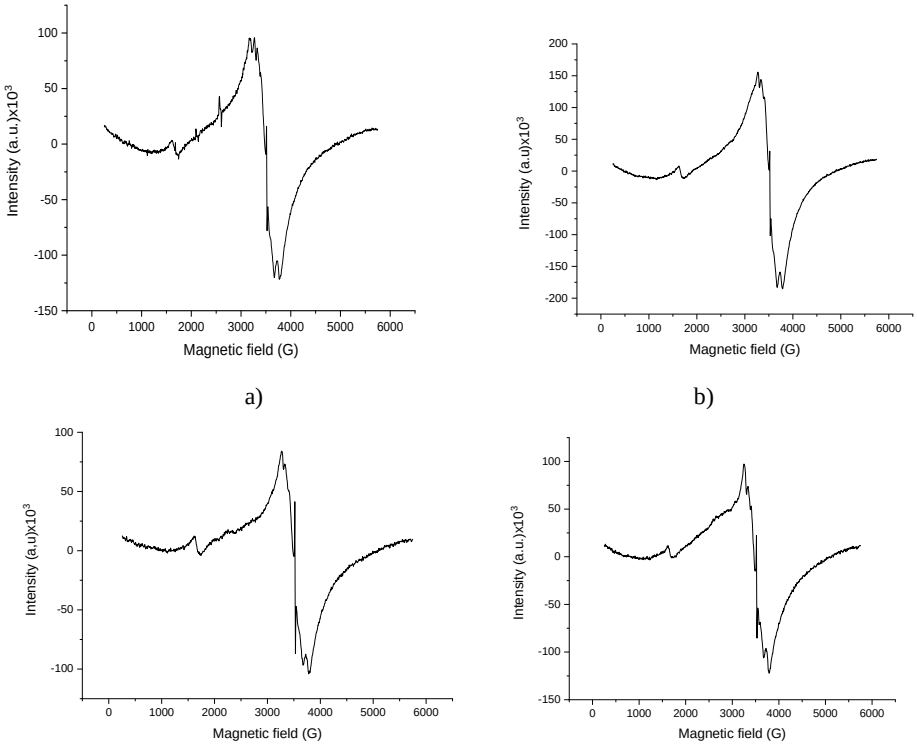

Figure 7. EPR spectras of roots *G. hirsutum* AP-317 samples in a wide range of magnetic field: a)-control, b) -0.01mM DNSA, c)- 0.1Mm DNSA, d)-1mM DNSA

