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ROLE OF CHITIN-SPECIFIC PEROXIDASE IN WILT-RESISTANT COTTON

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Chitin-specific isoforms of peroxidase with molecular weights 67 and 54.7 kDa that are involved in cotton wilt-resistance were obtained from sprouts of cotton variety AN-Bayaut-2 using chromatography. Electron microscopy showed that the morphology of the fungus V. dahliae *changed under the influence of the chitin-specific isoforms of peroxidase.*

Key words: cotton, peroxidase, chitin-specific, resistance, wilt.

Despite significant progress in various areas of phytoimmunity, the issue of unraveling the mechanisms underlying the resistance of cotton cells to the harmful action of pathogens remains unresolved. The mechanisms for overcoming cotton varietal immunity to virulent strains of wilt-inducing agents are also unclear.

Phytopathogens induce the expression of a whole series of genes that code for enzymes that catalyze the formation of covalent bonds between proteins of cell walls and polysaccharides. Peroxidase is such an enzyme. The literature confirms that any damage to plants by pathogens increases quickly the activity of this enzyme [1]. It is still not known why the activity increases or whether the enzyme is synthesized *de novo* or the activity of previously synthesized molecules already existing in the cell increases [2]. Based on numerous investigations, peroxidase is viewed as one of the most important catalytic systems among biochemical means of protecting plants from phytopathogens [3]. Isoforms of this enzyme can be used as a unique diagnostic method for biochemical research. It is known that peroxidase is necessary for the development of the plant cell and is always present in the plant. Its activity is increased in the presence of chitin [4].

In order to investigate the role of chitin-specific isoforms of peroxidase in the resistance of cotton to damage by wilt and to find resistant varieties, we analyzed peroxidase activity in 4-5-day sprouts of C-4727 and AN-Bayaut-2. Peroxidase activity was two times greater in AN-Bayaut-2 than in the control in sprouts damaged by the fungus *Verticillium dahliae* (Table 1).

It has been reported that peroxidase activity changed little upon infection with systemic damage in susceptible plants whereas the infection in resistant plants caused a significant increase in the enzyme activity and, possibly, the appearance of *de novo* isoforms [5]. According to our results, the high peroxidase activity in variety AN-Bayaut-2 and the low activity in C-4727 suggests that AN-Bayaut-2 is a variety that is resistant to the action of the phytopathogen whereas C-4727 is susceptible.

Then we used further variety AN-Bayaut-2 with high peroxidase activity in cotyledonous leaves of 5-day sprouts compared with other plant organs damaged by *V. dahliae* through the cotton root system (Table 1). We used the property of certain peroxidase isoforms to bind pathogen to chitin to elucidate the resistance mechanism of cotton to the action of *V. dahliae*. It is known that these isoforms participate in lignification of host plant cell walls, preventing the fungus from spreading through the plant. In order to obtain purified chitin-specific isoforms, starting material from cotyledonous leaves of AN-Bayaut-2 containing peroxidase activity was separated by gel filtration over LKB Ultrogel (20,000-30,000) into three fractions, of which the second fraction had peroxidase activity.

We used a column with chitin to isolate chitin-specific peroxidase isoforms from the active fraction based on the property of peroxidase to bind to chitin of the phytopathogen. The chitin-specific fraction obtained by chromatography over chitin had activity 24.2 units/mg. The results were repeated at least three times.

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TABLE 1. Change of Peroxidase Activity (units/mg-protein) in 5-Day Sprouts of Cotton Variety C-4727 (Wilt Susceptible) and AN-Bayaut-2 (Wilt Resistant) Caused by *V. dahliae* Kleb.

Variety	Variant	Rootlets	Hypocotyls	Cotyledonous leaves
C-4727	Healthy	8.30	5.40	4.51
	Infected	12.50	4.13	8.90
AN-Bayaut-2	Healthy	12.21	13.32	20.24
	Infected	23.60	24.20	26.51

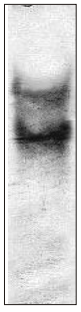

Fig. 1

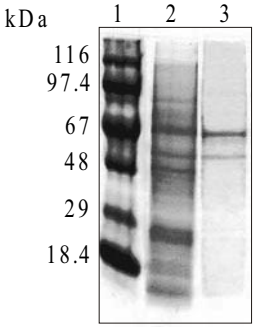

Fig. 2

Fig. 1. Electrophoresis in PAAG (10%) of chitin-specific peroxidase from cotyledonous leaves of variety AN-Bayaut-2 after purification (precipitation by 80% ammonium sulfate, filtration through sorbents ultragel, chitin, and Sephadex 75).
Fig. 2. Electrophoresis in PAAG (10-15%) with sodium DDS of chitin-specific isoforms of peroxidase: markers (1), starting peroxidase fraction (2), chitin-specific peroxidase isoform (3).

