The Asian and Australasian Journal of Plant Science and Biotechnology ©2013 Global Science Books

Some Aspects of the Interaction between Cotton Anionic Peroxidases and *Verticillium dahliae* Kleb.

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ABSTRACT

Verticillium dahliae Kleb. is a phytopathogenic fungi which causes wilt disease in a wide range of crops, including cotton. In this study, we have examined the role of the peroxidase (POX) enzymes in cotton response to pathogen. We examined the inducibility of POXs through the experimental explosion of cotton (*Gossypium hirsutum* L.) cultivars by *V. dahliae*. The results of this study indicate that POX activity was associated with resistance and that its activity was correlated with levels of different resistance of cotton cultivars. POX activity of 7-day-old cotton seedlings increased in the presence of *V. dahliae* chitin and conidia in comparison with its activity in crude extract. This activation occurred in the chitin-sorbed fraction. Isoelectric focusing shows that cotton seedlings of var. 'AN-Bayaut-2' are considered to be resistant and had two anionic isoforms (pI 3.4 and 3.9) that bind to *V. dahliae* while 'C-4727' is considered to be susceptible and had only one of these types of isoforms (pI 3.1). The treatment of cotton seeds and seedlings by soluble chitosan revealed that POX activity was induced among all the tested plants. The role of POX in disease resistance was examined through an anti-fungal activity test of chitin-binding anionic isoforms. Significant anti-fungal activity of chitin-binding POX isoforms of the resistant cotton variety was observed. Microscopic examination of the hyphal growth inhibition zone revealed that the anionic chitin-bound POXs of 'AN-Bayaut-2' were able to arrest the growth of the pathogen. The use of POX activity as a preliminary marker for resistance of cotton cultivars to *V. dahliae* is suggested.

Keywords: conidia, chitin, defense reaction, marker, resistance

INTRODUCTION

A pathogen attack and different stress conditions can cause the production of reactive oxygen species, which can damage DNA and proteins, and severely compromise the function of the membrane. In response to the increased production of oxygen radicals, plants activate a complex antioxidative system composed of antioxidant molecules able to scavenge these species, including a variety of enzymes. The main plant enzymes involved in detoxification of such radicals are peroxidase (POX) (EC 1.11.1.7) (Hiraga *et al.* 2001). POXs participate in a great number of physiological processes, biosynthesis of ethylene, lignification and suberinization of host cells during defense against pathogens, wound healing, and stress response (Cochrane *et al.* 2000; Conrath *et al.* 2001; Ross *et al.* 2005; Gorjanovic 2009). Although certain POX functions could be related to the specific isoforms, it is generally difficult to assign a specific function to the individual POX isoforms *in vivo*. Numerous studies have examined the functions of POXs and tried to attribute some function *in vivo* to the anionic, cationic, neutral, ionically or covalently bound POX isoforms (Sanchez *et al*. 1995; Quiroga *et al*. 2000, Minibayeva *et al*. 2009). Alternatively, there are several reasons in favour of factors other than enzyme structure being important in determination of POX activity: microenvironment, a high redundancy found in POX genes, very similar immunological properties of different isoenzymes, and failure to relate down-regulated specific POXs to phenotype (Cosio and Dunand 2009).

Plants have evolved complex physical and biochemical mechanisms to detect pathogens in their natural environments and to activate defense reactions. Among the numerous enzymes involved in plant defense mechanisms, POX occupies a crucial position in a plant's early response to pathogens (Lin and Kao 2001). Recognition plays a central role in the interaction between plants and pathogens (Wang *et al*. 2004). The initial stage of this process is poorly understood. Fungal cell walls are structurally unique and differ significantly from the cellulose-based plant cell wall. Fungal cell walls are comprised of glycoproteins and polysaccharides, mainly glucan and chitin. Chitin is a polymer of N-acetyl-D-glucosamine that is a major component of the cell wall of many fungi (Banks *et al.* 2005). Chitosan, a deacetylated form of chitin, is also important in maintaining cell wall integrity (Baker *at al.* 2011). The proteins, glucans and chitin are integrated into the wall by covalently cross-linking the chitin, glucans, protein-associated oligosaccharides and glucosyl-phosphatidylinositol anchors together (Cabib *et al.* 2007). Chitin perception by plants in response to microbial invasion plays an integral role in cell signaling during pathogenesis. During fungal infection chitin fragments (chitooligosaccharides or chitin oligomers) from fungal cell walls can act as an elicitor to induce plant innate immunity against the invading pathogen (Stacey and Shibuya 1997; Shibuya and Minami 2001). Different POX molecules may exhibit different interactions with various constituents of the extracellular matrix. One of the few known examples of a specific interaction between a cell wall protein and a cell wall polymer is the binding of some isoperoxidases to the homogalacturonan domains of pectins (Carpin *et al.* 2001). Khairullin *et al.* (2000) found that the pathogen *Tilletia caries* induced anionic POX isoforms in wheat seedlings, and that some of these POXs strongly interacted with chitin. They supposed that these anionic POXs participated in the lignification of the plant cell wall during pathogenesis. The authors found that an increase of POX activity occurred in the presence of chitin. Additionally, it was assumed that the affinity of POXs for carbohydrates is determined by the presence of zones with high

Received: 28 June, 2012. Accepted: 5 November, 2012.