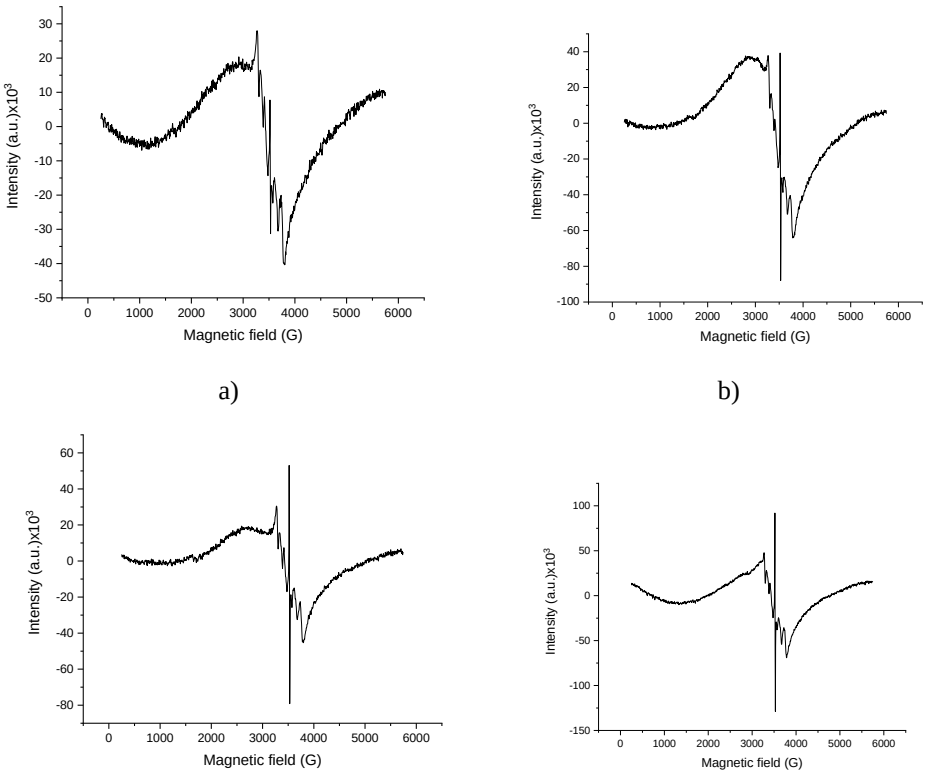

Fig. 8. EPR spectras of leaves *G. hirsutum* AP-317 samples in a wide range of magnetic field: a)-control, b) -0.01mM DNSA, c)- 0.1Mm DNSA, d)-1mM DNSA

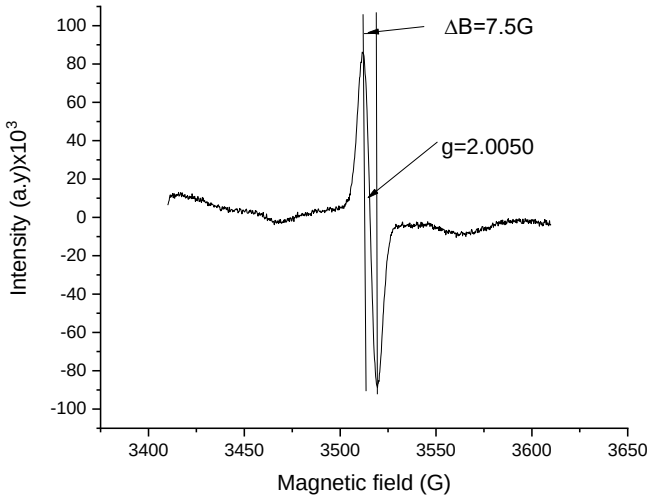

Figure 9. The singlet line of EPR spectra *G. hirsutum* AP-317 inacculated by 1mM DNSA leaf samples in the 200 G range of magnetic field

Table 1. Dependence of the intensity of the free radical spectrum in the root on the consentration of DNSA

Samples	g factor	ΔB	Intensity (a.u)
AP-317 -control	2,0057	7,50	1.00
AP-317- 0,01 mM DNSA	2,0057	7,50	1.19
AP-317- 0,1 mM DNSA	2,0058	7,50	1.24
AP-317- 1 mM DNSA	2,0057	7,50	0.96

Table 2. Dependence of the intensity of the free radical in the leaves spectrum on the consentrations of DNSA

Samples	g factor	ΔB	Intensity (a.u)
AP-317 -control	2,0050	8,25	1.00
AP-317- 0,01 mM DNSA	2,0045	7,94	3.84
AP-317- 0,1 mM DNSA	2,0043	7,94	3.93
AP-317- 1 mM DNSA	2,0043	7,50	6.24

4. Discussion

One of the interesting properties of DNSA is its ability to reduce sugars, converting itself into 3-amino-5-nitrosalicylic acid, the reaction wich underlies the determination of reducing sugars in various biological objects (Miller, 1959). The reducing activity of sugars converts them into organic acids, which can also affect the developing plant (Wood *el al.*, 2012; Khan *et al.*, 2020).

Taking into account the importance of SA as significant phytohormone in plant life we focused our attention on the capability of SA derivative, DNSA, to influence on some biochemical parameters, involved in defensive reactions of cotton seedlings. Based on the data obtained, it can be stated that the nature of the influence of DNSA on these