Then the chitin-specific fraction with peroxidase activity was additionally purified by gel filtration over Sephadex G-75 to produce three fractions in which peroxidase activity was detected in the second (10.2 units/mg) and third (1.43 units/mg) fractions.

Electrophoresis in PAAG (10%) established that the chitin-specific isoforms of the second fraction with high enzymatic activity from cotyledonous leaves had R_f 0.18 and 0.23 (Fig. 1). Electrophoresis of chitin-specific peroxidases was performed in a PAAG gradient (10-15%) to determine their molecular weights as 67 and 54.7 kDa (Fig. 2).

Capillary electrophoresis of chitin-specific isoforms showed two peaks corresponding to the presence of two peroxidase isoforms with binding affinity for chitin.

A comparison of the electrophoresis data revealed the presence of chitin-specific isoforms in the isospectrum of starting material, indicating that the activity of existing isoenzymes increased but their amount did not change upon interaction with *V. dahliae*.

The fungicidal properties of peroxidase and its chitin-specific isoforms were determined by placing paper disks at the boundary of a patch of a 4-day colony of *V. dahliae* (Fig. 3). Paper disks were wetted in a solution containing 30 μg of peroxidase after gel filtration over LKB Ultrogel (20,000-30,000) and purified chitin-specific peroxidase from cotyledonous leaves of AN-Bayaut-2. Fraction II and the chitin-specific isoform suppressed fungus growth at the boundary of the colony. The boundary of pathogen growth lagged by 2 mm under the influence of chitin-specific peroxidases.

Determination of fungicidal activity by a turbidometric method also showed that growth of the pathogen was inhibited by 18% by the chitin-specific isoperoxidase.

Chitin-specific peroxidase isoforms isolated from cotyledonous leaves of AN-Bayaut-2 sprouts infected by *V. dahliae* are able to suppress fungus growth according to our results from the determination of fungicidal properties.

Judging from the results, the isolated chitin-specific isoforms can act as an indicator of cotton resistance to a phytopathogen. The high peroxidase activity and the fungicidal properties of the enzyme isoforms confirm that AN-Bayaut-2 is resistant to wilt. The chitin-specific peroxidase isoforms are involved in protective lignification of the plant cell wall upon infection by the phytopathogen, which agrees with the literature [6].

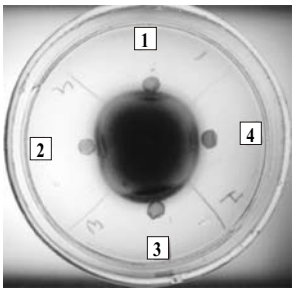

Fig. 3

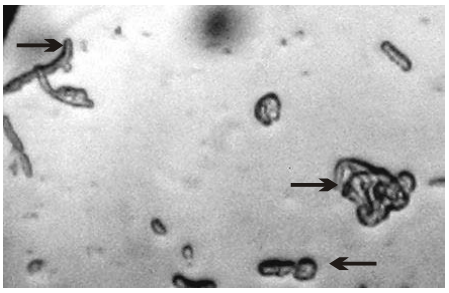

Fig. 4

Fig. 3. Fungicidal activity of peroxidase from 5-day cotyledonous leaves of wilt-resistant cottong variety AN-Bayaut-2 for *V. dahliae* colonies: fraction I from gel filtration over LKB Ultrogel from cotyledonous leaves of AN-Bayaut-2, 1-day infection by *V. dahliae* (1); fraction II from gel filtration over LKB Ultrogel from cotyledonous leaves of AN-Bayaut-2, 1-day infection by *V. dahliae* (2), fraction III from gel filtration over LKB Ultrogel from cotyledonous leaves of AN-Bayaut-2, 1-day infection by *V. dahliae* (3); chitin-specific isoform from fraction II after gel filtration over LKB Ultrogel from cotyledonous leaves of AN-Bayaut-2 (4).

