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EFFECT OF INDOLE-3- BUTYRIC ACID ON THE ANTIOXIDANT ENZYMES, NO AND CHLOROPHYLL CONTENT OF AGDASH-3 AND AP-317 GENOTYPES OF UPLAND COTTON (*GOSSYPIUM HIRSUTUM* L.)

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Abstract. The main objective of our research was to determine the effect of various concentrations of IBA on the activity of numerous prooxidant-antioxidant enzymes, NO content and on the components of the photosynthetic apparatus: chlorophyll a, b and carotenoids in cotton seedlings of Gossypium hirsutum Agdash-3 and Gossypium hirsutum AP-317 genotypes. Our study also focused on the impact of different concentrations of IBA on the dynamics of the activity of polyphenol oxidase, which plays an essential role not only in the metabolism of phenolic compounds but also in the involving of these compounds in the defense response of plants. The results indicate that IBA participated in modulating the activity of prooxidant-antioxidant enzymes (CAT, POX, SOD, and PPO), the formation of the NO and components of the photosynthetic apparatus. At the same time, the activity of SOD and catalase changed insignificantly, which may indicate lower effect of IBA on these two enzymes

Keywords: *IBA (indole-3-butyric acid), Gossypium hirsutum, photosynthetic pigments, antioxidant enzymes, NO-content.*

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1. Introduction

Various natural and anthropogenic changes in the environment negatively affect the viability of plants, or rather, weaken the immune system of plants, increasing their sensitivity to various types of stressors, both biotic and abiotic in nature. The combination of these negative stress factors is ultimately characterized by a decrease in the productivity of agricultural plants (Pandey *et al.*, 2017; Szőke *et al.*, 2021).

The system of acclimatization and adaptation of plants to negative environmental factors includes various metabolic pathways that reduce the harmful effects of stressors on plants. One of the initial stages of activation of adaptive mechanisms is the induction of the formation of reactive oxygen species (ROS), such as $O_2^{\cdot -}$, H_2O_2, $OH^{\cdot}$, $^1O_2$. ROS in stressful situations serve as signal molecules, transmitting an excitation signal both at

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the level of intracellular and intercellular interactions. On the other hand, as the effect of the stressor increases, so does the concentration of ROS, the excess of which is very toxic to cells. They cause oxidative stress, DNA damage, plasma membrane lipids and other cell components, and, therefore, there is a need to neutralize them (Considine *et al.*, 2015; Foyer & Noctor, 2016; Mignolet-Spruyt *et al.*, 2016). In this case, an important role is played by antioxidant enzymes involved in this process, which mitigate oxidative stress, and thereby stabilize and protect cellular metabolism, as well as cellular components (Mittler *et al.*, 2004).

The antioxidant system is a network of systems that includes both antioxidant enzymes (superoxide dismutase (SOD), peroxidase (POX), ascorbate peroxidase (APX), catalase (CAT), glutathione peroxidase (GPX), and peroxiredoxin (PRX)) and antioxidant substances (vitamins C, E, proline, glutathione, etc.). The constant generation of ROS (due to specific enzymes such as NADPH oxidase and other types of oxidases and peroxidases, NO synthetases) and the removal of excess occurs with the indirect participation of genes that control the concentration of ROS (Mittler *et al.*, 2004; Rashid *et al.*, 2021). Early studies have already reported a positive effect of phytohormones on plant resistance to free radical attacks (Denancé *et al.*, 2013; Barna *et al.*, 2012). Phytohormones such as salicylic and jasmonic acids, auxins, and gibberellins are described as regulators of resistance to various stress factors. In particular, the participation of auxins, namely, IAA (indole-3-acetic acid) and IBA (indole-3- butyric acid), as growth regulators and inducers of resistance to pathogens in various plants was established (Hurný *et al.*, 2020; Schepetilnikov & Ryabova, 2017; Pieterse *et al.*, 2012; Šípošová *et al.*, 2021; Fu & Wang, 2011; Amrahov *et al.*, 2022). In this case, the activating and inhibitory concentrations of these phytohormones played an important role. Therefore, controlling the concentrations of various phytohormones in the plant growth environment can provide an opportunity to enhance certain plant characteristics, which ultimately can increase the stress resistance of plants.

