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EFFECT OF VERTICILLİUM WILT ON THE ANTIOXIDANT SYSTEM AND FORMATION OF IRON NANOPARTICLES IN COTTON GENOTYPES

N. R. Amrahov[*1,3,4], V. Y. Allahverdiyev[5], Y. I. Agharzayeva[2], R. B. Mammadova[6], S. N. Omarova[7], F. A. Khudayev[3], A. N. Nasibova[7,8], M. Shoaib[9], R. I. Khalilov[7,8] and Z. M. Mammadov[2]

[1]Bioengineering Laboratory , Excellence Center for Research, Development and Innovations, Baku State University, Baku, Azerbaijan Republic
[2]Department of Molecular Biology and Biotechnology, Faculty of Biology,Baku State University, Baku, Azerbaijan Republic
[3]Azerbaijan Research Institute of Crop Husbandry, Ministry of Agriculture,Baku, Azerbaijan Republic
[4]GPOGC SRİ, Azerbaijan State Oil and Industry University, Baku, Azerbaijan Republic
[5]Department of Biotechnology, Faculty of Natural Sciences, University of Silesia in Katowice, Katowice, Poland
[6]Department of technical and forage plants, Genetic Resources Institute of ANAS, Baku, Azerbaijan Republic
[7]Department of Biophysics and Biochemistry, Faculty of Biology, Baku State University, Baku, Azerbaijan Republic
[8]Institute of Radiation Problems of ANAS, Baku, Azerbaijan Republic
[9]Department of Parasitology and Microbiology, Faculty of Veterinary and Animal Sciences, PMAS Arid Agriculture University Rawalpindi, Pakistan
Corresponding author's email: nurlan00686@gmail.com

ABSTRACT

The effect of a highly virulent fungus *Verticillium dahliae* Kleb. VD-11, with non-defoliating strain on polyphenol oxidase (PPO) and the antioxidant enzymes, such as guaiacol peroxidase (POX) and superoxide dismutase (SOD), was studied in double haploid cotton variety- Pima 3-79 (*Gossypium barbadense* L.), highly inbred line TM-1– (*Gossypium hirsutum* L.), as well as in genotypes CS B-15 and GS B-22, created by the substitution of one chromosome of TM-1 with the chromosome 15 and 22 of Pima 3-79 respectively. The treatment of plants with the pathogen led to an increase in the activity of PPO approximately 1.2 times in the case of TM-1 and CS B-15. However, these changes were insignificant in the case of Pima 3-79 variety, whereas the activity of the enzyme in the case of CS B-22 was decreased apparently. Similar effects were also obtained for POX. After inoculation, the activity of SOD in all genotypes, except CS B-15, was increased. On the contrary, the activity of SOD was decreased in the case of CS B-15 genotype. It was found that plant infection with the pathogen leads to a change in the concentration of free radicals, which, in turn, affects the activity of antioxidant enzymes. The fungal infection also led to the creation of new paramagnetic centers and nanophase crystal-like iron oxide particles in roots, but not in leaves.

Keywords: Cotton, *Verticillium dahliae*, polyphenol oxidase, antioxidant enzymes, Fe-nanoparticles.

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INTRODUCTION

Cotton is one of the most important spinning crops in the world. Cotton production provides income for more than 250 million people worldwide by employing almost 7% of all labor in developing countries (Voora *et al.,* 2020). The oil obtained from the seeds is used for food, and the oilcake is used as a high-protein feed for livestock. Its fiber serves as a raw material for the textile industry. In addition to productivity and fiber quality, disease resistance is a very important goal for cotton cultivation. One of the economically important disease of cotton is verticillium wilt caused by a soil-borne fungus *Verticillium dahliae*. Most of the commercial cotton cultivars are susceptible to this pathogen (Zhou *et al.,* 2014; Jabbar *et al.,* 2022). Verticillium wilt could cause great economic losses in cotton production (Land *et al.,* 2017). Verticillium wilt negatively affects lint yield and seed weight in cotton. Drastic change in the lint yield between healthy plants and plants with verticillium wilt was observed showing 32% to 46% reduction in lint yield in diseased plants (Ayele *et al.*, 2020). The fungus grows optimally at 27°C, which is also optimal for cotton development (Pegg *et al.*, 2002). Parts of dead plants maintain a high level of the fungus, which is very difficult to eliminate by fungicide treatment (Xu *et al.,* 2011). Pathogen's mycelia can survive in the soil for up to 10 years. Therefore, an

important target for cotton breeders is to incorporate fiber quality and verticillium wilt resistance (Naraghi *et al.,* 2019). The nature of cotton resistance to Verticillium pathogen, including non-defoliant and defoliant strains, was investigated (Shaban *et al*., 2018). In the most cases, resistance has biochemical nature and can be determined by the primary contact with the pathogen and the response to this contact. At the same time, the protective response includes the involvement of reactive oxygen species (ROS), free radicals which participate normally in oxidative stress, inhibit the development of the pathogen and are also important signalling molecules in the signal transmission system from the affected area (infection) to areas where there is still no contact with the pathogen (Rashid *et al.,* 2021). Oxidative stress is regulated by the specific prooxidant-antioxidant enzymatic system (Keshari *et al.*, 2015; Amrahov *et al.,* 2022), which on the one hand, requires the formation of ROS to trigger antioxidant defense, on the other hand, removes excess ROS since they can damage the cell.

Taking into account the above-mentioned facts, the aim of our research was to study the influence of the pathogen Verticillium dahliae Kleb. VD-11 on the enzymatic activity of the antioxidant system and polyphenol oxidase, as well as on the intracellular formation of iron nanoparticles in the leaves and roots of cotton plants. The results of our research will allow us to give recommendations on the possibility of using these genotypes in subsequent selections.

