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PHYSIOLOGICAL AND BIOCHEMICAL ANALYSIS OF ASA-GSH ANTIOXIDANT SYSTEM OF SEA-ISLAND COTTON IN RESPONSE TO *VERTICILLIUM DAHLIAE*

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Abstract

Sea-Island cotton (*Gossypium barbadense*) is a high-quality long-fiber cotton species and is mainly planted in southern region of Xinjiang. The primary disease is Verticillium wilt, which is caused by *V. dahlia* (*Verticillium dahlia*) affecting *G. barbadense* growth and development. That leads to reduction in quality and yield of the fibers and thus to the huge economic loss. AsA-GSH antioxidant system has been extensively studied and is seen as having a crucial function in plant's response to biotic and abiotic stresses. The goal of this study is to look into the resistant mechanism of AsA-GSH antioxidant system in *Gossypium barbadense* in response to *V. dahliae*. The two varieties, wilt-susceptible XH17 and wilt-resistant XH24, were incubated by V991 strain of *V. dahliae* and the treated leaves were collected for the physiological and biochemical analysis of AsA-GSH antioxidant system. The leaves were collected at day 0, 2, 6 and 9 after fungal inoculation and the functions of related enzymes of AsA-GSH antioxidant system including Superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and glutathione re-oxidase (GR), as well as the physiological indexes of malondialdehyde (MDA)

and proline were measured. The mRNA and protein expression levels of the genes of APX, dehydroascorbate reductase (DHAR), monodehydroascorbate reductase (MDHAR), SOD, CAT, and GR, that constitute AsA-GSH antioxidant system, were analysed by the transcriptomic and proteomic data. The enzyme activities of CAT, SOD, GR, and APX were found to be increased significantly after *V. dahliae* treatment in both XH24 and XH17. Interestingly, SOD and APX activities maintained at relative high values in wilt-resistant XH24 but a decrease in values was observed during the late stage of *V. dahliae* treatment in case of wilt-susceptible XH17. High proline accumulation and low MDA content were observed in wilt-resistant XH24. The results of AsA-GSH antioxidant system genes shows that four APX members of GbAPX1A, GbAPX1D, GbAPX10D, and GbAPX12A, one DHAR member GbDHAR2D, four MDHAR members of GbMDHAR1A, GbMDHAR1D, GbMDHAR3A, and GbMDHAR3D, two GR members of GbGR2A and GbGR2D, two CAT members of GbCAT1A and GbCAT1D, and six SOD members of GbCSD4A, GbCSD5A, GbCSD4D, GbCSD5D, GbMSD1D and GbMSD1A were all expressed induced accumulations significantly after *V. dahliae* treatment, implying their important potential functions for *G. barbadense* to resist *V. dahliae*. Generally, the protein expression and mRNA expression indicated a similar profile, while some unique expressions of these AsA-GSH antioxidant system genes were also been discovered. There were ten APX members of GbAPX1D, GbAPX2A, GbAPX2D, GbAPX3A, GbAPX3D, GbAPX6A, GbAPX6D, GbAPX8A, GbAPX12A, and GbAPX3D; all six DHAR members with the exception of GbDHAR2D; three GR members locating in A sub-genome containing GbGR 1A, GbGR 2A, and GbGR 3A; six MDAHR members including GbMDAHR1A, GbMDAHR1D, GbMDAHR3A, GbMDAHR3D, GbMDAHR4A, and GbMDAHR4D; two CAT members of GbCAT1A and GbCAT4D; as well as eight SOD members of GbCSD1A, GbCSD4A, GbCSD4D, GbCSD2A, GbMSD2D, GbMSD2D, GbMSD1A and GbMSD1D, to indicate steady high expressions. These results indicated that there exist some close link and consistency between the mRNA and protein expressions, and that the preferentially expressed proteins of AsA-GSH antioxidant system might perform important functions as enzymes to catalyze

the oxidation/reduction reactions and thus to maintain the redox balance and integrity of the cells in the process of *G. barbadense* plants to resist *V. dahliae*.

Keywords: ASA-GSH; *Verticillium dahlia*; Sea-Island Cotton; Antioxidant; Enzyme activities; Protein expression.

INTRODUCTION

V. dahliae is among the gravest vascular fungal diseases in cotton cultivation at present worldwide and is also called cancer of cotton. In 1914, *V. dahliae* was initially reported in the United States (U.S) and later it spread to many other cotton producing countries of the world. *V. dahliae* is a rod shaped fungus with a wide host range and it can infect about 660 species of plants including 184 species of crops (Bhat & Subbarao, 1999). Gramineae crops such as rice, wheat, corn, and sorghum are generally not harmed (Fradin & Thomma, 2006). It is an important obstacle to the growth of cotton and the sustainable development of cotton production in China as well as in the other parts of the world (Zhang *et al*., 2012). Numerous cotton-producing regions around the world experience enormous losses every year. According to a recent analysis on disease-related crop loss in the United States, between 1940 and 2014, there was a loss of about 480 million bales (Lawrence at al., 2016). While *V. dahliae* has infected more than 40% of the cotton-growing regions in China, causing direct economic losses of $250–310 million dollars (Wang *et al*., 2016; Gong *et al*., 2017). The soil-borne pathogen *V. dahliae*, which can infect cotton and many other plants, is a pathogenic fungus that can seriously harm agricultural production. One of the significant issues that must be resolved quickly in agricultural productivity is the study and treatment of this illness.

In recent years due to the changes in many factors such as climate regulation, promotion of disease resistant varieties cultivation system and conditions, the pathogen of *V. dahliae* has accumulated in a large amount in the soil further causing a sharp increase in *V. dahlia* infections. Although in recent years, with the breeding and planting of disease resistant varieties and the reasonable application of related cultivation measures, *V. dahliae* has been controlled to some extent, however, due to the frequent introduction in different places continuous cropping in cotton fields, differentiation of pathogenicity of pathogen and other factors, *V. dahliae* infects various cotton planting areas in China to varying degrees every year.

The pathogenic mechanism and pathogenesis of *V. dahliae* in cotton are very complicated, the terms "blockage theory" and "toxin theory" are generally the most discussed theories. Blockage

theory generally believes that the cause of cotton wilting after *V. dahliae* infection is due to the fact that *V. dahliae* colonizes in the vascular bundle and proliferates in large quantities, infecting the inside of cotton thus causing adjacent cells to produce secretory jelly and part of infiltrates. As a result the water and nutrition transportation of the plant cannot be transported to the whole plant after the catheter is blocked further leading to the wilting and yellowing of the plant (Yang *et al*., 2020). Street *et al* found that after tomato was infected with *V. dahlia,* the symptoms of leaf infection were positively correlated with the degree of xylem blockage (Street & Copper, 1984). Others believe that the wilting of plants caused by *V. dahliae* is due to the invasion of bacteria into the vascular bundle and the secretion of pectinase to brown the vascular bundle and further hydrolyse the middle layer and pectin of the vascular bundle of plants thus affecting the transportation of water. Cantu studied the physiological and biochemical characteristics of toxin secreted by *V. dahliae* and its toxicity to cotton microtubule system, and found that toxin secretion from *V. dahliae* could cause blockage of cotton vascular system, this further indicates that catheter blockage is probably a defensive reaction of cotton when it resists toxins (Cantu *et al*., 2008).

Cottons own tissue structure is different and directly leads to its differential resistance to *V. dahliae* infection, owing the mechanism directly related to vascular bundle. Studies have found that Egyptian cotton and U.S disease resistant cotton varieties almost are immune to *V. dahliae* and have solid xylem along with multiple rows of pith lines with a large amount of starch storage. On comparing the tissue structure, varieties of susceptible cotton have small xylem gaps and thick cell walls (Cantu *et al*., 2008).