Original Research Paper

electrostatic potential in the enzyme molecule (Dunand *et al.* 2002). The specific sorption of POX on infectious components of fungi containing chitin was observed as abundant scurf of phenol polymers on the haustoria of *Uromices viciae-fabae* (Medeghini *et al.* 1994). On the other hand, it has been shown that some POXs produced reduced oxygen species such as hydrogen peroxide (Bestwick *et al*. 1997) or the hydroxyl radical (Schweikert *et al*. 2000) through complex free radical reactions. The hypothetical role of POXs in pathogenesis is related to oxidative scission of chitin cell walls of the pathogen followed by production of oligosaccharide elicitors (Schweikert *et al.* 2002).

We hypothesize that anionic POXs participate in chitin perception and may serve a direct role in the anti-fungal defense mechanism.

MATERIALS AND METHODS

General

Cotton seeds (*Gossypium hirsutum* L.) of 'AN-Bayaut-2' and 'C-4727' varieties were grown in an experimental field of the Institute of Cotton Breeding and Seed Production (Tashkent, Uzbekistan). 'AN-Bayaut-2' is considered to be highly resistant to *V. dahliae* and 'C-4727' is considered as susceptible (Ibragimov 1993). *V. dahliae* Kleb. conidia were isolated from the diseased cotton (from the collection the Institute of Genetics, Academy Sciences of Republic of Uzbekistan, Tashkent, Uzbekistan). The pathogen was grown on Czapek's agar (Gautam *et al.* 2011). Chitin from crab shells (Fluka Chemical Co., Milwaukee, WI, USA) was used for chromatography. The chitin adsorption assay was performed on a chromatographic column (2 cm × 6 cm) packed with chitin previously equilibrated with 0.1 M sodium phosphate buffer (pH 6.6). Protein fractions were monitored at 280 nm using a flow cuvette on a Uvicord 2238 S II spectrophotometer (Pharmacia-LKB, Sweden). Cotton seeds were treated with chitosan 75% deacetylated (Sigma Chemical Co.). Isoelectric focusing of proteins was carried out on an LKB Multiphor-2117 apparatus (Pharmacia, Uppsala, Sweden). The IEF mix 3.5-10.6 (Sigma Chemical Co., St. Louis, MO, USA) was used as the isoelectric focusing marker. Isoelectrofocusing of POX was carried out on a horizontal plate containing 7% polyacrylamide gel, 0.016% *N*, *N*-methylene-*bis*-acrylamide, 10% glycerol, 1.5% ampholines at pH 3.5-10 (Pharmacia-LKB, Uppsala, Sweden) and 0.033% ammonium persulfate in 8 M urea. The anode was 0.5% HCI, and the cathode was 0.5% NaOH. POX activity was ascertained on a gel using 0.02 M guaiacol. POX isozymes were also quantified by a reaction with 0.02 M guaiacol (Sigma Chemical Co.) and 0.03% H_2O_2 in a 0.01 M sodium phosphate buffer (pH 5.8). After 1 min of reaction time the absorption maxima at 470 nm were determined on a SF-26 spectrophotometer (LOMO, St. Petersburg, Russia). The activity of each POX isoforms was determined by measuring the color intensity using a LKB 2202 Ultrascan laser densitometer (Pharmacia-LKB, Sweden). The total protein concentration was determined by the method of Lowry (15). Assays were performed using an optical microscope (Neofot-2, Carl Zeiss, Jiena, Germany) at 90X magnification. All experiments were repeated at least three times. Data was subjected to ANOVA (Stat Soft. 2008 ANOVA/MANOVA [Online: http://www.statsoft.com/textbook/stanman.html]) and differences between treatments evaluated by a Student's two-sample *t*-test at $P < 0.05$.

