American Journal of Plant Sciences, **2011, 2, 43-49**
doi:10.4236/ajps.2011.21005 Published Online March 2011 (http://www.SciRP.org/journal/ajps)

Participation of Chitin-Binding Peroxidase Isoforms in the Wilt Pathogenesis of Cotton

Egor Pshenichnov[1], Nigora Khashimova[1], Alik Akhunov[1], Zamira Golubenko[1], Robert D. Stipanovic[2]

[1]A.S. Sadykov Institute of Bioorganic Chemistry, Academy of Science, Tashkent, Uzbekistan; [2] USDA-Agricultural research Service, Southern Plains Agricultural Research Center, College Station, USA.
Email: bob.stipanovic@ars.usda.gov

Received December 17th, 2010; revised January 2nd, 2011; accepted January 19th, 2011.

ABSTRACT

Specific chitin-binding isozymes of peroxidase (*POX*) *play an important role in pathogenesis of plant diseases caused by fungi. We studied the dynamics of peroxidase activity in two varieties of cotton* (*Gossypium hirsutum L.*); *one was susceptible and the other resistant to the plant pathogen Verticillium dahliae. After infection with strongly and weakly virulent isolate of V. dahliae, we observed a correlation between the level of seedling tissue lesion and peroxidase activity. Thus, the first POX activity was observed in all infected plants* 2 *hours after inoculation, but POX activity of the resistant variety rapidly increased and maximized* 3 *days after infection*, *while POX activity in the susceptible variety showed a slow increase that continued to increase during the remaining* 8 *days of experimental observation. The increase of POX activity in the susceptible variety after infection may be explained by progressive fungal colonization of cotton tissues leading to irreversible senescence. Microscopic examination of plant tissues supports this hypothesis. The more virulent isolate caused more necrosis and significantly more POX activity than the mildly virulent in both susceptible and resistant plants. Control plants showed no changes in POX activity*; *however, the POX activity in the control resistant varieties was higher than the control susceptible varieties. These findings indicate the potential utilization of chitin binding POX as a biochemical tool to guide breeding programs to increase resistance to V. dahliae.*

Keywords**:** *Peroxidase*, *Plant Protection*, *Cotton*, *Gossypium Hirsutum*, *Verticillium Dahliae*

1. Introduction

Recognition of a potential pathogen is required to trigger a defense response in living organisms. Plants do not produce immunospecific antibodies; however, some constituents of pathogens can elicit the activation of plant defense pathways. Among these biogenic inductors (elicitors) are proteins, glycoproteins, polyenic fatty acids, and oligosaccharide fragments of fungi wall cells [chitin, β-(1,3)-glucans] [1]. Among the numerous enzymes involved in plant defense mechanisms, peroxidase (POX) occupies a crucial position in an early plant response to pathogens [2]. The pathogen related protein PR-9 POX belongs to an enzyme involved in lignin formation [3]. The increase of lignification processes is connected with active interactions between pathogen structures and POX. Lignin formation on the surface of fungal mycelia in the presence of this enzyme has been described for POX isolated from cucumber [4]. Aver'yanov *et al.* [5] showed an increase in POX activity in rice blast infected plants compared to healthy plants, and researchers have shown that cell wall bound POX produces H_2O_2 in response to fungal pathogen elicitors [6,7]. Hammond-Kosak and Jones [8] have published an extensive review of the interaction between specific plant pathogen elicitors and receptors that elicit plant resistant genes.

Hence, the race specificity of pathogens can be determined by their ability to activate the defense mechanisms of host plants. The varieties of agricultural plants differ in their range of resistance to phytopathogens. Recently, the ability of individual POX isozymes to bind chitin was discussed [9]. This specific sorption of POX on infectious components of fungi containing chitin was observed as abundant scurf of phenol polymers on the haustoria of *Uromices vicia-fabae* [10]. A polysaccharide binding domain was observed in POX anionic isozymes of *Arabidopsis thaliana* and *Cucurbita pepo* [11]. The hypothetical role of POX in pathogenesis relates to peroxidation of chitin fragments of cell walls of the pathogen followed by production of oligosaccharide elicitors [12]. Thus, the role of chitin-binding POX likely includes

initiation of plant defense mechanisms after pathogen attack. Herein we report our investigation of the dynamic activation of POX in seedlings from two cotton varieties (*Gossypium hirsutum* L.) varieties triggered by the action of two different strains of the fungal wilt pathogen *Verticillium dahliae* and discuss its POX activation.