When cotton gets infected with pathogen, cell wall gets thick, cells are closely arranged and produce lignification of cell walls. These changes play role in resistance to *V. dahliae* infection in cotton. At the same time, the infection of *V. dahliae* also thickens and compacts the xylogenesis around the vascular tissue thus making its internal tissue structure exhibit a high degree of lignification phenomenon to prevent further invasion of pathogenic bacteria. Lignification can not only strengthen the toughness of cell walls but also accumulates lignin in cells. Through the analysis of RNA-seq expression profile it was found that the accumulation of lignin in resistant varieties was significantly higher than that in susceptible varieties when both resistant and susceptible cotton varieties were inoculated with *V. dahliae* simultaneously (Xu *et al*., 2011).

Although many studies in other plants have found resistance resources to *V. dahliae* (Bailey *et al*., 2006; Shilei, 1995), the limitation of resistance genes to *V. dahliae* is still limited. Studies have found that the presence of *V. dahliae* resistance gene Vel, encoding extracellular leucine rich

receptor-like proteins, can improve the resistance of tomato plants to *V. dahlia*. This resistance gene can increase tomato plants' resistance to *V. dahliae* by promoting the production of PR and SA regulatory pathway genes. Also, Vel homologous gene mVel is related to *V. dahliae* resistance in other plants (Fradin & Thomma, 2006). It has also been reported that the plant induced gene silencing (PTGS19) plays a significant part in the defence mechanism to *V. dahliae* in *Arabidopsis thaliana* and mutant phenotype of gene silencing related genes is more sensitive to *V. dahliae* than wild type (Ellendorff *et al*., 2009).

MATERIAL AND METHODS

Screening of Sea-Island cotton susceptible and resistant varieties

Six different varieties of Sea-Island cotton, Xinhai 17, Xinhai 22, Xinhai 24, Xinhai 36, Xinhai 41 and Xinhai 44, were tested. The V991 strain of *Verticillium dahliae*, which was preserved by our laboratory China, Xinjiang, Shihezi University, was selected as the experimental pathogen.

Experimental reagents

The non-defoliating fungal strain V991 was activated in Potato Dextrose Agar (PDA) medium and expanded with Chas medium. 200g of potatoes were peeled and chopped boiled to obtain potato juice. 20g of sucrose and 15-20g of agar were added to 1000mL of water and solution was sterilized at 121℃. Sodium nitrate (3g), Dipotassium phosphate (1g), Magnesium sulfate ($MgSO_4.7H_2O$), Potassium chloride (0.5g), Iron sulfate (0.01 g), Sucrose (30g), were poured in water (1000mL solution) and the solution was heated and sterilized at 121℃ for 20 min. Hoagland nutrients solution was used (Akram & Rehman, 2018).

For EDTA Ferric salt solution (500mL) firstly, EDTA solution was prepared by adding 3.37g EDTA-Na_2 to 300mL of water and after dissolving volume was made 500mL by adding water. Then $FeSO_4 \cdot 7H_2O$ was added to the solution with EDTA-Na_2 while stirring. The solution was stored in dark flask at low temperature.

Experimental methods

Fungal strain and inoculum preparation

V991 was activated on potato dextrose agar and cultivated in liquid Czapek medium (1g K_2HPO_4, 2g $NaNO_3$, 1g KCl, 1g $MgSO_4.7H_2O$, 30g L-1sucrose, and 2mg $FeSO_4.7H_2O$), a non-defoliating isolate of *V. dahliae* at 25℃ for one week in a vibration incubator at the speed of 160rpm. To keep

the mycelia, the resulting fungal cultures were filtered through sterile gauze. A conidial suspension (1x107/ml) was made for root inoculations using distilled water.

Plant Growth and Pathogen Inoculation

Seedlings of the *G. barbadense* cultivar Xinhai 24 (XH24) and Xinhai 17 (XH17) inside the greenhouse conditions were grown in chambers. First of all, Sea-Island cotton seeds were washed with distilled water three times, soaked with alcohol (70%) for 5 min and then with hydrogen peroxide (5%) for 2 hours to get disinfections. Vermiculite, perlite and nutrient soil in 1:1:3 ratios were added to cultivation pots. Each pot contained four seeds to allow proper germination. All the culture pots were kept in a tray with proper water supply. Twelve pots were used for each variety of cotton and every pot contained four seedlings of cotton. Replant was done to ensure the number of plants in every culture basin. At the 2-4 leaf stage, seedlings of each cotton variety (XH24, XH17) were inoculated with 10mL of a liquid containing 1107 spores per pot for the purpose of inoculation with *V. dahliae* (Zhou, 2012).

Biochemical tests

Verticillium wilt (VW) resistance-related protective enzymes, including as CAT and SOD, were chosen to study activity variations during the early stages of V991 infection at 0, 2, 6 and 9 days after inoculation (DAI),. Fresh leaves (0.1g) per sample were ground in liquid nitrogen and suspended in 0.9mL of phosphate buffer. The homogenate tissues of CAT sample was centrifuged at 2500rpm for 10 min, whereas SOD samples were centrifuged for 15 min at 3500rpm. CAT and SOD enzyme activity were assessed in both supernatants using commercially available kits from Tiangen, Biotech, Beijing, China, and their spectrophotometric measurements were done at 240, 470 and 560nm.

At 0, 2, 6, and 9 DAI, the defence enzymes GR and APX, which are connected to VW resistance, were tested for activity. Fresh leaves (0.1g) were crushed in liquid nitrogen and then placed in a 0.9mL extraction solution over ice for each sample. The GR sample was centrifuged at 4°C at 8000rpm for 10 min, while the tissue homogenate from the APX sample was done so at 4°C and 10,000rpm. Spectrophotometric measurements at 290 and 340nm, respectively, were taken after the resulting supernatants had been further treated in accordance with the directions of the APX and GR assay kits from Tiangen Biotech, Beijing, China. Using biochemical substances relevant to VW resistance, such as MDA and Proline, and fresh leaves (0.1g), which were crushed in liquid nitrogen, the content variations during the fungal strain V991 infection process were assessed.

Analysis of Sea-island cotton root transcriptome following V. dahliae infection

After inoculation of these two varieties of Sea-Island cotton with *V. dahliae*, the roots were collected at three time intervals of 0 hours, 6 hours and 24 hours for RNA and protein extraction. RNA from roots of the selected varieties of infected cotton was extracted using Polysaccharide Polyphenol RNA Extraction Kit (DP441) and BPP phenol extraction protocol was followed for protein extraction. Extracted RNA samples were sent for transcriptome sequencing and the extracted protein samples were sent to the mass spectrum platform for proteome mass spectrum (qualitative and quantitative) analysis.