MATERIALS AND METHODS

Experimental objects: The experiments were carried out on double haploid cotton variety- Pima 3-79 (*Gossypium barbadense* L.), highly inbred line TM-1 (*Gossypium hirsutum* L.), as well as 2 genotypes of CS B-15 and GS B-22, created by the substitution of one chromosome of TM-1 with the chromosome of Pima 3-79. CS B-15 genotype was created by the transfer of chromosome 15 from *Gossypium barbadense* L. Pima 3-79 to *Gossypium hirsutum* L. TM-1, whereas the CS B-22 genotype created based on the transfer of chromosome 22 from *G. barbadense* L. Pima 3-79 to *Gossypium hirsutum* L. TM-1. *G. barbadense* L. specie has a natural resistance to verticillium wilt. CS lines are unique germplasm which doesn't exist in nature. The use of chromosome substitution lines (CSL) as a bridge should provide a more efficient way to introgress alleles from *G. barbadense* L. into Upland cotton (Jenkins *et al.*, 2018). The use of chromosome substitution lines in such experiments is essential to determine the role of alien substituted chromosomes in the improvement of verticillium wilt resistance in cotton. As a biotic stress factor, a highly virulent non-defoliating fungus strain *V.dahliae* VD-11 was used.

Field experiments: Field experiments were carried out in Absheron Experimental Station of Genetic Resources Institute of Azerbaijan, National Academy of Sciences. Disease symptoms were observed during all vegetation periods and stem ranking was carried out at the end of vegetation. The stem cut below the first internode was done for stem ranking. The ranking of each genotype was determined using 3 technical and 10 biological replicates.

Pathogen isolation: *Verticillium dahliae* VD-11 strain was isolated from wilted cotton plants by Göre (Göre, 2007). Inoculum of VD-11 was prepared by growing on plates containing potato dextrose agar medium (PDA) and plates were incubated at 24°C in a thermostat. Two weeks later conidia were collected, suspended by washing with sterile water and preserved on potato dextrose agar plates at 26°C (Afzaal *et al.,*2021). The concentration of conidia was determined using a hemacytometer and appropriate dilutions were made to obtain conidia suspension with a concentration of 3×10^6 conidia per 1 ml and used for inoculation (Joost *et al.,* 1995).

Inoculation technology: İn the stage of six true leaves, the cotton plants were stem-inoculated with *V. dahliae* suspension using the hanging drop method of Bugbee and Presley (Bugbee and Presley, 1967). The conidia suspension was drawn into a 3-mL syringe and with a 22-gauge needle, a single drop was applied to the plant surface on the first internode above the soil line. The needle then was used to stab through the drop into the cotton stele. Plants were inoculated twice, once just below the cotyledonary node, and on the opposite side of the stem halfway between the soil and the cotyledonary node. Controls were inoculated with sterile water. Each treatment consisted of 15 plants. Following inoculation, plants were incubated at 27ºC with light and watering conditions described by Hanson (Hanson, 2000).

Preparation of enzyme extract: One gram of fresh plant leaf tissue was homogenized in an ice-cold mortar in 50 mM K-phosphate buffer, pH 7.8, with 0.1 mM EDTA. Homogenates were centrifuged at 9,000 × g for 20 min at 4°C. Supernatants were used for POX and SOD enzyme activity assays.

Determination of enzyme activity: The activity of guaiacol peroxidase (POX, EC 1.11.1.7) was determined according to Chance and Maehly (Chance and Maehly, 1955). The reaction mixture contains 0.1 M of Na-phosphate buffer (pH 7.2), 1mM of EDTA, 30 mM of H_20_2, 50 mM of guaiacol, and 50 µl of enzyme extract in the final volume of 1 ml. The formation of tetraguaiacol was detected at 440 nm. The concentration of tetraguaiacol was calculated using the extinction coefficient of tetraguaiacol (26.6 $mM^{-1} \cdot cm^{-1}$). GPX activity was expressed as the amount of formed tetraguaiacol (µ katal mg^{-1} protein)

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 (Beauchamp and Fridovic, 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.

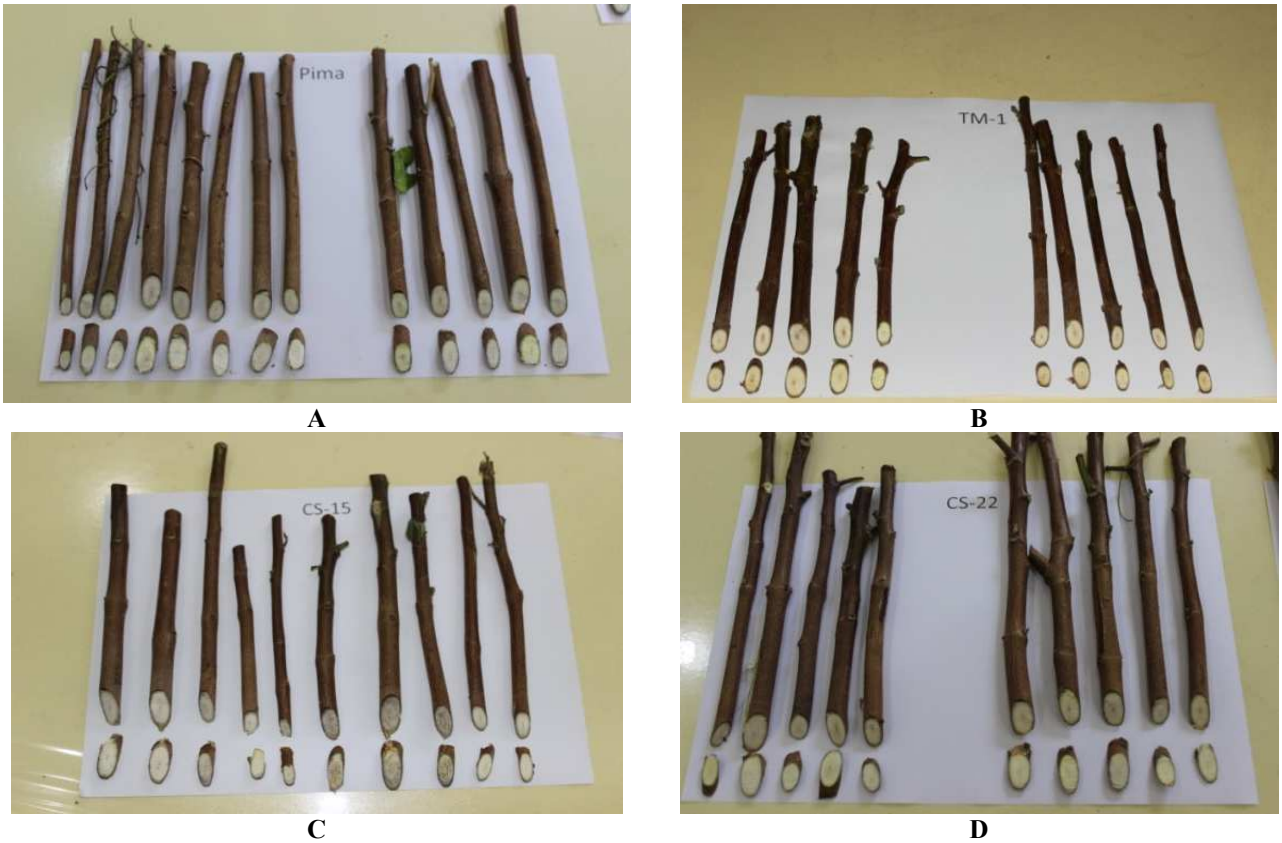
Preparation of crude PPO enzyme extract and analyse: The crude PPO enzyme extract was prepared by the modified method as in the procedures described by Sharma (Sharma *et al.,* 2001) and Ermakov (Ermakov *et al.,* 1987). One gram of cotton cotyledons, together with 5 ml of 100 mM phosphate buffer (pH 7.3) containing 10 mM of sodium ascorbate were homogenized in a cold mortal, filtered through four layers of gauze and centrifuged at 3,000 x g for 20 min. The final volume of the extract was made up to 25 ml with phosphate buffer (pH 7.3). The filtrate was then centrifuged at 15,000 x g for 20 min. The supernatant was used as an enzyme source.