Infection of cotton seedlings

Cotton seeds were cleansed with concentrated H_2SO_4, rapidly washed with running tap water and kept in water overnight. Seedlings were germinated in paper capsules during 7 days in darkness at 27°C. Infection of seedlings was performed by placing capsules in a flask (500 ml) containing 200 ml a conidial suspension (10^7 conidia per ml) of *V. dahliae*. Conidial concentration was quantified with a haemacytometer and the inoculums concentrations were adjusted to 10^7 conidia per ml before infection.

Treatment of cotton seeds and seedlings by chitosan

Cotton seeds were treated with chitosan (75% deacetylated). Uncovered seeds were moistened in water containing a 1 mg/l solution of chitosan during 24 h. Swollen seeds were wrapped in paper "cartridges" and germinated for 7 days at 27°C.

Crude enzyme preparation

7-day-old cotton seedlings were infected with *V. dahliae* conidia (10^7 conidia per ml). Control and infected cotton seedlings were cut into equal sections of about 4 to 5 mm in length. The plant material was ground in liquid nitrogen, and 1 g of material was extracted with 30 ml of 0.1 M sodium phosphate buffer (pH 6.6). The homogenate was then filtered through Whatman filter paper (M-3) and after that through a Sartolon polyamide filter pore size 0.45 mm (Sartorius AG, Göttingen, Germany). Experiments were replicated six times.

Chromatography of POXs on chitin and *V. dahliae* conidia

Chitin from crab shells was used for chromatography. Chitin-binding proteins were obtained as described by Maksimov *et al.* (2005). The *V. dahliae* conidia adsorption assay was carried out by the following method ("in volume"). Dried conidia of *V. dahliae* (500 mg) were previously treated with 1 N NaOH (5 min) and washed with 0.1M sodium phosphate buffer (pH 6.6). After centrifugation (6000 × *g*, 20 min), the desalinated total protein preparation was suspended with 500 mg conidia in a minimal volume of the same buffer (30 min), and conidia were washed with some portions of buffer to remove unbound proteins. A glass column (1.5 × 6 cm) was filled with the same sorbent (conidia after washing). The column was eluted with 1 M NaCl in 0.01 M sodium phosphate buffer (pH 6.6) at a flow of 20 ml h^{-1}. Fractions were monitored at 280 nm as indicated above. Purification of cotton anionic POX after chromatography on column with chitin was performed according to Mellon (1991). The anionic POX was purified by chromatography on Toyopearl DEAE – 650 M anion exchange media (Tosoh Bioscience LLC, Tokyo, Japan). The anion exchange media was equilibrated with 0.02 M sodium phosphate buffer (pH 7.0), and packed to form a 2.5 × 16 cm column (bed volume of 80 ml). The column was washed with 0.02 M sodium phosphate buffer (pH 7.0). The crude NaCl fraction was extensively dialyzed against sodium phosphate buffer and applied to the column. After sample application, the column was eluted with 100 ml sodium phosphate buffer (80 ml/h), followed by 400 ml of a 0 to 0.3 M NaCl linear gradient in sodium phosphate buffer. Final elution of the column was with 100 ml of 0.5 M NaCl in 0.02 M sodium phosphate buffer. The column fractions (2.5 ml) were monitored for protein at 280 nm and POX activity by the procedures described next.

Specific POX activity

POX (EC 1.11.1.7) activity was determined by diluting 0.1 ml of either the filtered plant homogenate or the fractions eluting from the chitin column with 1.9 ml of 0.01 M sodium phosphate buffer (pH 5.8) and 0.05 ml of a 0.02 M guaiacol in 0.01 M sodium phosphate buffer (pH 5.8). Then, 0.05 ml of 0.03% H_2O_2 was added. Assays were initiated by adding H_2O_2 and the change in optical density at 470 nm was measured for 1 min specific POX activity was calculated by the formula (Boyarkin 1951).

$$A = 2\Delta d / ab \text{ Unit/mg}^{-1}$$

where A = specific POX activity; Δd = difference in UV absorption from time zero to one minute; a = protein concentration in mg/ml^{-1}; b = volume of protein sample (0.1 ml).

The total protein concentration was determined by the method of Lowry (1951).

Fungal-growth-inhibition assay

The Petri dishes containing Czapek's standard solid medium (Golubenko *et al.* 2007) for cultivation of fungi were incubated with the hyphal mass of *V. dahliae* at 25°C for 2 days. Sterile paper discs (6 mm in diameter) containing 50 µg of filter-sterilized POX samples, or 20 µl sodium phosphate buffer solution (0.1 M, pH 6.6) as a control, were placed at the edge of the growing hyphae and incubated for two days under periodical observations.