Extraction of total RNA and protein from roots of G. barbadense

100 cleaned seeds of XH24 and XH17 each were selected and transferred to a mixed nutrition bowl (the ratio of nutrient soil to vermiculite is 2:1) with high temperature disaster bacteria and place them in an illumination incubator at (30℃, 14 h/25℃ light and 10h darkness, with 65% relative humidity) for cultivation with appropriate amount of water. As soon as the two cotyledons of the Sea Island cotton seedlings grew to be fully expanded, the seedlings were taken out of the soil and transferred to Hoagland nutrient solution for hydroponic culture (30°C, 16 h/25℃ light, 8h darkness, 65% relative humidity). Pathogenic fungal strain (V991) was activated in potato culture medium and placed in Cha's liquid medium at 25℃ and 160rpm for 5-7 days. After 5-7 days of culture, the resultant fungal cultures were filtered with four layers of sterile gauze counting with a blood cell counting plate, the spore suspension were diluted until the concentration of fungal spores got up to $1x10^6$ cfu/mL. Inoculation of the selected varieties of Sea Island cotton was done right after growth of 3-4 true leaves. During inoculation healthy seedlings, with consistent growth, were selected. The Sea Island cotton seedlings were carefully taken out and placed in a clean tray, the roots were abraded and the treated seedlings were placed in spore liquid with adjusted spore concentration for inoculation. Root samples of XH24 and XH17 were collected after 0 hours, 6 hours and 24 hours of inoculation, and for additional analyses, they were kept at -80°C and frozen in liquid nitrogen.

The RNA extraction kit DP441 was used to extract the RNA from 1g of ground-up root tissue under liquid nitrogen. Quality of extracted RNA was analysed by Agarose Gel Electrophoresis. Following quality check, extracted RNA samples were sent to Bio-Tech Co.Ltd for transcriptome sequencing analysis. BPP phenol extraction method was used to extract the protein.

RESULTS

Detection results of physiological indexes related to antioxidant system in resistant and susceptible varieties of sea-island cotton

Variety selection

Island cotton infection yellow withering disease index statistics results of six different island cotton varieties, XH17, XH22, XH41, XH44, XH36, and XH24, were used to screen out disease-resistant and disease-sensitive cotton varieties (Table 1). By observing the incidence of island cotton, one month after the infection of Dali rote bacteria, the disease index was calculated, and varieties were categorized from heavy to light. XH17 was the most severely infected in sixth islands, while XH24 had the least incidence in several cotton types (Table 1). Therefore, XH17 was selected as the disease-sensitive variety, while the XH24 was selected as disease-resistant variety of Sea-Island cotton for subsequent experimental analyses.

Table 1: Disease Index (DI) of V991 infection on six sea-island cotton varieties.

Varieties	**Disease degree**				
	Grade= 0(%)	**Grade= 1(%)**	**Grade= 2(%)**	**Grade= 3(%)**	**Grade= 4(%)**
XH17	0	0	9	23	68
XH22	0	0	0	17	83
XH41	0	0	18	26	56
XH44	0	1	34	16	49
XH36	0	10	29	10	51
XH24	10	74	14	2	0

Note: National standards for disease index (DI) or disease grade: Grade 0 indicates that Cotton seedlings were heal cotyledons and true leaves were not infected; Grade 1 indicates that cotyledons showed symptoms such as macular d or dehydration while true leaves showed no symptoms; Grade 2 indicates the disease of cotyledon and 1 true le indicates that all the leaves showed signs of disease; Grade 4 indicates that all the leaves showed severe symptoms, abscission, apical die or even plant death.

Four is the highest Disease degree if the whole plant dies and zero is the lowest Disease degree with no visible symptoms. Pathogenicity evaluation of the disease incidence level 15 days after inoculation and the number of cotton seedlings at that disease level were recorded respectively according to the investigation results the index of disease was calculated and the formula is as follows: DI = [(R disease grades x number of infected plants)/ (total checked plants x 4)] x 100). Finally, the disease index of each cotton variety was counted and detected the disease resistance of Sea-Island cotton.

The left picture shows the phenotype of XH24 and XH17 before infection, while the right picture shows the phenotype observation of XH24 and XH17 after infection with *V. dahliae*. Further observation, on the phenotype of Sea Island cotton one month after infection with *V. dahlia*, showed that the infection symptom of XH24 were the mildest, with wilting and yellowing leaves in a small number, while XH17 got infected severely with *V. dahlia* i.e., almost all leaves of the plant got wilted and yellow (Figure 1). For further experiments in this study, XH24 was selected as a resistant cultivar, while XH17 as a sensitive as per their response to *V. dahliae*.

Figure 1: Phenotype of the resistance and sensitive variety after V991 infection.

Determinations of antioxidant enzymes

Catalase enzyme assay

CAT (EC 1.11.1.6) is the most important H_2O_2 scavenging enzyme in plants, microorganisms, animals, and cultured cells. The accumulation of H_2O_2 is harmful to plant body, so CAT is the main H_2O_2 removing enzyme and also has a significant impact on the scavenging of ROS species. The CAT activity was determined using the change in absorbance rate. In our investigations, the activity of CAT was determined at 240nm since H_2O_2 exhibits a distinctive absorption peak at that wavelength. Figure 2 showed that the CAT activity of XH24 cultivars of Sea-Island cotton were significantly increased at 0, 2, 6 and 9 days of fungal infection (*V. dahliae* V991 strain), while the CAT activity of XH17, which are susceptible to *V. dahliae*, decreased significantly with the increase of infection time.

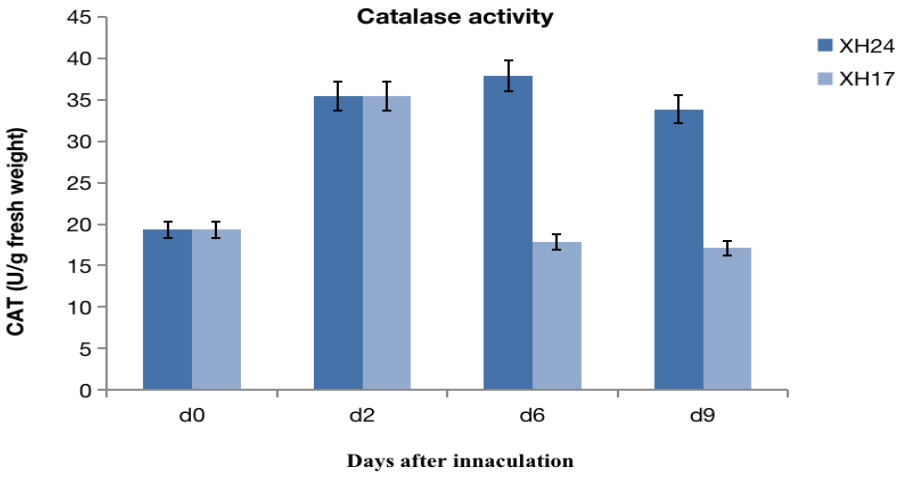


Figure 2: Analysis of CAT activity of XH24 and XH17 after *V. dahliae* infection.

Glutathione reductase assay

GR is a flavoprotein oxidoreductase widely existing in eukaryotes and prokaryotes. One of the important enzymes in the glutathione redox cycle, GR, catalyzes the conversion of GSSG to GSH. In order to maintain the GSH/GSSG ratio, GR catalyzes the reduction of GSSG to GSH from NADPH. In the oxidative stress reaction, GR in vivo scavenges active oxygen to a large extent. In addition, GR also participates in ascorbic acid 1-glutathione cycle pathway. GR can catalyze NADPH to reduce GSSG and regenerate GSH. Meanwhile, NADPH is dehydrogenated to generate $NADP^+$: NADPH has a characteristic absorption peak at 340nm, while NADP has no absorption peak at this wavelength: determine the rate of NADPH dehydrogenation by measuring the rate of absorbance decline at 340nm, so as to calculate GR activity. Glutathione reductase (GR) is an important antioxidant enzyme. Figure 3 shows that the activity of GR in XH24 increased gradually at day 0, 2 and 6, after treatment, while that of XH17 were significantly decreased in all four time intervals.