Polyphenol oxidase (PPO, EC 1.10.3.1) activity was determined by measuring the oxidation of 0.05 M catechol at 590 nm in 0.1 M potassium phosphate buffer (pH 7.2). Enzymatic activities were expressed as enzyme $\Delta A_{590}\ g^{-1}\ min^{-1}$.

EPR analyses: The leaves and roots of all the studied cotton genotypes (Pima 3-79, TM-1, CS B-15, CS B-22) were dried at room temperature (23-25°C) and powdered for EPR spectroscopy research. The spectra of powders were recorded at room temperature by Bruker-EMX EPR spectrometer (Germany). The registration was carried out in a wide range of magnetic fields (500-5500 G). The microwave frequency: 9.870 GHz, capacity: 2.189 MV been.

RESULTS

Field experiment results: The results of field experiments on inoculation cotton plants with *V. dahliae* Kleb suspension are given below (Fig. 1 A- D). The analysis of stem sections showed that the genotypes Pima 3-79, TM-1 and CS B-22 did not have any significant damage, while several stems of CS B-15 were affected by the pathogen, which is visible on the stem ranking.



A **B** **C** **D**

Figure 1. Stem ranking of *Gossypium barbadense* L. Pima 3-79 (A), *Gossypium hirsutum* L. TM-1 (B), CS B-15 (C), CS B- 22 (D), exposed to *Verticillium dahliae* Kleb. *in vivo*

Enzymes activity: The activity of enzymes – PPO, GPO and SOD was determined in TM-1, Pima 3-79, CS B-15 and CS B-22 cotton varieties infected with *Verticillium dahliae* fungus. The tests were performed 7 days after inoculation of the pathogen. Data represents mean±SE (standard error) from 6 independent samples. The experiment was done in three biological replicates with similar results.

The results obtained on the change in the activity of polyphenol oxidase are shown in Fig.2. As can be seen from the presented data, in the control variants of the tested varieties, the lowest polyphenol oxidase activity was observed in varieties of TM-1 and Pima 3-79, and the relatively high activity of this enzyme was observed in the case of CS B-15 and CS B-22 (Figure 2). After inoculation with the pathogen, the activity of the enzyme was increased approximately 1.2 times in the case of TM-1 and CS B-15 in comparison with the control. However, in the case of the Pima 3-79 variety, these changes were insignificant, whereas the activity of the enzyme in CS B-22 decreased obviously.

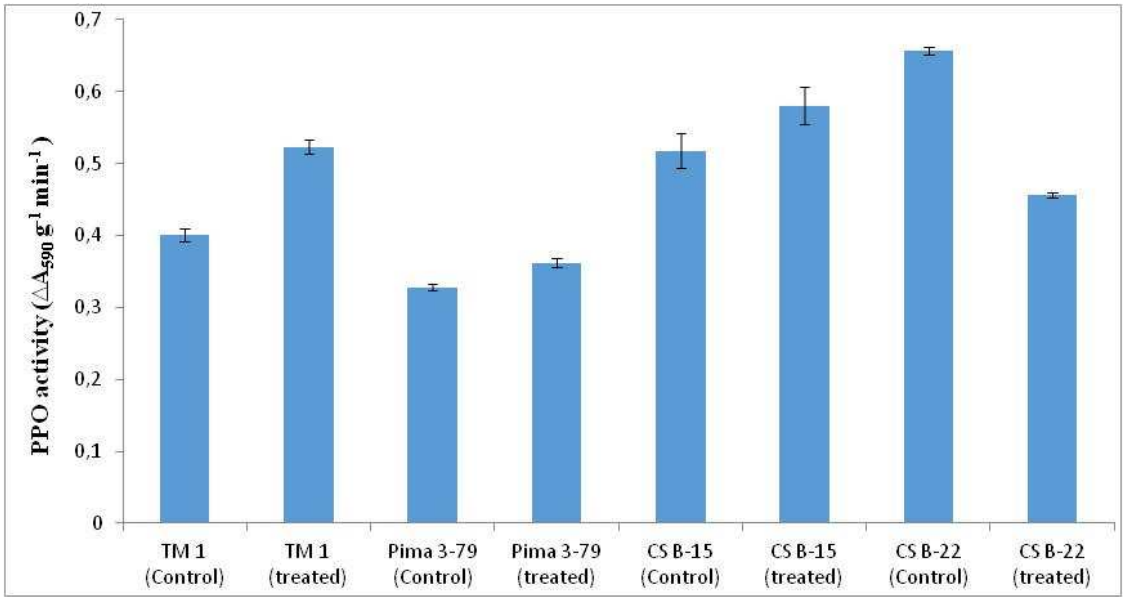


Fig. 2. Polyphenol oxidase activity of TM-1, Pima 3-79, CS B-15 and CS B-22 of cotton varieties after 7 days of treatment with *Verticillium dahliae* Kleb.