The aim of our study was to determine the effect of various concentrations of IBA on the activity of some prooxidant-antioxidant enzymes, NO content and on the components of the photosynthetic apparatus: chlorophyll a, b and carotenoids in cotton seedlings of *Gossypium hirsutum* Agdash-3 and *Gossypium hirsutum* AP-317 genotypes We also studied the effect of different concentrations of IBA on the dynamics of the activity of polyphenol oxidase, which plays an essential role not only in the metabolism of phenolic compounds but also in the involving of these compounds in the defense response of plants.

2. Materials and methods

The experiments were carried out on cotton *Gossypium hirsutum* Agdash-3 and *Gossypium hirsutum* AP-317 genotypes, received from the Genetic Resources Institute of the Ministry of Science and Education, Republic of Azerbaijan. The seeds of cotton genotypes were pre-treated with 0.2% potassium permanganate for 8 minutes, then, they were planted in 7 cm plastic cups containing perlite. The seedlings were grown in a phytotron (Taisite, GZX-300 E) at temperatures of 22–24°C, humidity of 65–75%, and light intensity of 4800 lux. The light length was 14/10 hours day/night. As a nutrient medium was used Steiner's solution with the addition of 0.25, 2.5, and 25 mM of IBA during the entire growth period, starting from the first days of seedling formation. Two-week-old embryonic cotyledons were used for analyses.

Catalase activity was determined by the Mosheva gasometric method (Mosheva, 1982). First, 0.5 g of plant material was measured, which was homogenized with the gradual addition of 0.5 g of $CaCO_3$ and 20 ml of distilled water. Subsequently, the extract was transferred to a Landolt vessel (Erlenmeyer flask) with a neck on the side and catalase activity was measured. To start the reaction, 5 ml of 3 % H_2O_2 was added to the extract and during the entire reaction, it was stirred on a magnetic stirrer. The measurement was carried out after 2 minutes. After this time, the amount of oxygen released could be read on the burette scale. The enzyme activity was expressed in ml/second*g (fresh sample).

Peroxidase activity (POX, EC 1.11.1.7) was determined according to Chance and Maehly (Chance & Maehly, 1955). The reaction mixture contained 0.1 M Na-phosphate buffer, pH 7,2, 1mM EDTA, 30mM H_2O_2, 50mM guaiacol, and 500µl of enzyme extract in the final volume of 4 ml. The formation of tetraguaiacol was detected at 440 nm. The concentration of tetraguaiacol was calculated using the extinction coefficient of tetraguaiacol (26.6mM $^{-1}$*cm $^{-1}$). POX activity was expressed as $\Delta A590_* g^{-1} * min^{-1}$.

Superoxide dismutase (SOD, EC 1.15.1.1) activity was determined spectrophotometrically by measuring its ability to inhibit photochemical reduction of nitroblue tetrazolium (NBT) as reported by Beaucham and Fridovic (Beauchamp & Fridovich, 1971). The reaction mixture contained 50 mM K phosphate buffer, pH 7.8, with 0.1mM EDTA, 150µM NBT, 26mM methionine, 8 µM riboflavin, and 50 µl of enzyme extract in the final volume of 2,05 ml. Reaction mixtures were incubated for 8 min under light condition. 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 (Yermakov *et al.,* 1987). The activity of polyphenol oxidase was expressed as U/min*g (FW).

The amount of NO in cotyledons was determined by using the Griess reagent with the modified method of Zhou et al. and Karpets et al (Zhou *et al.,* 2005, Karpets *et al.,* 2015). Leaves (1 g) were ground in a mortar and pestle in 5 ml of 50 mM cool acetic acid buffer (pH 3.6, containing 2% zinc diacetate). The homogenates were centrifuged at 8 000g for 15 min at 4^o C. After centrifugation 0.250 g of activated charcoal was added to the supernatant. After vortex and filtration through white filter paper (pore size- 8-12 µm), the filtrate was collected. The mixture of 2 ml of filtrate and 1 ml of the Greiss reagent was incubated at room temperature in dark for 30 min. Absorbance was determined at 548 nm. NO content was calculated by comparison with a standard curve of $NaNO_2$. This method is based on the conversion of endogenous NO to nitrite and the determination of the amount of nitrite by the Griess reaction.