RESULTS AND DISCUSSION

Chitin binding POXs

Chitin, a polymer of N-acetyl-D-glucosamine, is an important component of fungal pathogenicity, since fungal pathogens with defects in chitin synthesis are significantly less virulent on the original susceptible hosts (Soulie *et al.* 2006). These crystalline polymers have an enormous tensile strength and significantly contribute to the overall integrity of the cell wall (Bago *et al*. 1996). When chitin synthesis is disrupted, the wall becomes disordered and the fungal cell becomes malformed and osmotically unstable (Specht *et al.* 1996). Chitin is not present in vertebrates or plants, but is otherwise abundant in the environment. Chitin is a major constituent of arthropod exoskeleton, fungal cell wall, and helminth eggshell, pharynx and/or the cuticle (Whitrman *et al*. 2011). Infiltration of chitin oligomers into vertebrate or plant tissue causes massive and sometimes analogous innate immune responses, including upregulation of chitinases (Zhang *et al*. 2002; Reese *et al*. 2007) that are detrimental to parasite development (Lawrence end Novak 2006), and likely cause allergies in humans. Thus, elicitors such as chitin that are common to parasites of animals and plants can be recognized by and cause parallel immune responses in plant and animal hosts. Plant cells are equipped with chitin-degrading enzymes to digest fungal cell walls and are capable to perceive chitin fragments (chitin oligosaccharides) released from fungal cell walls during fungal infection (Wan *et al.* 2008). Plants pretreated with chitin showed resistance to *Scaptomyza flava* larvae (Whitrman *et al*. 2011), consistent with the fact that chitin elicits a major defence response in Arabidopsis and other plants, including production of chitinases and protease inhibitors (Zhang *et al*. 2002) that are ecologically important plant defences against insects (Lawrence and Novak 2006). Chitin recognition results in the activation of defense signaling pathways. Although chitin is a well recognized pathogen-associated molecular pattern, little is known about the molecular mechanism of chitin signaling. Concerning to the receptor for chitin oligosaccharide elicitor, Kaku *et al*. (2006) was identified a high-affinity binding protein for this elicitor in the plasma membrane of rice cells by affinity labeling. Similar binding proteins were also detected in various plant cells that could respond to the elicitor (Day *et al*. 2001; Okada *et al*. 2002). Correlation between the presence of the binding proteins and the elicitor responsiveness of these cells, correlation between the binding specificity and the preference of the structure of chitin oligosaccharides in defense responses, strongly indicated that the binding proteins function as a receptor, or a part of receptor complex, for chitin oligosaccharide elicitor (Kishimoto *et al*. 2011). In suspension-cultured rice cells, the action of chitin fragments has been extensively studied. These studies showed the induction by chitin fragments of enzyme activities involved in the biosynthesis of terpenoid phytoalexins (Ren end West 1992; Yamada *et al*. 1993), membrane depolarization (Kuchitsu *et al*. 1993; Kikuyama *et al*. 1997), Cl^- and K^+ efflux, cytoplasmic acidification (Kuchitsu *et al*. 1997), generation of reactive oxygen species (Kuchitsu *et al*. 1993), biosynthesis of jasmonic acid (Nojiri *et al*. 1996) and expression of unique early responsive genes and typical defence related genes (Nishizawa *et al*. 1999; Taylor *et al*. 2001) by chitin fragments. Previous DNA microarray studies suggested plant cells can reprogram gene expression in response to chitin elicitation (Day *et al*. 2001; Okada *et al*. 2002). Recently, the use of quantitative transcriptase-polymerase chain reaction in conjunction with DNA microarrays, revealed 118 transcription factors (TF) genes responsive to chitin (Libault *et al*. 2007). The induction of a number of the chitin-responsive TF genes (Eulgem 2007) was previously shown to depend on the activation of the mitogen-activated protein kinase (MAPK) cascade (Wan *et al*. 2004), suggesting that these TFs may play an important role in regulating other chitin responsive genes. Indeed, the mRNA levels of approximately 900 *Arabidopsis* genes were shown by DNA microarray analysis to respond to chitin elicitation (Ramonell *et al*. 2005; Wan *et al*. 2008). Likewise, in rice, a large number of genes were also shown to be regulated by chitin (Day *et al*. 2002). Consistent with the elicitor role of chitin, many of these regulated genes are defense-related genes, such as those encoding pathogenesis-related proteins, and disease resistance proteins (Ramonell *et al*. 2005; Wan *et al*. 2008).