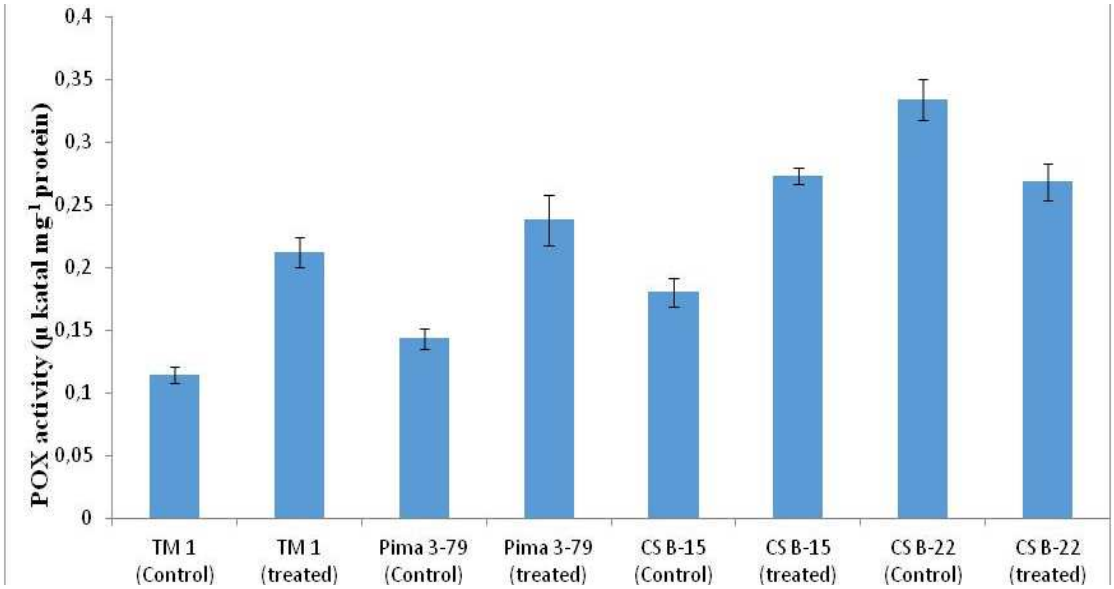


Fig.3. Guaiacol peroxidase (POX) activity of TM-1, Pima 3-79, CS B-15 and CS B-22 of cotton varieties after 7 days of treatment with *Verticillium dahliae* Kleb.

As in the case of polyphenol oxidase, the lowest activity of guaiacol peroxidase in the control variants was observed in the case of the TM-1 variety, whereas the highest one is in the case of CS B-22. The treatment of plants with the pathogen has led to an increase by 1.8 times in the activity of the enzyme compared to the control in the case of TM-1 and Pima 3-79, whereas in the case of CS B-15, it was 1.6 times higher. On the contrary, the activity of guaiacol peroxidase in the case of CS B-22 seedlings decreased markedly compared to the control (Fig. 3).

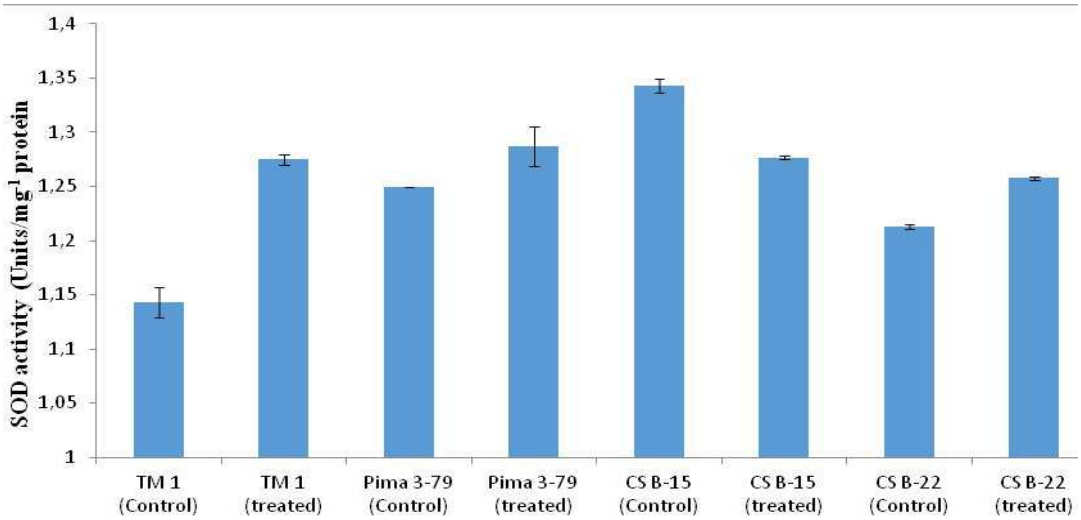


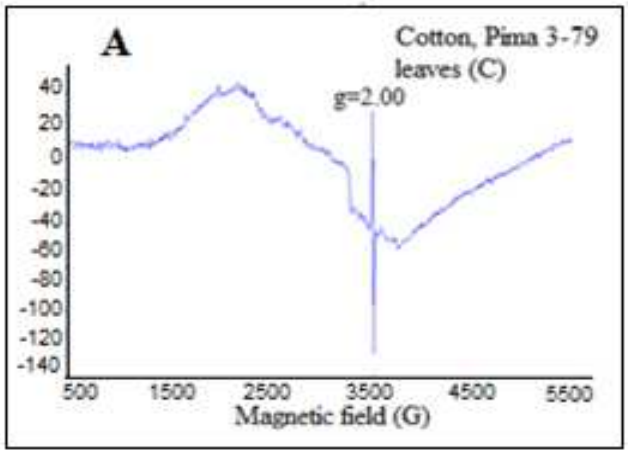
Fig. 4. Superoxide dismutase activity of TM-1, Pima 3-79, CS B-15 and CS B-22 of cotton lines after 7 days of treatment with *Verticillium dahliae* Kleb.