2. Materials and Methods

2.1. Biological Material

Cottonseed (*Gossypium hirsutum* L.) of AN-Bayaut-2 and C-4727 varieties were grown in an experimental field at 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 to be susceptible [13]. Conidia of *V. dahliae* Kleb. (weakly virulent T-4 and highly virulent AN-3 isolates) were originally isolated from the diseased cotton and are from the collection housed at the Institute of Genetics, Academy Sciences of Republic of Uzbekistan, Tashkent, Uzbekistan. The pathogen was grown on Czapek's agar.

2.2. General

Chitin from crab shells (Fluka Chemical Company) was used for chromatography. Isoelectric focusing of proteins was carried out on an LKB Multiphor-2117 apparatus. Sigma Chemical Company's IEF mix 3.5-10.6 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 pH 3.5-10 (LKB, Sweden) and 0.033% ammonium persulfate in 8 M urea. The anode was 0.5% HCI, and the cathode was 0.5% NaOH. The POX activity was ascertained on the gel using a 0.1% solution of benzidine dihydrochloride. POX isozymes were quantitated by reaction with a 0.01% benzidine dihydrochloride solution (Reachim Company, Russia) and 0.005% H_20_2 in a 0.1% sodium acetate buffer (pH 4.7), and the absorption maxima at 620 nm was measured after one minute of reaction time. The POX activity of each isozyme was determined by measuring the color intensity using a LKB Densitometer. The total protein concentration was determined by the method of Lowry [15]. Assays were performed using an optical microscope [Neofot-2 (Carl Zeiss, Germany)] at 90 x magnification. All experiments were repeated at least three times. Data were subjected to analysis of variance (ANOVA), and differences between treatments assessed by Student's two-sample *t*-test at $P < 0.05$.

2.3. Infection of Cotton Seedlings

Cottonseed were cleansed with H_2SO_4, rapidly washed with running water and kept in water overnight. Seedlings were wrapped in paper towels for 7 days at 27˚C. Infection of seedlings was initiated by placing the towels in flasks containing a conidial suspension (10^7 conidia per mL) of *V. dahliae*.

2.4. Lesion Assay

Cottonseeds were sterilized by treatment with a solution containing 36 % H_2O_2 and 96 % ethanol (1:1). *V. dahliae* (AN-3 and 4-T strains) conidia were incubated in separate tubes (2.0x20 cm) with Czapek's agar (1×10^6 per tube; 2.0-2.5 cm columns of media) for 10 days. Then a warm agar solution was added to the tubes and a single sterilized seed was placed in this layer in each of six tubes for each treatment. The lesion on seedlings was evaluated by visual observation and plant damage was assayed by measuring the height of the seedlings and the size of the necrotic zones on the surfaces of damaged seedlings.

2.5. Crude Enzyme Preparation

The enzyme preparation was a modification of that previously described [14]. That is, raw plant material was ground in liquid nitrogen and stirred with 0.1M sodium phosphate buffer (pH 6.6), and 0.5M NaCl (5 mL per 1 g of plant tissue) with a magnetic mixer 1 h at 4˚C. The precipitate was removed by centrifugation (6000 g, 20 min). The supernatant solution was used for further investigations.