indicators depended significantly on DNSA concentration. Thus, treatment of plants with DNSA at a concentration of 0.01 mM led to a decrease in NO content, a decrease in the activity of PPO and catalase, as well as a decrease in the absorption of phosphorus-containing anions, while the activity of SOD and peroxidase remained virtually unchanged. However, an increase in DNSA concentration was accompanied by an increase in peroxidase activity, a decrease in catalase activity, restoration of PPO activity, partial restoration of phosphate absorption and complete restoration of NO formation. Since there is evidence that NO can participate in signal transduction, that is, it is a molecular messenger that interacts with ROS and is capable of modulating the expression of some genes involved in hormonal signaling (Astier *et al.,* 2018), based on our data, we can assert that either the effect of DNSA at low concentrations is not mediated by this oxide or NO enters into redox reactions with some components (possibly ROS) formed at low concentrations of DNSA, which is responsible for the decrease in its concentration. The fact that the activities of SOD and peroxidase remained virtually unchanged and catalase activity decreased, also allows us to conclude that low concentrations of DNSA do not lead to the accumulation of ROS. And even if they do, they are immediately neutralized by NO. However, the increase in NO content and peroxidase activity under the influence of high concentrations of DNSA may indicate that the concentrations of ROS increase and the neutralization of them is mainly carried out by peroxidase (Wang *et al.,* 2018).

The physiological role of PPO in plants is still under study (Taranto *et al.,* 2017). However, the literature suggests that PPOs may act in plant protective reactions through: (1) direct quinone toxicity; (2) reducing the bioavailability and alkylation of pathogen cellular proteins; (3) cross-linking of quinones with protein or other phenolic compounds, forming physical barriers and (4) the production of reactive oxygen species (ROS), which are known to play an important role in defense signaling (Taranto *et al.,* 2017). To contribute to the clarification of this issue, we investigated changes in the activity of this enzyme and we did not observe any changes in polyphenol oxidase activity under the influence of DNSA. These data suggest that DNSA is not involved in mediating defensive processes, activated by the PPO or as derivate of SA in cotton seedlings (Kunito *et al.,* 2009).

As for the results we obtained regarding a significant decrease in the absorption of phosphate ions by plant roots at low DNSA concentrations, since, as is known, to absorb phosphate ions, plant roots use specialized membrane proteins called phosphate transporters, which are located in the plasma membrane of root cells and these transporters actively pump the phosphate ions from the soil into the root cells, using the energy from ATP hydrolysis (Bechtaoui *et al.,* 2021).

We can assume that at low concentrations of DNSA the absorption of phosphorus ions is inhibited and the function of phosphate transporters is disrupted. In other words, at low concentrations, DNSA either directly blocks the work of specialized membrane proteins responsible for the absorption of phosphates or interferes with their work, disrupting the process of ATP hydrolysis and thereby depriving them of the energy necessary for the active transport of phosphate ions. However, to clarify this, it is necessary to carry out several experiments in this direction.

The EPR spectra obtained from the studied samples are characteristic spectra for samples of plant remains taken from the geographical area of Azerbaijan, especially Absheron. The singlet (g=4.25) observed in a small magnetic field is attributed to iron (Fe^{3+}) ions. The absence of this line in the leaf spectra indicates that iron metal ions

easily diffuse from the soil to the root, but very little is transported to the leaf during the vegetative growth process. A very broad signal was observed in the central part of the spectrum (B=600 G, g=2.035). The chemical source of this signal is related to the trivalent iron ion (Nasibova *et al.,* 2023). From Figures 1 and 2, it can be seen that the intensity of the broad line decreases significantly when moving from the root samples to the leaf samples, but the intensity of the central singlet increases and this line becomes more prominent in the overall spectrum. The sextet observed in the spectrum belongs to Mn^{+2} ions, which are widely distributed in nature through water and since the nuclear spin is I=5/2, it gives a spectrum with an superfine structure consisting of six lines. An easily visible feature of the obtained spectra is that the intensity of the central singlet line changes when dinitrosalicylic acid is given to the cotton plant as an artificial inducer.

It was found that in root tissues with the addition of DNSA, the concentration of lipid peroxide radicals increased. They reached their maximum value of 0.1 mM of DNSA. The same was observed in leaf tissue. The data is correlated with the result of peroxidase activity.

The tables 1 and 2 shows the intensity variation of the central singlet line in both root and leaf samples. The measurements were calculated according to the spectrum at 200 G. The noted intensity refers to the signal of lipid peroxides. It can be seen from the table that the intensity of the line in both root and leaf samples is greater in the inducer-incorporated plant. Especially this growth is observed in the leaf. From here, we can say that the response given by the plant to the introduction of the inducer is related to the increase in the amount of the biochemical paramagnetic centre belonging to this line. In modern scientific literature, the semiquinone radical is accepted as the biochemical source attributed to the line.

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