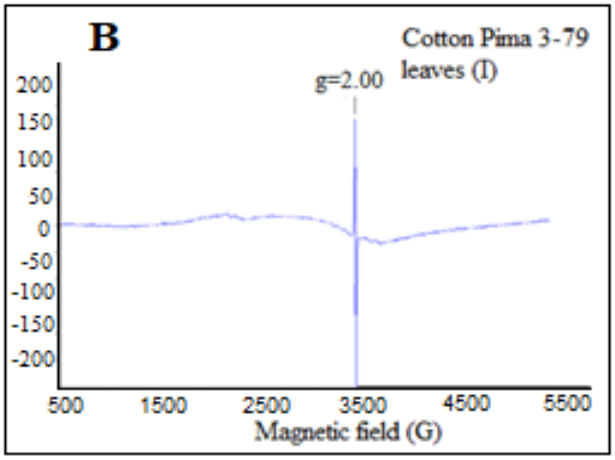
As a result of a comparative analysis of the activity of SOD in control and pathogen-inoculated varieties (Fig. 4), it was revealed that:

1. The highest activity of SOD was in the case of CS B-15, whereas TM-1 genotype was characterized by the lowest activity. In Pima 3-79 this activity was higher by 9.1 %, whereas in the case of CS B-15 - 17.5 % in comparison with TM-1.

2. After inoculation, the activity of SOD in all genotypes, except CS B-15, was increased. In the CS B-15 genotype, on the contrary, the activity of SOD was decreased.

Results of EPR analyses: In a wide range of magnetic fields, EPR spectra of leaves and roots of cotton belonging to the Pima 3-79 genotype were recorded (Fig. 5 A, B ,C, D). In the root samples, a free radical signal with a width δ=10 Qs, g-factor g=2,0023, and a broad EPR signal with a width δ=340 Qs, g-factor g=2,34 were detected. This signal characterizes magnetic nanoparticles of iron oxide with its parameters (Khalilov *et al.* 2011; Khalilov *et al.,* 2015). In the Pima 3-79 genotype, treated with ***Verticillium dahliae*** fungus the intensity of free radical signalling was higher. Unlike root samples, the signal characterizing magnetic nanoparticles of iron oxide in the leaves was not observed.





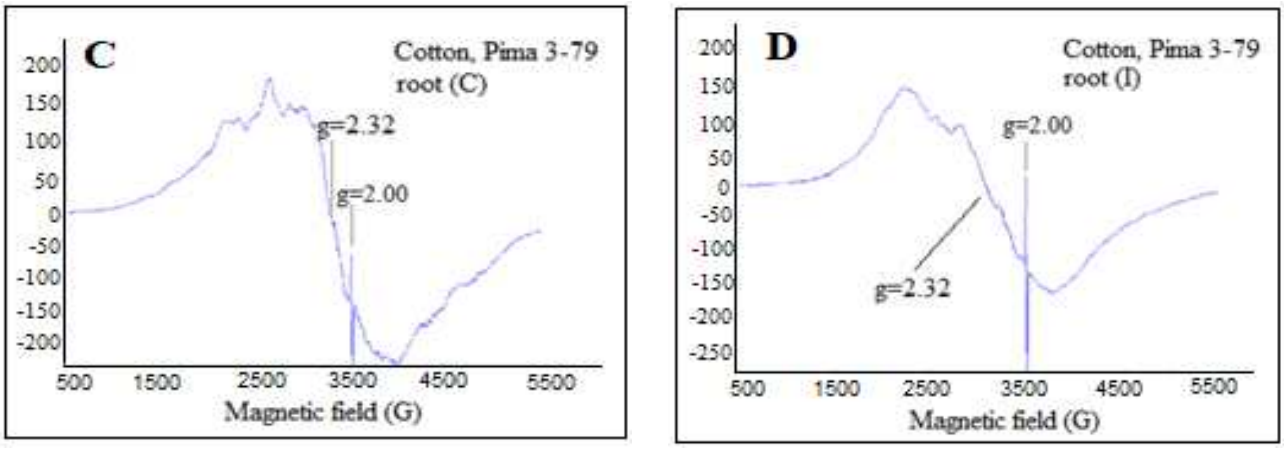


Fig. 5. Leaf and root EPR spectra of the Pima 3-79 genotype.
A-leaves, control; B-leaves, inoculated; C-roots, control; D-roots, inoculated.

EPR spectra of root and leaf samples of cotton belonging to the TM-1 genotype are given in the Fig.6. In TM-1 (patient) samples, an increase in the intensity of the signal characterizing magnetic nanoparticles of iron oxide (G=2,32; δ=400 Qs) was observed (Fig. 6 A, B, C, D).