Fig. 4. Change of morphology of *V. dahliae* Kleb. caused by chitin-specific peroxidase from wilt-resistant cotton variety AN-Bayaut-2. Arrows show destruction of fungus morphology.

Electron microscopy studies of the action of chitin-specific peroxidases from the wilt-resistant cotton variety on the morphology of *V. dahliae* showed that it changed under the influence of chitin-specific peroxidase isoforms isolated from AN-Bayaut-2 sprouts. The changes included abnormal thickening of the fungus mycelium, rolling up into a ball, and disruption of the polarity in mycellar growth (Fig. 4). Such properties of chitin-specific peroxidases in resistant plants indicate that they are involved in protective responses against chitin-containing phytopathogens.

Thus, our research showed that peroxidase activity increased under the influence of *V. dahliae*. Peroxidase activity in cotyledonous leaves of sprouts grown in the presence of pathogen was significantly higher in AN-Bayaut-2 than in susceptible variety C-4727. Chromatography produced from AN-Bayaut-2 chitin-specific peroxidase isoforms with molecular weights 67 and 54.7 kDa. Electron microscopy showed that the morphology of *V. dahliae* changed under the influence of the chitin-specific peroxidase isoforms. The changes included abnormal thickening in mycelium, rolling up into a ball, and disruption of the polarity of mycellar growth.

EXPERIMENTAL

We used 4-5-day sprouts of varieties AN-Bayaut-2 and C-4727 for comparative biochemical studies. Sprouts were infected with the phytopathogen *V. dahliae* through the root system at a concentration of 10^6 conidia/mL.

Ground plant material was triturated in phosphate buffer (PB, 0.1 M, pH 6.0) for 1 h on a magnetic stirrer at +4°C. The solid was separated by filtration through filter paper with subsequent centrifugation at 5000 rpm for 20 min. The supernatant was transferred to a clean container. Proteins were precipitated by ammonium sulfate (80%) and separated over LKB Ultragel (20,000-30,000).

Protein content in the extract was measured by the Lowry method [7].

Specific peroxidase activity was determined by the Boyarkin method [8].

Chitin-specific isoforms were isolated over a column of chitin with elution by PB. Crab chitin was demineralized and purified by treatment with HCl (0.5 M) and NaOH (0.5 M) and washed with distilled water until the pH was neutral after the acid and base.

Chitin Preparation. Ground dry boiled crab shell purified of meat was boiled for 2 h in a flask with water. The aqueous solution was poured off. The solid was treated at room temperature with HCl solution (5%). The acid was removed. The shells were washed several times with water, transferred to a flask, treated there with NaOH solution (5%), and boiled again

for 2 h. The dark alkaline solution into which proteins from the shells dissolved was filtered. The solid was washed with water and treated alternately with acid and base two more times.

Chromatography of PO isoforms was carried out in a glass column (1.2 × 25 cm). The column with sorbent was equilibrated with buffer before the analysis in order to extract peroxidase. The supernatant was placed on the chitin column. The sorbent was rinsed with PB at 20 mL/h to remove nonbinding proteins. Then the column was rinsed with PB (0.01 M). Chromatographic fractions were detected in a flow cuvette on a spectrophotometer using absorption at 280 nm. The resulting chitin-specific isoforms were purified by gel filtration over Sephadex G-75 using PB.

Component composition of proteins was determined by electrophoresis in a PAAG gel gradient (from 10 to 15%) with sodium DDS according to Lammly [9].

Capillary electrophoresis was carried out on an Agilent Technologies "capillary electrophoresis" instrument. The conditions were buffer separation, borate buffer (20 mM), pH 9.3; capillary, total length 64.5 cm, effective length, 56 cm, inner diameter, 50 μm; temperature 15°C; potential 30 kV; positive polarity; injection, 200 mbar·s; sample concentration, 0.1 mg/mL.

Fungicidal properties were determined in Petri dishes on Chapek solid medium [10] by placing paper disks near a patch with *V. dahliae*.

Turbimetric determination of fungicidal properties of peroxidase isoforms was performed by the literature method [11]. The change of fungal conidia concentration was measured on a Hospitex Diagnostic microplate reader.

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