Plant POXs are believed to function in diverse physiological processes including disease resistance and wound response. It has been shown that some POXs produced oxygen species as signal mediators and antimicrobial agents (Schweikert *et al*. 2002). For example, tiol POXs have been implicated in cell signaling due to their ability to reduce intracellular levels of hydroperoxides and to serve as floodgates of H_2O_2 signaling (Wood *et al*. 2003). However, studies have also revealed that *Saccharomyces cerevisiae* Gpx3/Hyr1/Orp1 can serve as an H_2O_2 sensor and activate the transcription factor Yap1 by forming a disulfide in this protein (Delaunay *et al*. 2002), and a *Schizosaccharomyces pombe* thiol peroxidase Tsa1 was found to stimulate signaling through a MAPK pathway (Wan *et al*. 2004; Veal *et al*. 2007).

The function of individual POXs may differ from each other, as suggested by their organ-specific or characteristic stress responsive expression profiles. Cell signaling and the perception of small phytoactive compounds comprise the basis for communication between plants and microbes in a number of widely studied systems (Day *et al*. 2001). An apoplastic isoperoxidase from zucchini was shown to bind strongly to polygalacturonic acid in their Ca^{2+}-induced conformation (Carpin *et al.* 2001). Apoplastic isoperoxidase anchoring to homogalacturonan-rich domains of the cell wall is likely to control the spatial distribution of apoplastic isoperoxidase and the orientation of reaction products released by POX. Carpin *et al.* (2001) hypothesized that pectins, in addition to being a constituent of the physical frame that surrounds the plant cell, may exert a biological activity through the localization and stabilization of interacting proteins. It has been shown that in addition to some POXs, there are other proteins that interact with pectins (Penel and Greppin 1996). Modified chitin oligosaccharides, similarly, play a central role in the establishment of a host-specific symbiosis between legumes and their rhizobial symbionts (Cohn *et al.* 1998). Although much has been done to elucidate the numerous responses evoked upon ligand recognition, relatively little is known about how these signals are perceived by the host plant. However, it is apparent that chitin perception by diverse plant species shows some similarities (Stacey and Shibuya 1997). To further define the structural requirements of chitin recognition in cotton variety, we investigated the binding of POXs with chitin.

We conducted experiments on a modeling of interaction between fungi pathogen with cotton POX using chitin as chromatographic matrix. Affinity column chromatography using chitin revealed that some POXs of 7-day-old cotton seedlings of both susceptible cotton variety 'C-4727' and resistant 'AN-Bayaut-2' absorbed to the column. These POXs, which were eluted with 1 M NaCl, occurs at markedly higher level of POX activity in the infected seedling extracts, and it is specifically bound by the chitin column (**Table 1**). POX activity of the resistant cotton variety was significantly higher in chitin-bound fractions than in the susceptible variety. The maximum increase was observed in

Table 1 Activity of fractions unbinding and binding to chitin from 7-day-old resistant ('AN-Bayaut-2') and susceptible ('C-4727') cotton seedlings infected with *V. dahliae* conidia.

Seedling part	Peroxidase activity, Unit/mg^{-1}					
	Control plants			Infected plants		
	Crude extract	Chitin non-binding	Chitin binding	Crude extract	Chitin non-binding	Chitin binding
'AN-Bayaut-2'						
Roots	12.3 ± 1.8 a	14.4 ± 0.3	17.2 ± 0.8	20.8 ± 0.3	16.1 ± 0.1	23.6 ± 0.6
Hypocotyls	1.5 ± 0.5	1.8 ± 0.3	2.6 ± 0.3	8.2 ± 0.4	6.3 ± 1.3	10.2 ± 2.3
Cotyledons	15.6 ± 3.1	15.1 ± 3.1	20.6 ± 0.6	20.2 ± 1.2	18.5 ± 0.6	24.2 ± 2.5
'C-4727'						
Roots	8.7 ± 1.7	10.3 ± 0.4	3.0 ± 0.5	16.0 ± 0.1	20.0 ± 1.8	12.5 ± 0.4
Hypocotyls	1.3 ± 0.5	4.5 ± 0.8	2.4 ± 0.3	6.5 ± 0.3	13.2 ± 0.9	4.1 ± 0.2
Cotyledons	10.2 ± 2.7	12.2 ± 0.3	8.0 ± 0.6	13.3 ± 4.0	11.5 ± 0.9	8.9 ± 0.5

[a] Values are mean ± SD, experiment repeated at least three times.
Presented averages and standard deviations according to Student's *t*-test ($P < 0.05$)

cotyledons – 24.2 ± 2.5 Unit/mg^{-1} and the less activity was in hypocotyls – 10.2 ± 2.3 Unit/mg^{-1}. In comparison, the POX activity of the susceptible 'C-4727' infected with *V. dahliae* conidia was lower in the chitin-bound fraction in all parts of the seedling on average 2.3 ± 0.4 times than levels observed in the resistant variety.