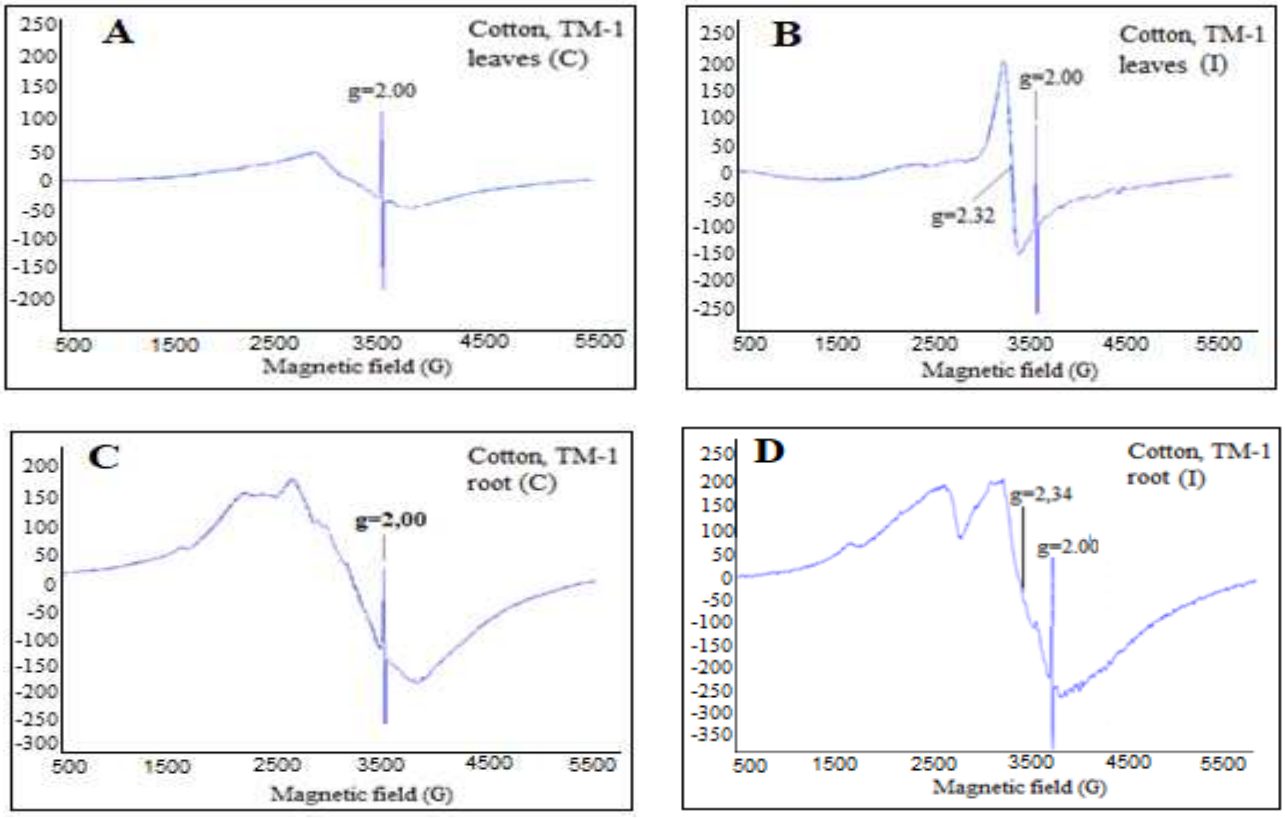


Fig. 6. Leaf and root EPR spectra of the TM-1 genotype.
A-leaves, control; B-leaves, inoculated; C-roots, control; D-roots, inoculated.

A similar result was also recorded in the cotton leaf samples belonging to the TM-1 genotype. That's why, as can be seen from the picture, the intensity of the signal characterizing iron oxide magnetic nanoparticles in the inoculated sample was 5 times higher than the intensity of the signal received from the control sample (Fig. 6 A, B).

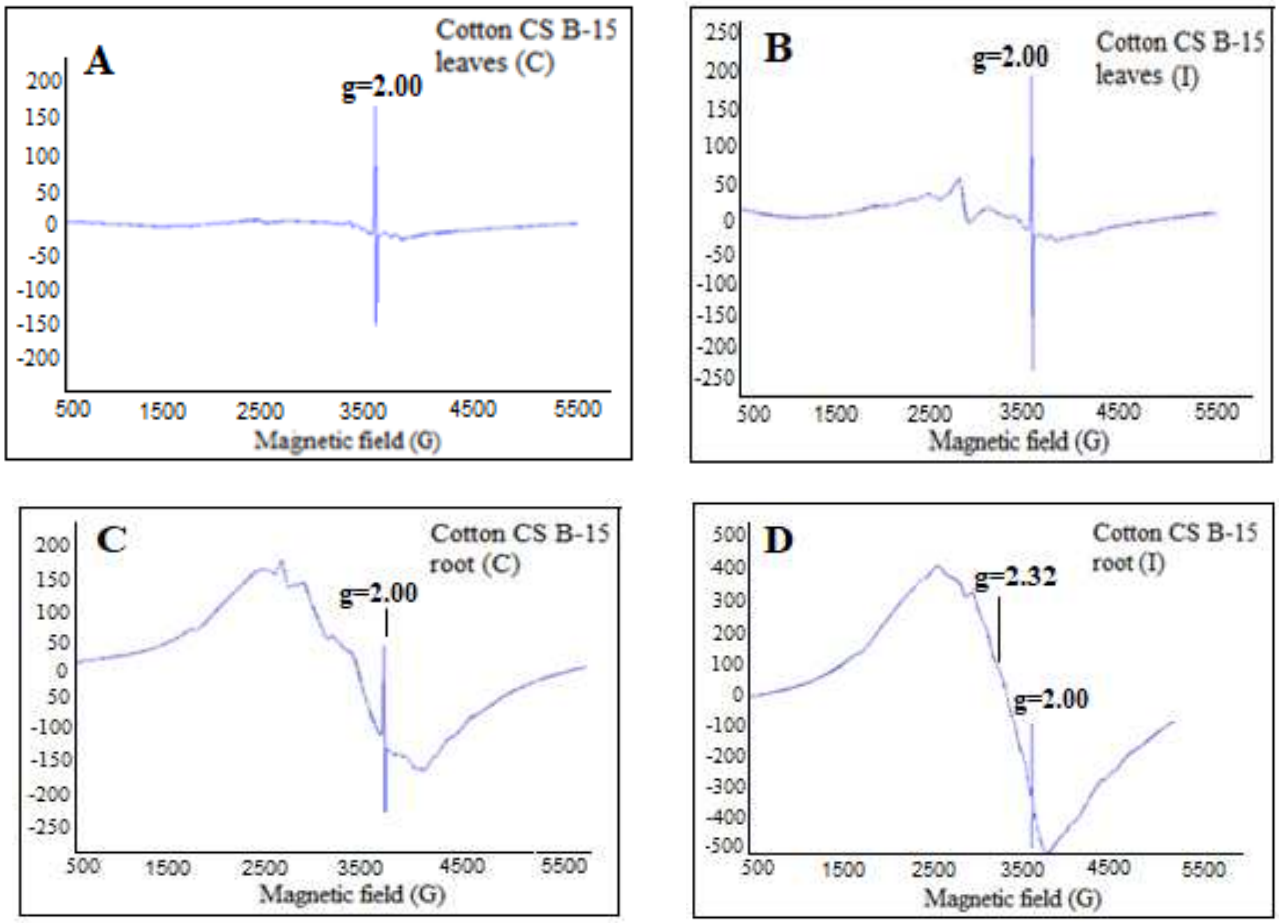
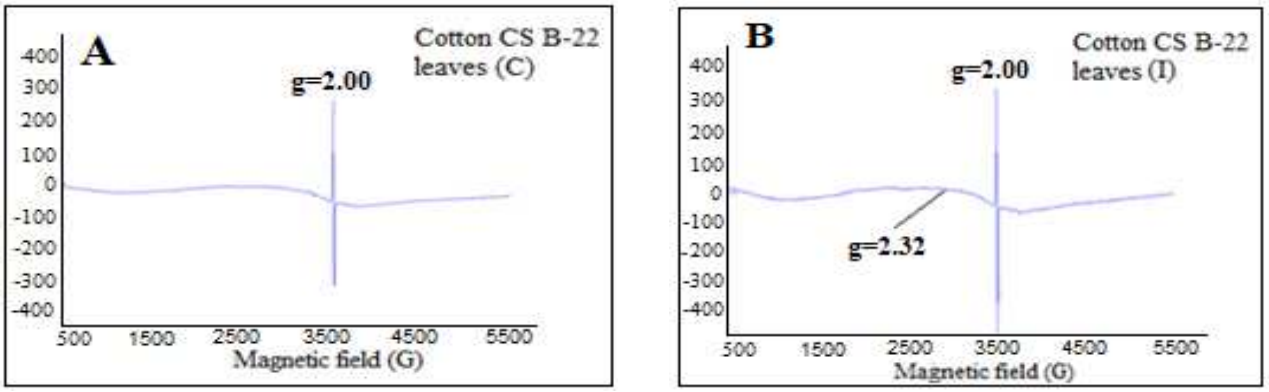