Chlorophyll a, b, and carotenoids determinations were carried out using the Wellburn method (Wellburn and Lichtenthaler, 1984). 1 gram of fresh leaf tissue was taken, cut into small pieces, and homogenized in a mortar in 20 ml of 80% acetone. Then the homogenate was passed through an F-grade filter paper. The optical density was measured in the resulting filtrate at wavelengths 440, 644, and 662 nm. Results were calculated in mg/g of fresh sample.

Statistical analysis

The adsorption experiments were performed in triplicate and data was reported as mean ± SD. The regression coefficient (R2) values were calculated using statistical functions of Microsoft Excel (version Office 7, Microsoft Corporation, USA).

3. Results

The effect of different concentrations of IBA on the catalase activity of Agdash 3 and AP-317 cotton genotypes was shown in figure 1. As can be seen from the figure, a low concentration of the IBA (0.25 μM) did not affect the activity of the enzyme in both genotypes. An increase in the IBA concentration up to 2.5 μM in both cotton genotypes was accompanied by a decrease in catalase activity. At a high concentration of IBA (25 μM) in the Agdash-3 genotype, the activity remained unchanged, while in the AP-317 genotype it slightly increased. These experiments indicated that, firstly, the basic level of catalase activity in Agdash-3 is higher than in AP-317, and secondly, IBA is able to influence the level of catalase activity in the leaves of the studied cotton seedlings.

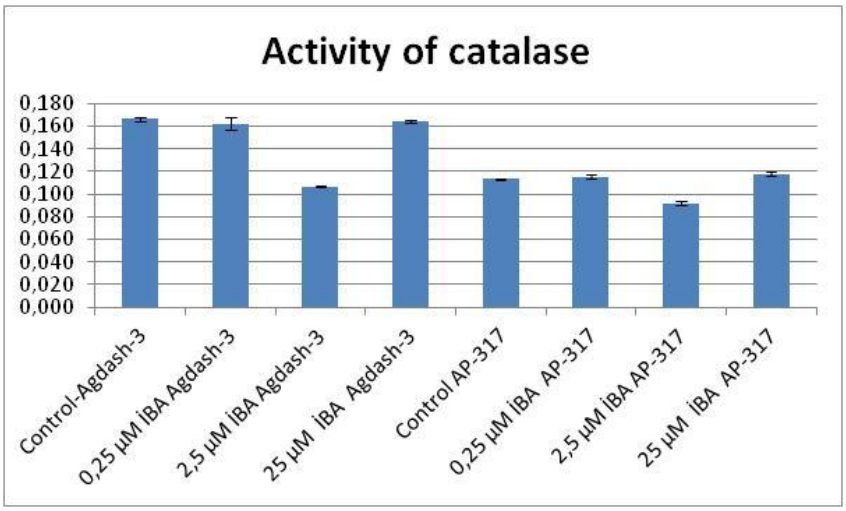

Fig 1. Catalase activity in leaves of cotyledons of genotypes *Gossypium hirsutum* Agdash-3 and *Gossypium hirsutum* AP-317 exposed to different concentrations of IBA

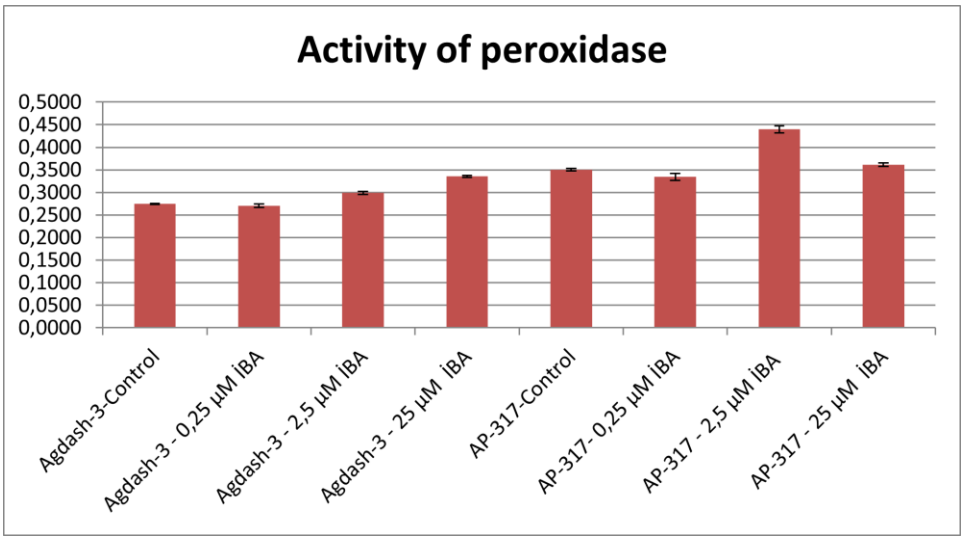

Fig.2. Peroxidase activity (POX) in cotyledons of genotypes *Gossypium hirsutum* Agdash-3 and *Gossypium hirsutum* AP-317 exposed to different concentrations of IBA