Superoxide dismutase activity measurement

SOD (EC 1.15.1.1) is a type of metal enzyme found in many different types of organisms. It is a crucial oxygen self-maintaining scavenger that has the ability to catalyse the superoxide anion's oxidation to produce H2O2 and O2. SOD is a key enzyme in the biological antioxidant system since it not only scavenges superoxide anion but also produces the majority of H2O2.For detection of SOD activity, the selected sea island X24(resistant), and X17 (susceptible) cotton varieties were tested at day 0, 2, 6 and 9 after infection of *V. dahliae* strain V991.

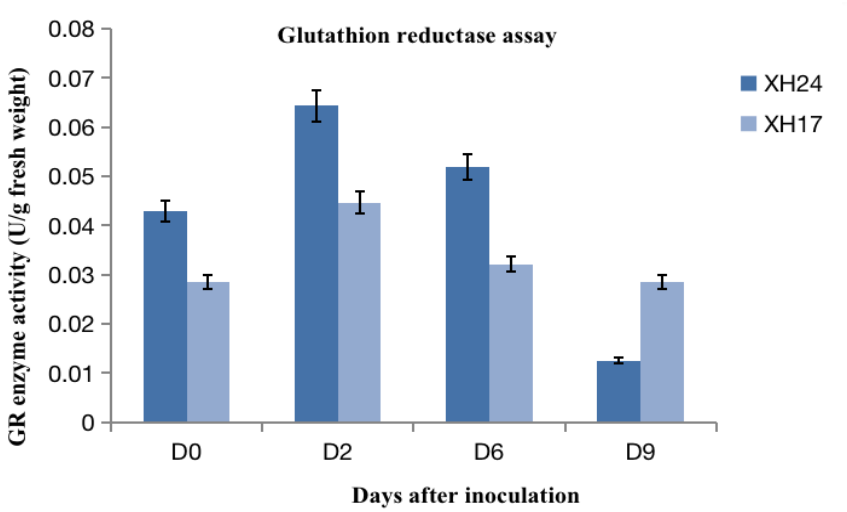


Figure 3: Analysis of GR activity of XH24 and XH17 after *V. dahliae* infection.

Figure 4 showed that the activity of SOD in XH24 rose noticeably as infection duration increased. However, the XH17 SOD activity, which are susceptible to V. dahliae, decreased significantly with the time.

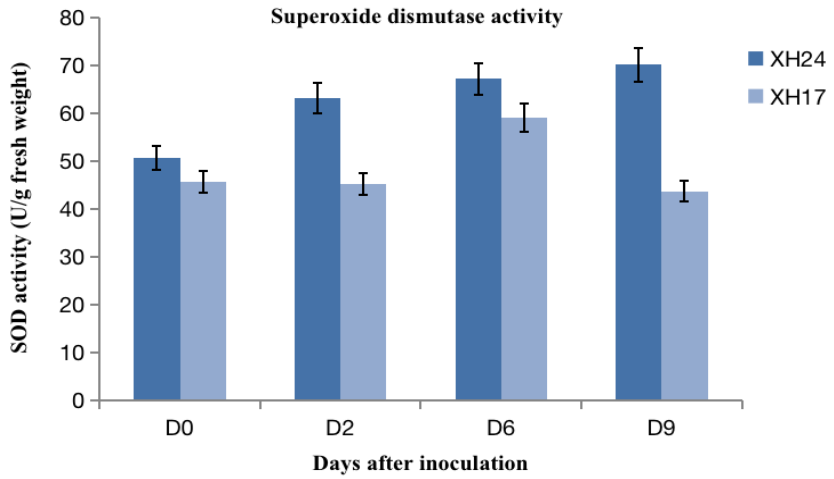


Figure 4: Analysis of SOD activity of XH24 and XH17 after *V. dahliae* infection.

Ascorbate peroxidase detection

One of the most important and necessary antioxidant enzymes is APX, for scavenging active oxygen in plants, and also one of the main crucial enzymes for metabolism of ascorbic acid. Similarly, we detected the APX of the selected Sea Island cotton varieties at 0, 2, 6 and 9 days

after *V. dahliae* infection. The APX activity of XH24 which are resistive to *V. dahliae* increased significantly, while the APX activity of XH17 (Figure 5), which are susceptible to *V. dahliae* showed significantly decreased with the extension of the infection time.

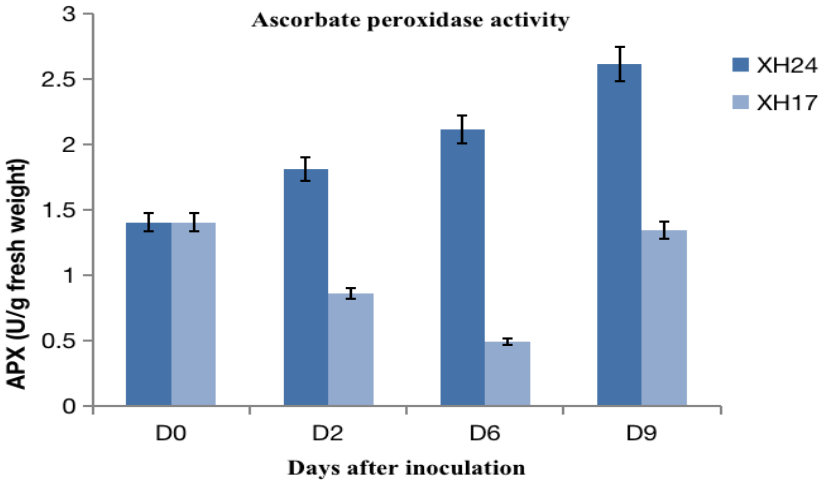


Figure 5: Analysis of APX activity of XH24 and XH17 after *V. dahliae* infection.

Permeable substance index detection

Proline content assay

Proline (PRO) is widely found in plants, animals, cultured cells and in microorganisms. Under adverse conditions, the content of PRO in plants increased significantly. To some extent, the increase of PRO reflects the resistance to adversity. The varieties with strong early resistance tend to accumulate more Proline. Therefore, Proline increase can be used as one of the physiological indexes of stress resistant breeding. PRO was extracted with (SA), the absorbance was measured at 520nm. After the infection of *V. dahliae*, the content of pro in XH24 gradually increased and reached the maximum concentration on the sixth day after the inoculation, and decreased on the ninth day. The content of pro in XH17 gradually decreased with the time of infection (Figure 6).

Malondialdehyde (MDA) assay

Lipid peroxide is created when oxygen free radicals react with the unsaturated fatty acids in lipids. Lipid peroxide eventually breaks down into a number of complicated chemicals, including malondialdehyde MDA.As shown in the Figure 7, the content of MDA in resistant and susceptible cultivars increased from 0 to 6 days, and decreased at 9th day.

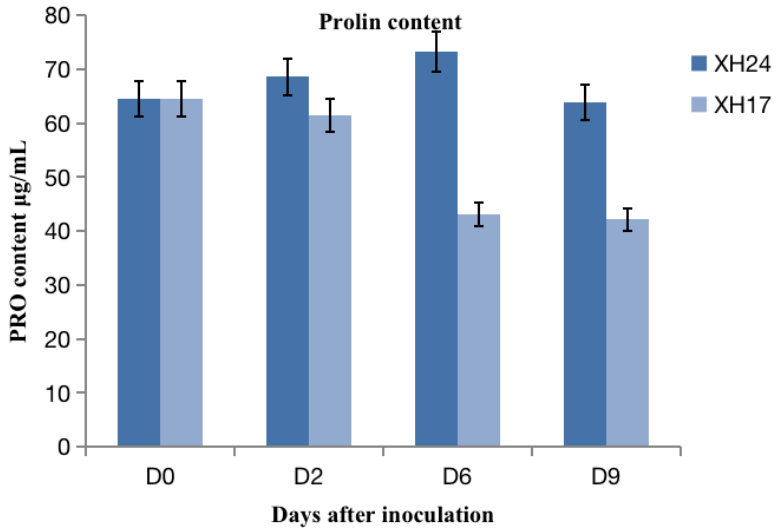


Figure 6: Analysis of pro content of XH24 and XH17 after *V. dahliae* infection.