Fig.7. Leaf and root EPR spectra of CS B-15 cotton genotype.
A-leaves, control; B-leaves, inoculated; C-roots, control; D-roots, inoculated

The EPR spectra of root samples of the CS B-15 cotton showed once again that the formation of magnetic nanoparticles in the CS B-15 (inoculated) sample is very intensive compared to the control (Fig.7 A, B, C, D).



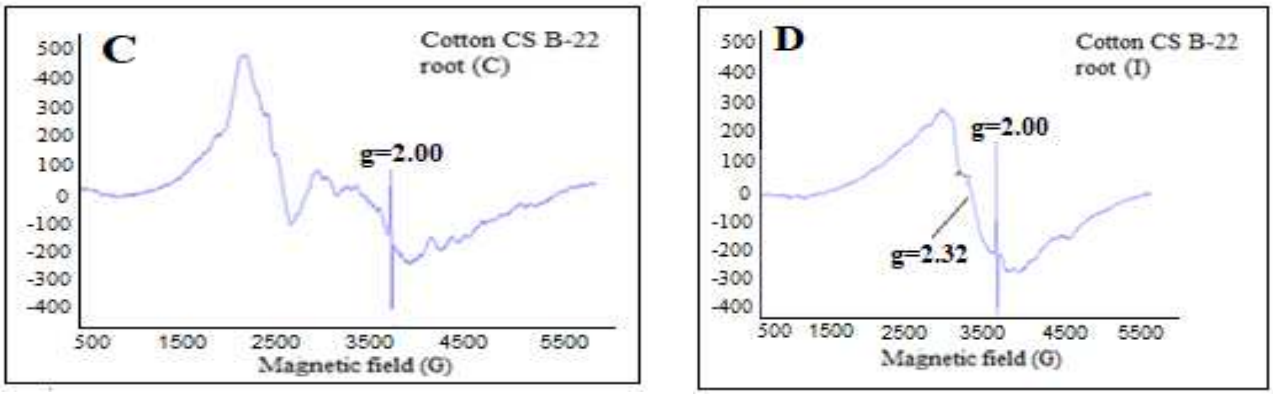


Fig.8. Leaf and root EPR spectra of CS B-22 cotton genotype.
A-leaves, control; B-leaves, inoculated; C-roots, control; D-roots, inoculated

EPR spectra of leaves and roots were also studied comparatively in the case of CS B-22 cotton genotype (Fig. 8 A, B, C, D) . Free radical signals were observed in the spectrum of control and inoculated leaf samples recorded over a wide range (g=2.0023) of magnetic fields. A very weak signal characterizing the magnetic nanoparticles (g = 2.32) was found in the inoculated leaf sample. The study of paramagnetic centers in a wide range of magnetic fields of root samples of cotton genotype CS B-22 showed that in comparison with a control sample, the inoculated sample had a high-intensity signal (g = 2.32) characterizing iron oxide magnetic nanoparticles, along with a free radical signal (g = 2.0023) . This may be due to the formation of a reducing environment as a result of disruption of the electron transport chain in mitochondria as a result of stress in root samples (Nasibova *et al.,* 2016; Nasibova *et al.,* 2021).