A low concentration (0.25 µM) of IBA had almost no effect on the level of peroxidase activity in both cotton genotypes either (Fig. 2). However, an increase in the content of the phytohormone in the seedling germination medium caused the activation of the enzyme. In the Agdash-3 genotype, a direct relationship was observed between the concentration of IBA and the induction of activity, and in the AP-317 genotype, the maximum activity was recorded at a concentration of 2,5 µM. It should also be noted that, in contrast to catalase, in this case, the base level of peroxidase in the AP-317 genotype was much higher than in the Agdash-3 genotype.

At low (0.25 µM) and medium (2.5 µM) concentrations, IBA did not show any significant changes in SOD activity in both cotton genotypes. However, at its high concentration (25 µM), the activity of SOD relative to the control in the Agdash-3 genotype significantly decreased, while in the AP-317 genotype, on the contrary, it increased (Fig 3).

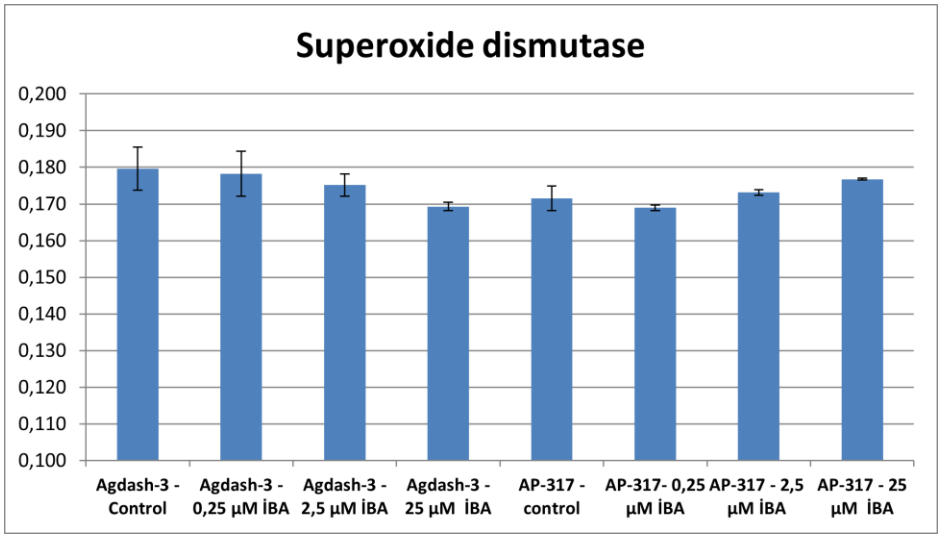

Fig.3. SOD activity in the cotyledons of genotypes *Gossypium hirsutum* Agdash-3 and *Gossypium hirsutum* AP-317 exposed to different concentrations of IBA