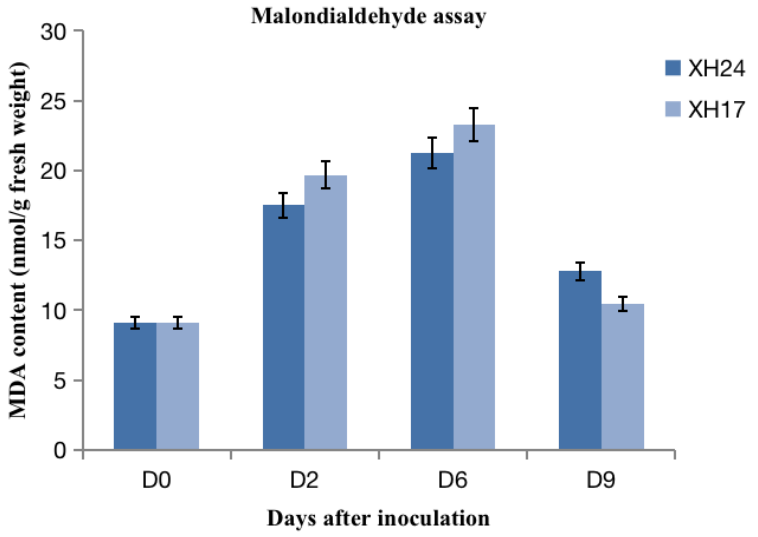
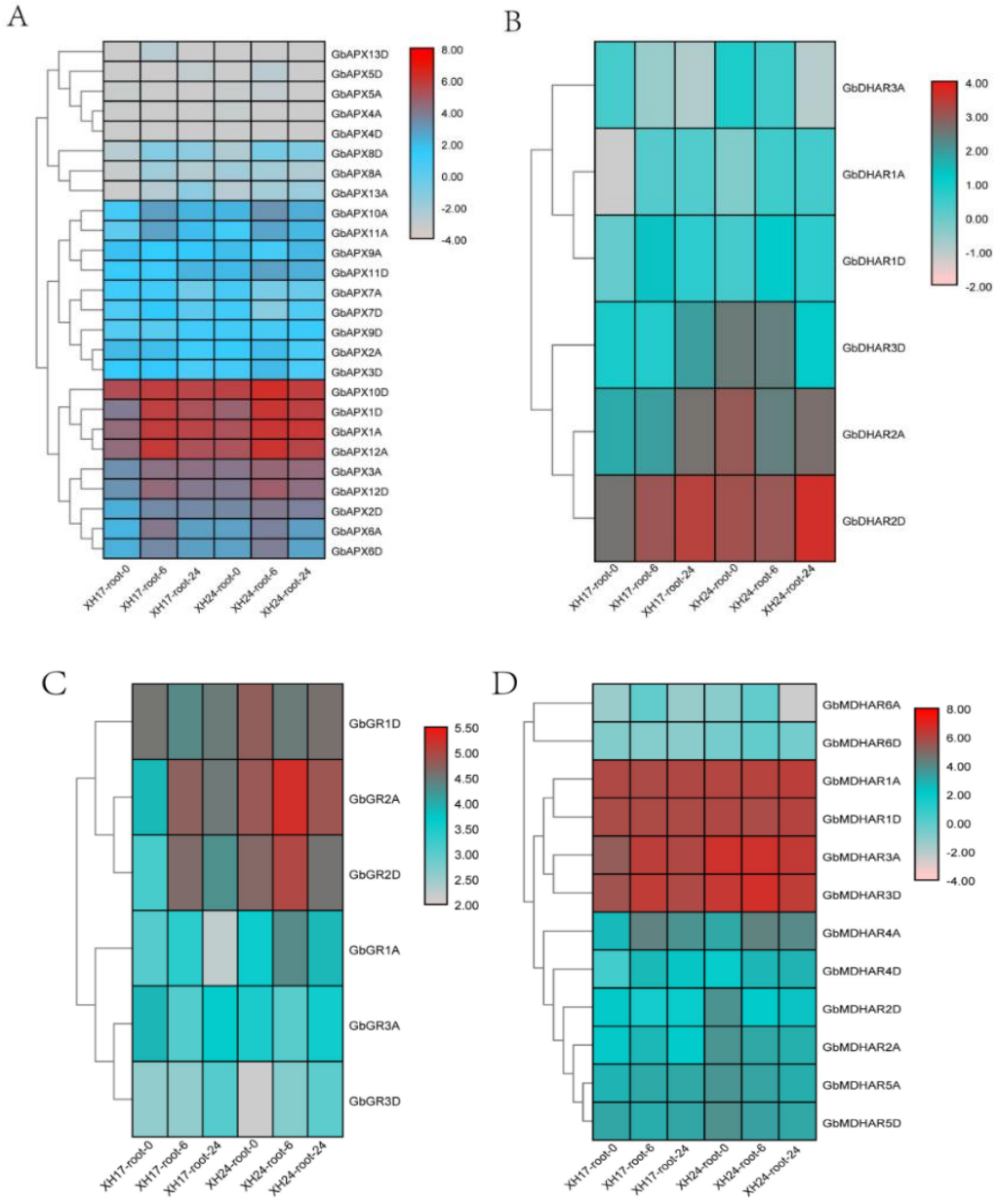


Figure 7: Analysis of MDA content of XH24 and XH17 after *V. dahliae* infection.

Transcriptional level analysis of antioxidant system genes

A total of twenty-six *GbAPX* members were identified in Sea Island cotton. The members expressed in the roots of cotton infected by *V. dahlia* are *GbAPX1A*, *GbAPX1D*, *GbAPX12A*, *GbAPX12D*, *GbAPX2D*, *GbAPX3A*, *GbAPX6A*, *GbAPX6D*, *GbAPX10A*, *GbAPX10D*, *GbAPX11A*, and *GbAPX11D* (Figure 8A). Six *GbDHAR* were identified in Sea Island cotton

(Figure 8B), twelve *GbMDHAR* (Figure 8D) and six *GbGR* (Figure 8C) genes. After inoculation with *V. dahlia*, *GbDHAR3A* and *GbDHAR3D* kept low expression profile; *GbMDHAR1A*, *GbMDHAR1D*, *GbMDHAR3A*, *GbMDHAR3D* were with high expression profile, and *GbGR1A*, *GbGR1D*, *GbGR2A*, *GbGR2D* were also with high expression profile (Figure 8C, D). Meanwhile, the expression levels of *GbCAT1A*, *GbCAT1D*, *GbCAT3A* and *GbCAT4D* genes in the CAT family were significantly increased after infection with *V. dahlia* (Figure 8E). The SOD family members: *GbCSD4A*, *GbCSD5A*, *GbCSD4D*, *GbMSD1D* and *GbMSD41A*, all showed significant changes in expression following the infection (Figure 8F). Alterations in regulating gene expression in ascorbic acid synthesis pathway indicate that these genes in AsA-GSH synthesis pathway may participate in stress response of Sea Island cotton after infection by *V. dahliae*.