2.6. Adsorption of POX Isozymes on Chitin and *V. dahliae* Conidia

All procedures were carried out at 4˚C. The supernatant obtained as described above was treated with ammonium sulfate to 30% saturation. The supernatant solution was collected and brought to 70% $(NH_4)_2SO_4$ saturation. The residue was collected by centrifugation (6000 g, 20 min), resuspended in a minimal volume of twice-distilled water and desalted in 0.01M sodium phosphate buffer (pH 6.6) using "Vivaspin 20" tubes (Sartorius, Germany) at 6000 g. The chitin adsorption assay was performed on a chromatographic column (2 cm × 6 cm) packed with chitin previously equilibrated with 0.1M sodium phosphate buffer (pH 6.6). The crude protein preparation was applied to the top of the column and the column was washed with 0.1 M phosphate buffer with a flow of 20 mL/h to remove the unbound protein. Fractions were collected and protein fractions were detected by monitoring the UV absorption at 280 nm using a flow cell on an Uvicord system (LKB, Sweden). When all of the unbound protein had been eluted, the bound protein was eluted with 1 M NaCl in 0.01 M phosphate buffer (pH 6.6). The *V. dahliae* conidia adsorption assay was carried

out in "volume". Conidia (500 mg) were previously treated with 1N NaOH (5 min) and spun down (6000 g, 20 min.) and then washed with 0.1M sodium phosphate buffer (pH 6.6). After a second spin (6000 g, 20 min.) the desalinated total protein preparation was suspended with conidia in a minimal volume of the same buffer (30 min), and conidia were eluted with some portions of buffer. The column was eluted with 1M NaCl in 0.01M sodium phosphate buffer (pH 6.6). Fractions were monitored at 280 nm as indicated above.

3. Results and Discussion

3.1. Peroxidase Activity in Seedlings

Segments of the two varieties of 7-day-old cotton seedlings were harvested each hour after infection with *V. dahliae* conidia (AN-3 strain) up to 6 hour (**Figure 1(a)**) and then daily for 8 days (**Figure 1(b)**). POX activity in control samples was not statically different at any period compared to time zero. In the case of the AN-Bayaut-2 cotton variety, we observed an increase in POX activity at 2 hours after infection in all segments (**Figure 1(a)**). At 3 hours the POX activity had slightly decreased and further significant increase was observed after 3 days (**Figure 1(b)**) with a gradual lost of activity up to 8 days. The POX activity of the C-4727 was not significantly different than the control after 2 hour and 8 days in any of the time segments; there was a slight increase in POX activity beginning 3 days after infection in C-4727 (**Figure 1(a), (b)**) and continuing until 8 days. This is probably due to the additional tissue that is infected with time. In the case of AN-Bayaut-2, the pathogen infection is quickly contained and additional tissues are not infected; thus, the level of POX decreases.

3.2. Lesion of Seedlings

The effect of the *V. dahliae* isolates on the two cotton cultivars are shown in **Table 1**. The C-4727 cotton seedlings were 1.8 cm shorter than the control seedlings when they were treated *V. dahliae* isolate 4-T, while those for AN-Bayaut-2 were only 0.5 cm shorter than the control when treated with the same isolate. The *V. dahliae* AN-3 isolate had a greater effect on these cotton varieties. That is, C-4727 was 2.6 cm shorter than the control and AN-Bayaut-2 was 2.2 cm shorter.

Surface symptoms of infection appeared for C-4727 cotton seedlings after 4-5 days as small dark necrotic lesions on stems, cotyledon leaves, and roots. These lesions were about 1 cm in length on stem and cotyledon tissues with desiccation of tissues. Fifteen to eighteen days after infection of the C-4727 cultivar with the highly virulent *V. dahliae* isolate AN-3 the seedlings were dead. Thus, the C-4727 cultivar was highly susceptible to the AN-3 isolate. In comparison, when the AN-Bayaut-2 cotton seedlings were infected with the AN-3 isolate, some growth inhibition was observed as well as the appearance of necrotic zones in hypocotyl tissue (about 2 mm length) 15 to 18 days after infection. The isolate 4-T did not cause any surface symptoms except a small inhibition in growth for AN-Bayaut, and only a 1.8 cm inhibition in growth in the C-4727.