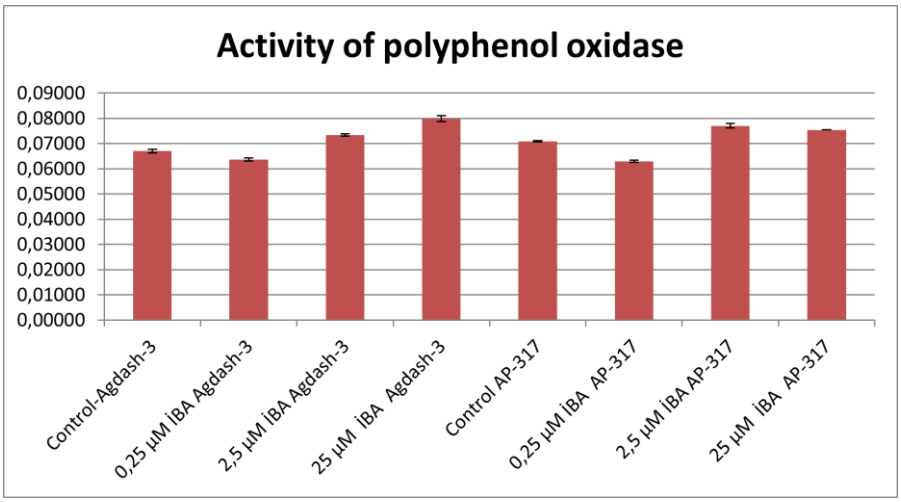

Fig 4. The activity of polyphenol oxidase in the cotyledons of genotypes *Gossypium hirsutum* Agdash-3 and *Gossypium hirsutum* AP-317 exposed to different concentrations of IBA

The low concentration of IBA decreased the activity of PPO, while its medium and high concentrations caused an increase in the activity of the enzyme, which may indicate increased oxidation of polyphenols (Fig 4).

As it is known, the synthesis of NO is realized both due to NO synthase, as well as a complex of nitrate and nitrite reductases/ It was found that, as the concentration of IBA increased, the content of NO also increased in the leaves of cotton seedlings. This pattern was somewhat more pronounced in the Agdash-3 genotype than in the AP-317 genotype compared to the control ,in 25 µM IBA finally 1.50 and 1.25 times, respectively (Fig.5).

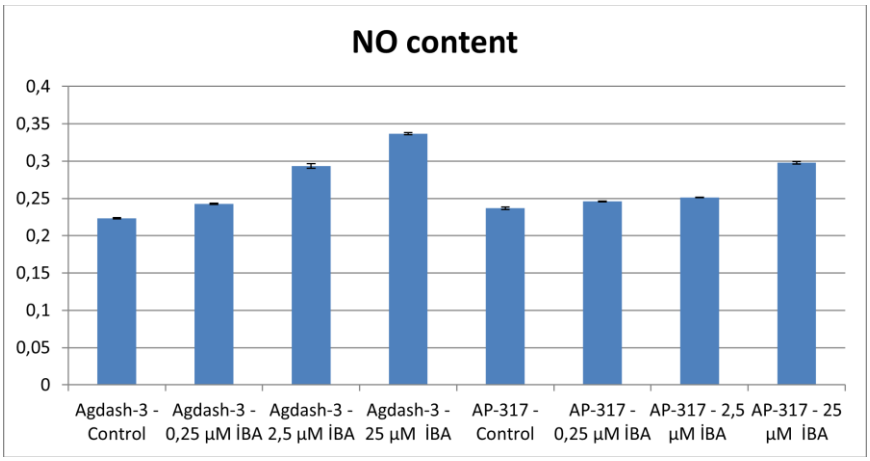

Fig. 5. Effect of different concentrations of IBA on the dynamics of NO formation in the cotyledons of *Gossypium hirsutum* Agdash-3 and *Gossypium hirsutum* AP-317 genotypes

IBA at all concentrations had a positive effect on the content of chlorophyll a in both genotypes. The concentration of chlorophyll b remained practically unchanged in the Agdash-3 genotype, but in the AP-317 genotype, as the IBA concentration increased, it noticeably increased. IBA also stimulated the formation of carotenoids in both cotton genotypes, but to a greater extent in the Agdash-3 genotype than in the AP-317 genotype (Fig. 6).

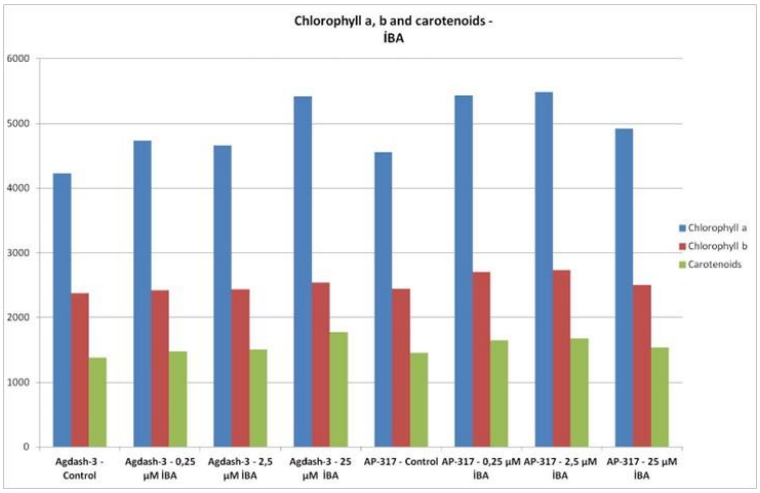

Fig. 6. Effect of different concentrations of IBA on the concentration of chlorophyll a, b and carotenoids in the cotyledons of *Gossypium hirsutum* Agdash-3 and *Gossypium hirsutum* AP-317 genotypes