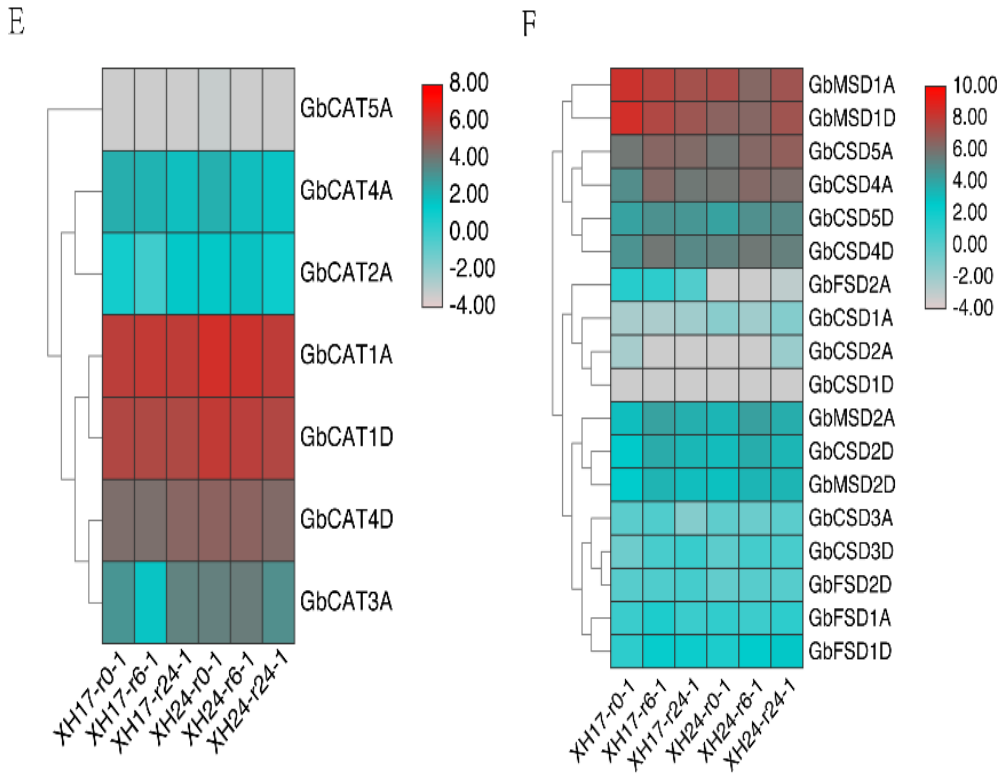
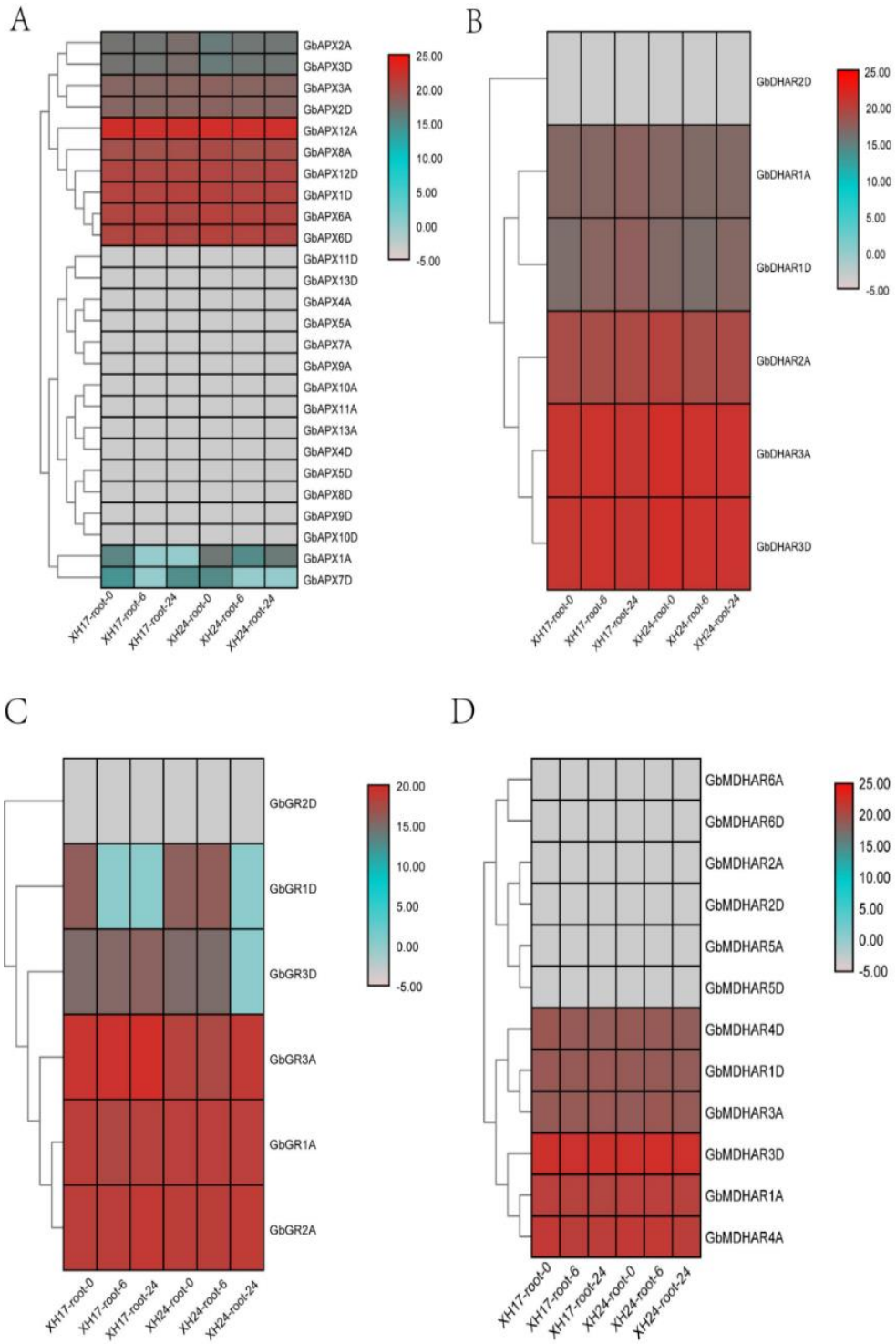


Figure 8: Expression analysis of *APX, DHAR, MDHAR, GR, CAT* and *SOD* family genes at the transcription level.

Protein level analysis of antioxidant system enzymes

Among the 26 GbAPX protein members of Sea Island cotton, GBAPX2A, GBAPX3D, GBAPX3A, GBAPX2D, GBAPX3A, GBAPX12A, GBAPX12D, GBAPX8A, GBAPX1D, GBAPX6A, GBAPX6D, GBAPX1A, and GbAPX7D were expressed in the roots after infection by *V. dahliae* (Figure 9A). Among the GbDHAR family, GbDHAR1A, GbDHAR1D, GbDHAR1D2A, GbDHAR1D3A, and GbDHAR3D were found to be expressed in in Sea Island cotton (Figure 9B). The five GbGR proteins GbGR1D, GbGR3D, GbGR3A, GbGR1A and GbGR2A were expressed in the root after infection by *V. dahliae* and showed higher expression levels (Figure 9C). GbMDHAR4D, GbMDHAR1D, GbMDHAR3A, GbMDHAR3D, GbMDHAR1A, and GbMDHAR4A, the members of GbMDHAR family, were all highly expressed (Figure 9D). The expression levels of GbCAT1A and GbCAT4D proteins of the CAT family changed significantly after *V. dahliae* infection (Figure 9E). SOD family members, GbCSD1A, GbCSD4A, GbCSD4D, GbCSD2A, GbMSD2D, GbMSD2D, GbMSD1A and GbMSD1D, also showed significant changes after *V. dahliae* infection (Figure 9F). The expression results of genes regulating protein expression in ascorbic acid synthesis pathway are consistent

which further indicates that these genes and proteins in AsA-GSH synthesis pathway are likely to participate in stress response of Sea Island cotton after being infected by *V. dahliae*.