DISCUSSION

The results of experiments conducted to study the effects of biotic stress on the components of the cotton defense system are presented above. As is known, *V. dahliae* is a soil-borne vascular fungus (soil vascular fungus) that infects plant roots and affects a wide range of plants - about 400 types of fruits, and vegetables, including cotton plants (Pegg *et al.,* 2002). Under influence of various damaging factors (physical damage, nematode bite), losing their integrity at different points (root tip, lateral root zones) the roots of the plant become susceptible to conidia and microsclerotia of the fungus (Coque *et al.,* 2020). The effect of the pathogen on cotton is due to its ability to cause obvious symptoms on the surface of leaf tissue in the form of necrosis and chlorosis (Fradin *et al.,* 2006).

Based on the vascular browning of cotton stems, Pima, TM-1 and CS B-22 plants showed resistance to the pathogen, while CS B-15 was sensitive to the pathogen. This fact partially revealed the inability of the root system of the CS B-15 genotype to resist the natural infection by pathogen .

The ability of different plant tissues to develop protection against pathogens involves the participation of specific protective enzymes as well as the synthesis of oxygen free radicals which damage and kill directly pathogen (Torres *et al.,* 2006). The presence of mechanisms for transmitting activation signals from the pathogen contact zone throughout the plant cell is also an important component of the pathogen-plant interaction. This signal transmission mechanism involves the ROS molecules, such as H_2O_2, NO and Ca^{2+} ions (Mellersh *et al.,* 2002). The activity of guaiacol peroxidase was increased in Pima 3-79, TM-1, and CS B-15 genotypes. This can be explained by the ability of these genotypes to stimulate an aggressive protective reaction by producing O_2-free radicals, which gives rise to the formation of a H_2O_2, which is then neutralized by peroxidase (Lichtenthaler, 1998). On the contrary, the guaiacol peroxidase activity of CS B-22 decreased, which can be explained by the development of other forms of protection against this pathogen. Due to the absence of any damage of CS B-22 during native experiments in the field, this factor is crucial.

The 1-week pathogen influence has led to an increase in the activity of cytosolic SOD in the Pima, TM-1, and CS B-22 genotypes. This functional manifestation of SOD activity, which was considered the primary boundary in the protection of the cell, could be explained by its localization and the ability to neutralize the more aggressive form of oxygen ($O_2^{\bullet -}$) to a less dangerous one, that is H_2O_2 (Apel *et al.,* 2004). On the contrary, there was a small decrease in the SOD activity in the case of CS B-15. At the same time, the high enzyme activity in the control plants of CS B-15 indicates the native enhanced expression of SOD in this genotype even in the absence of a pathogen. Perhaps that constitutive SOD activity was enough for the evaluation of sufficient fungus defence reaction.

It is believed that in plant protection against biotic factors among enzymes polyphenol oxidase also plays a peculiar function (Xin *et al.,* 2019). Using free oxygen and phenolic compounds, polyphenol oxidases produce quinones (Mayer, 1986). The activity and induction of this enzyme are highest in plants which are more resistant to infection. The effect of this enzyme in this process is twofold. It is assumed that an increase in the concentration of quinones leads to substrate inhibition of polyphenol oxidases, and subsequently to necrosis of the infected tissue and adjacent areas, thereby creating a barrier against pathogens (Linskens *et al.,* 2012). Quinones that have obvious antimicrobial activity also inhibit the development of microorganisms in the affected area and in adjacent tissues (Sommer *et al.,* 1994).

In our experiments, it was found that the impact of the pathogen on the polyphenol oxidase activity in different species, and their genotypes was different. In Pima, TM-1 and CS B-15 genotypes, the activity of this enzyme was positively stimulated under this biotic stress factor. The quinones formed under the action of this enzyme could behave as natural inhibitors of the pathogen (Sommer *et al.,* 1994). On the contrary, CSB-22 demonstrated results distinguishable from other studied genotypes. The introduction of the pathogen into CS B-22 led to a slight decrease in the activity of PPO. However, the CS B-22 genotype was naturally characterized by the high activity of the enzyme. Obviously, the high level of activity could allow this genotype to overcome complications associated with the pathogen by PPO. At the same time, there was no correlative relationship between the level of PPO activity and resistance to infection. For example, in CS B-15 the activity of this enzyme was high, while the infection was observed in field experiments. On the other side, the activity of the tested enzyme was the least in the case of Pima 3-79, whereas this species showed resistance against the pathogen in field experiments. In various previous studies, similar response of PPO to the pathogen inoculation was recorded (Jabeen *et al.,* 2021; Sharf *et al.,* 2021).

It can be assumed that a high level of antioxidant potential plays a certain role in the protection of cotton against a fungus pathogen (Javaid *et al.,* 2021; Shoaib *et al.,* 2021). This could be seen in the CS B-15 cotton genotype, where the level of POX, SOD and PPO activity was high. In the field experiments, this genotype demonstrated a weak pathogen protective reaction which perhaps can be explained by a primary pathogen-host interaction, where the main role is assigned to a more aggressive form of oxygen, which is synthesized by NADPH oxidase and then involves SOD in this process.

It was established that resistance to infection was not associated with genes located on the 15th chromosome, since no resistance to infection was detected for the CS B-15 variety in field experiments, as well as on the basis of data on the activity of antioxidant enzymes, in particular SOD. Chromosome substitution also did not significantly affect on the activity of antioxidant enzymes, in particular SOD.

The study of the influence of stress factors on different plant samples by the EPR method has shown that during stress, new anomalous magnetic properties are formed in them (Nasibova *et al.,* 2021). Our experiments with leaf and stem samples of different cotton genotypes also showed that the biotic stress factor in samples leads to the creation of new paramagnetic centers and nanophase crystal-like iron oxide particles in roots, but not in leaves. The results obtained can be considered very relevant and promising both in terms of environmental assessment and agricultural application.

Conclusion: It is concluded that with the participation of the pathogen, a change in the concentration of free radicals was revealed, which in turn was reflected in a shift in the activity of antioxidant enzymes. all genotypes except CS B-15 showed tolerance to the pathogen. However, the CS B-15 genotype showed low activity of the SOD enzyme, which may also influence on the low resistance of the pathogen. After inoculation, the synthesis of iron nanoparticles was observed in all variants, both in leaf tissue and in roots, which assumes the influence of the pathogen on the synthesis of iron nanoparticles.

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