4. Discussion

Phytohormones play an important regulatory role in metabolism and, consequently, in plant ontogeny. IAA is one of the natural forms of phytohormones called "endogenous auxins". The active form of IBA is IAA, i.e., for the manifestation of its hormonal activity, it must be converted into IAA (Uzunova *et al.,* 2016; Strader & Bartel, 2011).

Like other phytohormones, IBA is involved not only in the processes of cell proliferation and differentiation (Enders & Strader, 2015), but also in the defense response of plants to biotic and abiotic environmental factors (Navarro *et al.,* 2006; Chen *et al.,* 2007). At the same time, the question of the influence of IBA on such an important component of the defense system of the cotton plant remains unresolved.

Our experiments showed that exogenous IBA, as a phytohormone, is involved in the prooxidant-antioxidant activity of plants, and affects polyphenol oxidase activity and the synthesis of some components of the photosynthetic apparatus. Moreover, the nature of the action of this phytohormone on the listed processes largely depends not only on its applied concentration but also on the cotton genotype.

Catalase, as an intracellular regulator of H_2O_2 concentration in the cell, is important in the antioxidant defense response of plants. Our studies revealed a decrease in catalase activity at the concentration of 2.5 μM IBA in both genotypes (Fig. 1). However, at the same concentration of IBA, the activity of another H_2O_2 consuming (i.e., neutralizing) enzyme, POX, was positively stimulated (Fig. 2). Moreover, the basic level of catalase activity in the Agdash-3 genotype was higher, and peroxidase activity was lower than in the AP-317 genotype (compare Fig. 1 and Fig. 2). These results could indicate that these enzymes work together in cotton leaves and the insufficient activity of one of them is compensated by the other. Li and colleagues reported that IBA at 25 μM caused an increase in POX activity 48 hours after treatment in Mung bean (*Vigna radiata*) (Li *et al.,* 2018). They also demonstrated that this phytohormone had no effect on SOD activity at 25 μM. In our studies, at the similar concentration, IBA showed an increase in SOD activity compared to the control in the AP-317 genotype, while it did not cause any noticeable changes in Agdash -3 (Fig. 3). These data prove the varietal features of the effect of IBA.

An important point in the defense reactions of plants is that an increase in the concentration of phenolic compounds also affects the increase in the activity of PPO and POX since phenols are polymerized with the participation of both enzymes (Lavid *et al.,* 2001, Sherman *et al.,* 1991; Can *et al.,* 2014). It is believed that the involvement of polyphenol oxidase in the polymerization of phenols and the accumulation of polyphenols in the zone of localization of unfavorable factors play a certain role in the defense reactions of plants (Lavid *et al.,* 2001). An increase in PPO activity at IBA concentrations from 2.5 to 25 μM may indicate its involvement in polymerization, which is consistent with the increase in POX activity (Fig. 4).

Activating and inhibitory concentrations of IBA in both genotypes had a positive effect on the synthesis of NO, which is considered a multifunctional molecule and performs various functions in a plant cell, including a protective one. (Fig.5). Similar data were also obtained in previous experiments (Della Rovere *et al.,* 2019).

Previous studies on the leaves of *Brássica júncea* have shown the involvement of IAA and some of its derivatives in the formation of chlorophylls (Ahmad *et al.,* 2001). An increase in the concentration of chlorophyll a, b and carotenoids at certain

concentrations of IBA may indicate its participation in the synthesis of these components in cotton leaves (Fig. 6). Thus, they can play a critical role in adverse environmental conditions.

5. Conclusion

The data obtained indicate the participation of IBA in the modulation of the activity of prooxidant-antioxidant enzymes (CAT, POX, SOD, and PPO), the formation of the NO and components of the photosynthetic apparatus. At the same time, the activity of SOD and catalase changed insignificantly, which may indicate lower effect of IBA on these two enzymes.

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