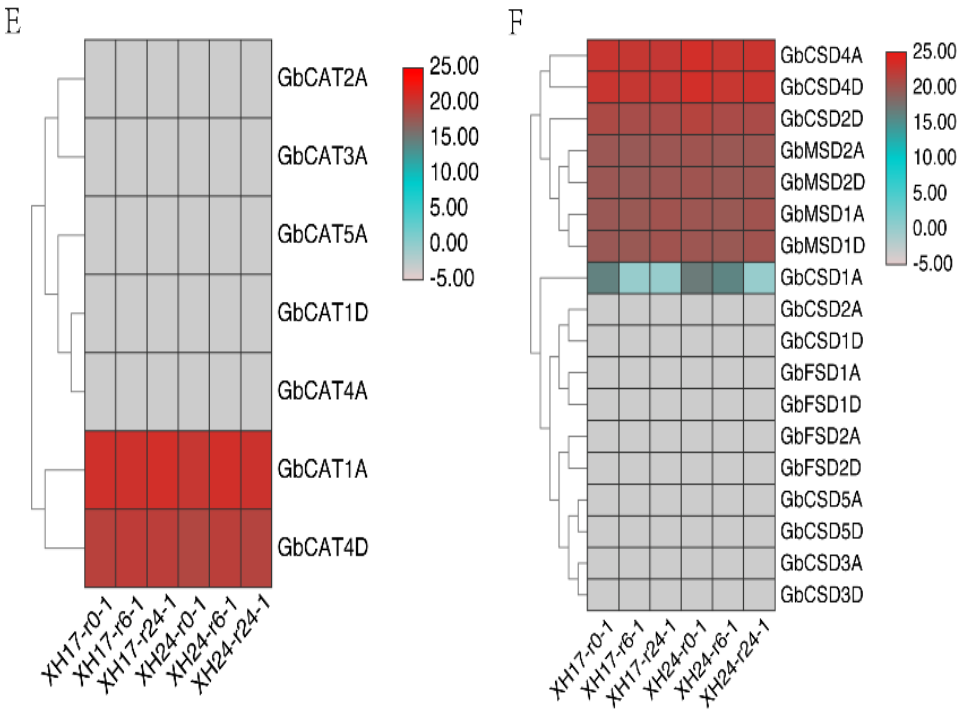


Figure 9: Expression analysis of *APX, DHAR, MDHAR, GR, CAT* and *SOD* family genes at the protein level.

DISCUSSION

Different kinds of antioxidant enzymes generally showed increased or maintained expression levels of Sea-Island (*Gossypium barbadense*) cotton in response to *V. dahliae* pathogen, also the decreased activities of certain enzymes were also observed in some of the samples. Summarizing the results, the enzyme activities such as those of CAT, GR, SOD and APX, in resistant cultivars XH24 increased significantly, while in case of XH17 decreased gradually. In this study, candidate *V. dahliae* resistant and susceptible *G. barbadense* varieties were screened and the transcriptome and proteomic analyses of in response to *V. dahliae* infection in the roots of *G. barbadense* cotton were detected after fungal inoculation in the two different cotton varieties. The pathway enrichment was screened through the all transcriptome and proteomics analyses showed that cell well related candidate genes might be involved in *G. barbadense* resistance to *V. dahliae*. Silencing the candidate genes in *G. barbadense* resistant varieties, found that the transgenic cotton has significantly show low resistance to *V. dahliae*, which providing a certain theoretical and candidate target genes for the application research of *G. barbadense* in against *V. dahliae*.

In plants, the antioxidant system has a significant impact on disease resistance function, which will contribute to the further study of disease resistance mechanism of Sea-Island cotton. This work

provides a theoretical basis for further studying the resistance mechanism of *G. barbadense* after *V. dahliae* infection and also provide the reference for further cultivation of disease resistant varieties of cotton.

The regulation of antioxidant enzymes and the synthesis of biochemical substances are two major examples of the numerous strategies that plants have evolved to combat damage or invasion by microbes as a result of prolonged interactions with various pathogens. These strategies include physiological defence responses and physiological and biochemical resistance. High quantities of ROS have been demonstrated to disrupt membranes, destroy cellular organelles and biomolecules, and govern homeostasis as an important secondary messenger, ultimately leading to cell death (Liu *et al*., 1999). Plants increase the activity of antioxidative enzymes such CAT, SOD, POD, GR, and APX to repair the damage caused by ROS in response to pathogen infection. POD, PPO, and APX activities in relation to *V. dahliae* were discovered to be positively connected with *V. dahliae* resistance in eggplant (Xu *et al*., 2011). The previous two also got better after cotton was inoculated with the V991 strain in our studies. *Gossypium barbadensis* (Sea-Island cotton) cultivars XH24 (resistant cultivars) and XH17 (susceptible cultivars) were examined in leaves samples at 0, 2, 6, and 9 days after infection (DAI) to determine the correlations between protective enzymes (CAT and SOD) and the two defence enzymes (APX and GR). The activity of VW-resistant and VW-susceptible cultivars at the same stage demonstrated a progressive, significant variation in CAT and SOD activity, according to the results of the protective enzyme. VW resistance and the CAT activity and SOD activity found in this study are correlated, as evidenced by the progressive rise in VW-resistant cultivars and decline in VW-susceptible cultivars. H_2O_2 is broken down and detoxified by CAT as it catalyses the oxidation of substrates by H_2O_2 (Mittler *et al*., 2004). As a result, a decline in CAT activity would lead to an accumulation of H_2O_2, which might then interact with (O_2) to form hydroxyl-free radicals through the Herbert-Weiss reaction (Zhou, 2012). The membrane might suffer direct harm from the hydroxyl-free radicals when they target the lipid's unsaturated fatty acids and cause lipid peroxidation (Asada, 1992). SOD's increased activity during the first stages of fungal stress may shield plants from oxidative damage (Bowler *et al*., 1992). The varied times of sampling of *G. barbadense* revealed increased, decreased, and unchanged antioxidant enzyme activity, which point to a distinct antioxidant metabolism in response to *V. dahliae* illness. The results of the study show that the activity of CAT in XH24 increased with prolong period of fungal stress, while in case of XH17, the CAT activity shows gradual decline up to day 6 but at day 9 the results showed a little increase in activity (Figure 2). These findings suggest that during initial stress in the VW-resistant cotton cultivars, CAT's