It should be noted that POX activity of chitin non binding fraction from seedlings of susceptible cotton variety 'C-4727' has been increased in comparison with the chitin-bound fraction. This observation indicates that POX activity in presence of chitin among the examined cotton varieties in comparison with its activity before the binding with chitin in crude extract is increased. In this case, POX activation took place both in a fraction sorbed on chitin and in fraction not bound to it. According to these results, it possible to argue that some plant isoperoxidases had ability to bind with chitin of phytopathogen cell walls. Therefore, it can be assumed that cotton POX, which adsorbed on chitin, contain sites that specifically interact with its acetyl residues. According to the literature data, a polysaccharide binding domain was observed in anionic POXs of *Arabidopsis thaliana* and *Cucurbita pepo* (Dunand *et al.* 2002). Probably, chitin- binding isozymes of POX are signaling molecules in plant defense mechanisms which identify the oligosaccharide containing phytopathogens. Thus, spreading pathological process probably depends on the rate of chitin-binding POX activation in infected cells and on the intensity of enzyme sorption on mycelium of chitin containing pathogen.

Effect of chitosan on POX activity

Chitosan is known to have eliciting activities leading to a variety of defence responses in host plants to microbial infections, including the accumulation of phytoalexins, pathogen-related (PR) proteins, and proteinase inhibitors, lignin synthesis, and callose formation. Chitosan is the deacetylated form of chitin. Chitin can be enzymatically deacetylated to chitosan by chitin deacetylases (EC 3.5.1.41) (Adams 2004; Baker *et al.* 2011). Small, diffusible chitin oligosaccharides can initiate a wide range of biological responses in plants, and inducing POX activity. In view of their well-defined chemical nature and the presence of highly sensitive perception systems in plants, some of these elicitors have provided good model systems to study how plant cells recognize such chemical signals and transduce them for activation of the defence machinery.

Therefore, we studied the effect of soluble chitosan in POX activity in roots and cotyledons of 7-day-old seedlings of resistant cotton variety 'AN-Bayaut-2' and susceptible one 'C-4727'. Results of studies are given in tables (**Table 2**). It was found that treatment with soluble chitosan had induced increasing POX activity in healthy seedlings of resistant cotton variety 'AN-Bayaut-2' and susceptible one 'C-4727'. It should be noted, that treatment with chitosan led to multiple rise of POX activity in seedlings of susceptible variety compared with control (13 times in roots and 4 times in cotyledons). Pretreatment with chitosan and subsequent infection with *V. dahliae* of resistant and susceptible varieties cotton seedlings also led to an increase of enzyme activity compared with control. In this variant of the experiment POX activity was insignificantly increased in roots of susceptible cotton variety than in infected with *V. dahliae*. However POX activity in cotyledons infected only with *V. dahliae* was not significantly differ from the variant pretreatment with chitosan and subsequent infection with *V. dahliae*.

Table 2 Peroxidase activity in roots and cotyledons of 7-day-old resistant ('AN-Bayaut-2') and susceptible ('C-4727') cotton seedlings infected with *V. dahliae* conidia and treated with chitosan.

Cultivar	Peroxidase activity, Unit/mg^{-1}			
	Untreated control	Chitosan treatment	*V. dahliae* conidia	Chitosan + *V. dahliae* conidia
'AN-Bayaut-2'				
Roots	28.2 ± 1.6 a	176.1 ± 0.8	45.5 ± 1.5	77.0 ± 0.6
Cotyledons	20.5 ± 1.9	75.0 ± 2.6	76.0 ± 3.0	45.5 ± 2.5
'C-4727'				
Roots	8.9 ± 0.6	117.0 ± 2.4	25.1 ± 1.6	56.8 ± 2.5
Cotyledons	14.2 ± 1.2	58.0 ± 2.8	16.8 ± 1.8	23.2 ± 3.0

[a] Values are mean ± SD, experiment repeated at least three times.
Presented averages and standard deviations according to Student's *t*-test ($P < 0.05$)

Pretreatment of susceptible variety with chitosan mimics defense responses which is typical for resistant forms. Probably the induction of POX synthesis increases defense potential in the susceptible plant and keeps high level POX activity during infection with *V. dahliae* than in infected only with them. Some *POX* genes are activated by infection with pathogens such as fungi (Harrison *et al*. 1995; Curtis *et al*. 1997). Additionally, recent gene expression profiling studies demonstrated that chitooligosaccharides were a potent regulator of plant gene expression (Zhang *et al*. 2002; Ramonell *et al*. 2005; Wan *et al*. 2008). Many reports showed that *V. dahliae* infection, or treatment with the fungal elicitor, activated the expression of defense response and *PR* genes in cotton (Meyer *et al.* 1994; McFadden *et al*. 2001; Zhou *et al*. 2002; Delannoy *et al*. 2006). All this suggests that a chitin perception and signal transduction pathway is presented in cotton to mediate plant disease resistance.