capacity to degrade active oxygen increased, but after persistent fungal stress, it reduced. For Kentucky bluegrass and Tall fescue, a decline in CAT activity has been seen following a period of fungal infection (Bowler *et al*., 1992). The relationship between CAT and MDA was significantly positively correlated. The antioxygen scavenger system's primary enzyme, SOD, catalyses the conversion of superoxide free radicals into H_2O_2 and (O^{2-}) (Okuda *et al*., 1991). It's probable that the increase in (O^{2-}) in the leaves accompanied the shift in SOD activity (Fu & Huang, 2001). Our study reported that the activity of SOD in XH24 increased significantly from D0-D9. On the other hand, the activity of SOD in XH17 decreased at all period of infection. According to several earlier investigations, SOD activity rose in the early days of fungal stress and then fell after 8 to 15 days of cotton plant infection (Blokhina *et al*., 2003; Liu & Huang, 2000). Previous studies have demonstrated that the sensitivity of SOD activity to various stresses varies with the intensity, duration, and species of the stressor. Huang hypothesised that this stress had no impact on SOD activity in sorghum under moderate stress (*Sorghum bicolor* L) (Huang *et al*., 2001). SOD activity rose in disease-resistant maize varieties, according to Zhang (*Zea mays* L.) (Zhang *et al*., 1995). SOD activity in wheat (*Triticum aestivum* L.) increased or remained unchanged throughout the initial stage of fungal inoculation before declining with additional prolonged stress (Jagtap & Bhargava, 1995). Jagtap noticed that osmotic stress led to a reduction in SOD activity in upland cotton (*Oryza sativa* L.) (Zhang *et al*., 1995). After a protracted period of infection, cotton plants' SOD activity declined, indicating that the scavenging abilities of these enzymes were compromised. The drop in SOD activity would encourage the buildup of O_2. This suggested that the equilibrium between active oxygen generation and the scavenging mechanism could become off after a lengthy period of infection (Reddy & Vajranabhaiah, 1993). H_2O_2-dependent oxidation of substrate is catalyzes by peroxidase. Other studies have reported increase (Zhang *et al*., 1995), decrease and no change in POD activity in response to *V. dahliae* infection (Reddy & Bhargava, 1995; Van Breusegem *et al*., 1998). Because of its greater affinity for H_2O_2, APX may have a more significant function in controlling ROS under stress (Fangmeier *et al*., 1994). The activity of APX in leaves of VW-resistant cultivars increased gradually but significantly deceased in VW-susceptible cultivars of Sea-Island cotton. Additionally, Kentucky bluegrass (*Poa pratensis* L.) leaves exposed to extend *V. dahliae* stress had increased APX activity (Gill & Tuteja, 2010). These outcomes are consistent with what we discovered. Increase in recovery potential of cotton plants may be due to the increase in APX levels. The stable and increased activities of these five enzymes observed in four cultivars of *Gossypium barbadense* under V991 fungal infection, could decrease H_2O_2 detoxification. Depending on the plant type, the degree of the stress, and the level of ROS production, various enzyme activity may react differently to a fungus infection. With the exception

of SOD, all physiological indices substantially correlated one another when subjected to fungus stress. Increased CAT, APX, and SOD activity can detoxify accumulated H_2O_2. Some biochemical compounds in plants give them the energy they need to respond defensively or are the results of harm and injury brought on by pathogen invasion, including MDA (Bian & Jiang, 2009; Chaoui *et al*., 1997), Proline (Radwan *et al*., 2006; Trapnell *et al*., 2010), and soluble sugar (Suriyan & Chalermpol, 2010) which are correlated with plant resistance. The leaves of *V. dahliae*-susceptible and resistant cotton cultivars for MDA concentrations exhibited notable variations at the same stage of peroxidation. However, none of the four cotton cultivars showed any kind of consistent relationship between *V. dahliae* resistance and the Proline content at the same time. Our results corroborated with previous studies (Bell, 1969; Xu *et al*., 2011). Zhou suggested that the Proline accumulation responded to fungal infection injury rather than being positively connected with stress tolerance (Zhou, 2012).

H_2O_2 has a vital and key role in plant immune response, ROS production and calcium ion channelization. The flow is recognized by receptors on the surface of plant cells, activating the plant's immune defense process (Mittlet *et al*., 2011). Generally, when plants suffer from adverse mechanical damage or pathogen invasion, the active oxygen in their bodies will increase to help the plants stimulate immunity against external invasion. However, too high ROS can also cause damage to cells. In order to deal with this problem plants themselves have evolved a mechanism to eliminate superoxide so as not to be harmed by ROS (Liu *et al*., 2017). Based on the data analysis of the transcriptome and proteome in the root of Sea Island cotton, we studied the functional enrichment of high-profile genes in the root of high-resistant and susceptible varieties of Sea Island cotton with the increase of infection time of *V. dahliae* at the same level of transcriptome. It was found that at the transcription level, these genes responding to pathogen stimulation had 25.93% gene enrichment in flavonoid metabolism, 25.93% gene enrichment in peroxidase activity function, 14.81% gene enrichment in flavonoid biosynthesis, 11.11% gene enrichment in juniperene synthesis and 11.11% gene enrichment in phenylpropanoic acid metabolism. Therefore, peroxidase was selected as the research objective in this study for post-infection expression profile analysis and it was found that five genes did increase slowly with the increase of infection time. All of these show that the active oxygen-related gene pathway inside the Sea Island cotton is obviously activated after *V. dahliae* infection; the response degree of active oxygen in high resistance Sea Island cotton varieties is stronger than that of susceptible Sea-Island cotton. The expression of GbAPX at 6 hours was significantly higher in highly resistant cultivar of *Gossypium barbadense* than susceptible cultivar. It is speculated that in order to activate the plant immune

system rapidly within 6 hours, a large amount of reactive oxygen species ROS broke out from highly resistant Sea Island cotton to activate the immune system. Therefore, more superoxide scavenging mechanisms are needed to prevent the plant itself from being invaded by superoxide. It is presumed that the immune response of susceptible plants is slower than that of highly resistant varieties. Long-term superoxide is very harmful to the plant itself so the hydrogen peroxide content of the two cotton varieties falls back to the normal level 24 hours after infection. However due to slow immune activation of susceptible, Sea Island cotton the expression of GbAPX used for scavenging superoxide will increase 24 hours after infection, after late superoxide accumulation for this part of superoxide scavenging. These can also reflect the possible reasons for achieving high resistance by Sea Island cotton at the molecular level and at the same time it also shows that superoxide generation is a strategy for plants to activate plant immune response in the short term. Plant cell wall is a structure used to support plant cells and is related to various life processes. However, in order to invade the plant, the pathogen must first enter through the cell wall, so, it is the first physical defence line that the pathogen invades (Cantu *et al*., 2008). When the pathogen invades the cell wall can sense the pathogen signal and transmit this signal to the inside of the cell to stimulate the plant immune response. Therefore, cell wall plays a very important role in plant resistance against pathogens; this is also called cell wall resistance. As enrichment of cell wall-related genes are quite obvious from the enrichment analysis of transcriptome function and the functional enrichment of transcriptome combination, therefore, these cell walls related genes may play a crucial role in the resistance of Sea Island cotton to *V. dahliae*.

CONCLUSION

This study concludes that the AsA-GSH antioxidant system has a key and significant role in the plant disease resistance function, which will contribute to the further study of disease resistance mechanism of sea-island cotton. This work provides a theoretical basis for better understanding the resistance mechanism of *G. barbadense* after *V. dahliae* infection and also offers the reference for further cultivation of disease resistant varieties of cotton.

Author Contributions

Conceptualization: Amjad Ali., Waqas Safir., Li Hongbin., Li Rong., Sara Zahid., Nasir Uddin. Tariq Masood and Anis Safir.;
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Formal analysis: Amjad Ali., Li Hongbin., Li Rong. and Waqas Safir.; investigation, Li Hongbin., Li Rong., and Amjad Ali.;
Data curation: Amjad Ali., Li Hongbin., Li Rong. and Waqas Safir.;
Writing-original draft preparation: Amjad Ali., Li Hongbin., Li Rong. and Waqas Safir.;
Writing-review and editing: Amjad Ali., Waqas Safir., Nasir Uddin., Sara Zahid. and Anis Safir .;
Supervision: Li Hongbin., and Waqas Safir.;
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All authors have read and agreed to the published version of the manuscript.

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