Adsorption of POX isoforms on *V. dahliae* conidia

Chitin biosynthesis is localized in the apical zone of hyphae and its fragments may penetrate to the intercellular space inducing activity of extracellular anionic isozymes of POX. Thus, these POXs may bind with and localize the fungal pathogens. We designed an experiment which modeled the interaction between the fungal pathogen and cotton POX enzymes using *V. dahliae* conidia as a chromatographic matrix. Analysis of fraction obtained "in volume" by elution 1 M by NaCl solution showed that POXs from cotton seedlings were capable to bind with conidia of *V. dahliae*. Isoelectric rofocusing shows that cotton seedlings of 'AN-Bayaut-2' had two anionic isoforms with pI 3.4 and 3.9 that bind to *V. dahliae* conidia (**Fig. 1**, lane 4) while 'C-4727'

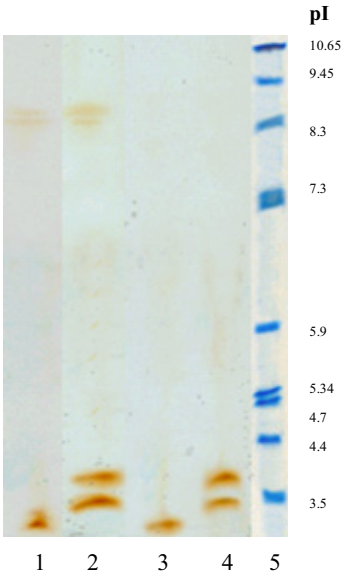

Fig. 1 Peroxidase isoenzyme from cotton seedlings of susceptible 'C-4724' and resistance 'AN-Bayaut-2' (visualized with guaiacol). 1 – total peroxidase fraction of C-4724 before absorption on *V. dahliae* conidia; 2 – total peroxidase fraction of 'AN-Bayaut-2' before absorption on *V. dahliae* conidia; 3 - conidia binding peroxidase fraction of 'C-4724'; 4 – conidia binding peroxidase fraction of 'AN-Bayaut-2'; 5 – pI markers (visualized with CBB G-250).

had only one of these types of isoperoxidase with pI 3.1 (**Fig. 1**, lane 3). A similar effect was obtained in pea root with the widely used elicitor, chitosan, which induced POXs with pI 5.3 and 5.7, which may be specifically related to pathogen defence (Kukavica *et al*. 2012).

The comparative study of chromatographic fractions eluted from conidia of *V. dahliae*, revealed differences in POX activity in both resistant and susceptible seedlings. Enzyme activity in resistant cotton variety with substrate presence in a fraction eluted from fungi conidia appeared to be 30.3 ± 2.5 Unit/mg^{-1}, in susceptible variety was 16.6 ± 3.1 Unit/mg^{-1}.

From this it follows that localization of chitin-binding POX in infection zone and enzyme ability to bind with conidia of phytopathogen fungi allow us to conclusion that POX can effect on growth and development of pathogens directly as well as be involved into synthesis of lignin in zone of contact with mycelium surface of fungi. A histochemical analysis of a non-fixed preparation of cotton vascular cells infected with fungi shows that POX activity is localized, in external and internal surfaces of cell wall and in the zones of necrosis (Pshenichnov *et al.* 2011).

Taken together, the results of the present study strongly indicate that the ratio of anionic peroxidases in the cell wall may be an important parameter for determination of disease resistance and can be considered as enzyme marker of cotton resistance to fungi infection.

Fungal growth inhibition

The anti-fungal properties of peroxidases are not well characterized, although their oxidative products such as indoles, oxygen radicals and halides are well described as having cytotoxic effects on host cells and pathogens (Nappi and Ottaviani 2000).

In this connection, we investigated inhibitory activity of partially purified chitin-binding anionic POX isoforms from 7-day-old seedlings of cotton plant 'AN-Bayaut-2' and 'C-4727' by the method of application of paper disks to lawns of fungi *V. dahliae*. Experiments have shown the direct dependence of inhibitory activity of chitin-binding anionic POXs from roots, hypocotyls and cotyledons in both resistant and susceptible cotton varieties from its enzymatic activity (**Table 3**). The fraction from the column with chitin

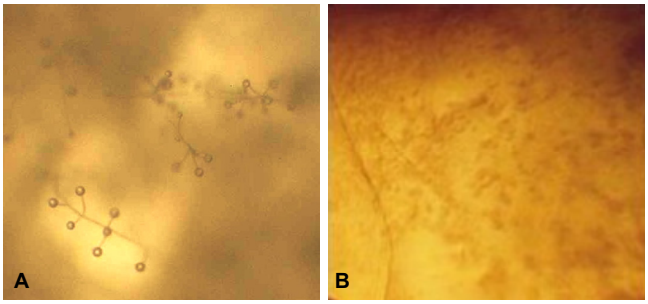

Fig. 2 Optical microscopy of hyphal growth inhibition zones of *V. dahliae*. A – normal state of *V. dahliae* growth: conidia end spores; **B** – morphological changes in *V. dahliae* under the action of purified chitin-binding peroxidase from resistance ('AN-Bayaut-2') cotton seedlings.

Table 3 Fungi growth inhibitory and enzyme activity of chitin-binding peroxidase from cotton seedlings.

Sample	Zone of *V. dahliae* growth inhibition, mm	Peroxidase activity, Unit/mg^{-1}
'AN-Bayaut-2'		
Roots	4.0 ± 0.5 a	23.6 ± 2.8
Hypocotyls	2.0 ± 0.3	17.2 ± 3.0
Cotyledons	6.0 ± 0.2	24.2 ± 2.0
'C-4727'		
Roots	0	12.5 ± 4.0
Hypocotyls	0	4.1 ± 3.8
Cotyledons	0	8.9 ± 2.2

[a] Values are mean ± SD, experiment repeated at least three times. Line correlation between zone of inhibition and enzyme activity of chitin-binding peroxidase is authentic at $r = 0.69$; $P < 0.01$

that had the highest POX activity also exhibited the highest anti-fungal activity, in this fraction the ratio of POX to other proteins was higher.

Microscopy studies of hyphal growth inhibition zone revealed that the purified chitin-binding anionic POX has challenged the swelling of conidia and their atypical accumulation. Such accumulation of fungi conidia (**Fig. 2B**) is typical for development in unfavorable conditions – conidia are accumulating and coating with protective cover. Perhaps, this case confirms localizing effect of chitin-binding anionic POXs in resistant cotton variety.

The mechanism of growth inhibition by the plant chitin-binding proteins is not known. There is a hypothesis given in literature that chitin-binding proteins selectively bind to sites where cell wall synthesis takes place: at conidia which grow isotropically by adding new wall material uniformly in every direction and to hyphal tips of fungi (Wessels 1994). Bormann *et al.* (1999) showed that antifungal proteins interfered with polarized growth at hyphal tips of *Paecilomyces variotii*, leading to abnormal branching and swollen hyphae with weakened walls that did not resist internal turgor pressure upon mechanical stress. Therefore, it might be speculated that chitin-binding POX greatly influences on synthesis of cellular walls of fungi by binding with newly-formed chitin and breaks polarity of growth of filamentous fungi. It can lead to atypical changes of fungi morphology. Primary protection mechanism of resistant cotton variety against fungi *V. dahliae* is, perhaps, bound with agglutination of conidia under effect of chitin-binding anionic isoperoxidases. Caruso *et al.* (2001) isolated a basic POX 36 kDa in size, from wheat kernels, that slowed growth in three fungal species. Their experiments demonstrated that the POX inhibited germ tube elongation and conferred direct anti-fungal activity. Alternatively, the oxygen radicals generated by POXs may indirectly inhibit the fungus. In tobacco plants, POX-generated hydrogen peroxide was shown to prevent germination of fungal spores *in vitro* (Peng and Luc 1992).

This supports the conclusion that among other cellular roles, chitin-binding anionic POXs serve an important role of arresting fungal growth.

CONCLUSION

Thus, molecular mechanisms of resistance in susceptible and resistant cotton varieties in the presence of chitin and chitosan have been revealed. It was shown that pretreatment with chitosan promote the protective potential of on infecting with fungi *V. dahliae* cotton plant due to activation of anion POXs. Inhibitory effect of chitin-binding POXs of resistant cotton variety on growth of *V. dahliae* has been revealed. The changes were shown in fungi morphology evidencing of direct participation of chitin-binding anionic POXs in pathogen localization processes. Investigation of POX role in molecular mechanisms of phytoimmunity to pathogens allows proposing to use this chitin-binding anionic POX as marker resistance cotton varieties to *V. dahliae*.

ACKNOWLEDGEMENTS

This work was supported by a grant FA-A10-T152 from the Department of Science and Technologies Development, Republic of Uzbekistan. The authors thank Dr. Jaime A. Teixeira da Silva for improving the grammar.

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