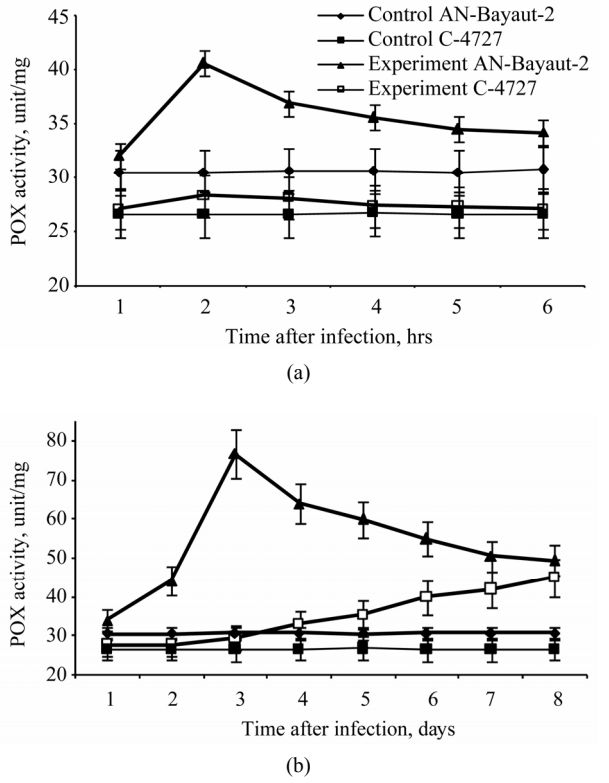


Figure 1. Dynamics of POX activity in 7-days seedlings of two cotton varieties susceptible (C-4727) and resistant (AN-Bayaut-2) after infection (a – in hours, and b – in days) with *V. dahliae* (AN-3 isolate) conidia.

Table 1. Growth inhibition of cotton seedlings after infection with different strains of *V. dahliae* (AN-3, highly virulent; 4-T weakly virulent).

V. dahliae isolate	Height of seedlings after 8 days (cm)	
	C-4727	AN-Bayaut-2
Control	14.5 ± 0.6	13.5 ± 1.2
4-T	12.7 ± 0.8	13.0 ± 1.6
AN-3	11.9 ± 0.5	11.3 ± 1.3

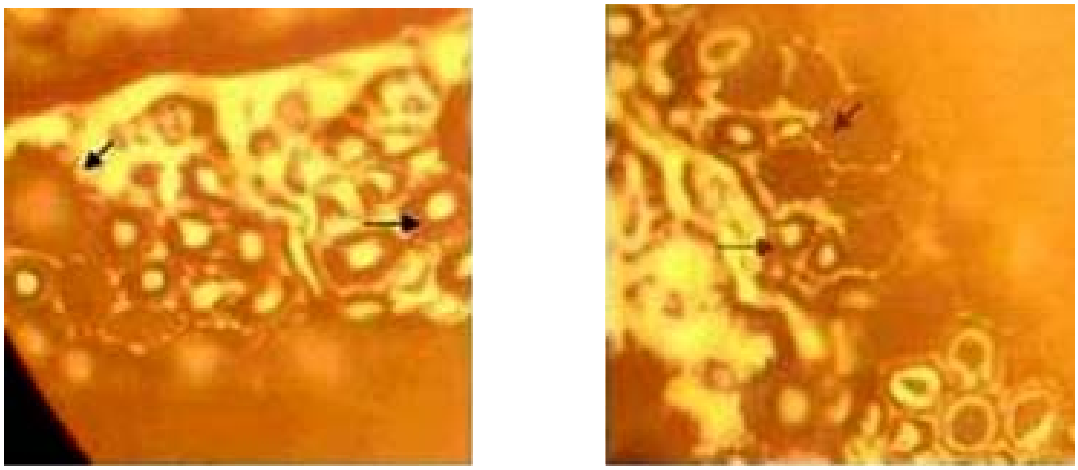
3.3. Optical Microscopic Assay of Cotton Seedling Tissues after Infection with *V. dahliae*

Pathogen penetration sites on root rhizoderma of AN-

Bayaut-2 cotton seedlings appeared under the microscope as zones of necrosis around live cells. **Figure 2(a)** shows the cytoplasm of root cortex cells of plants inoculated with isolate 4-T. These cells were filled with optically dense material. In the case of AN-3 isolate, the mycelia extended to the root cortex parenchyma (**Figure 2(b)**).

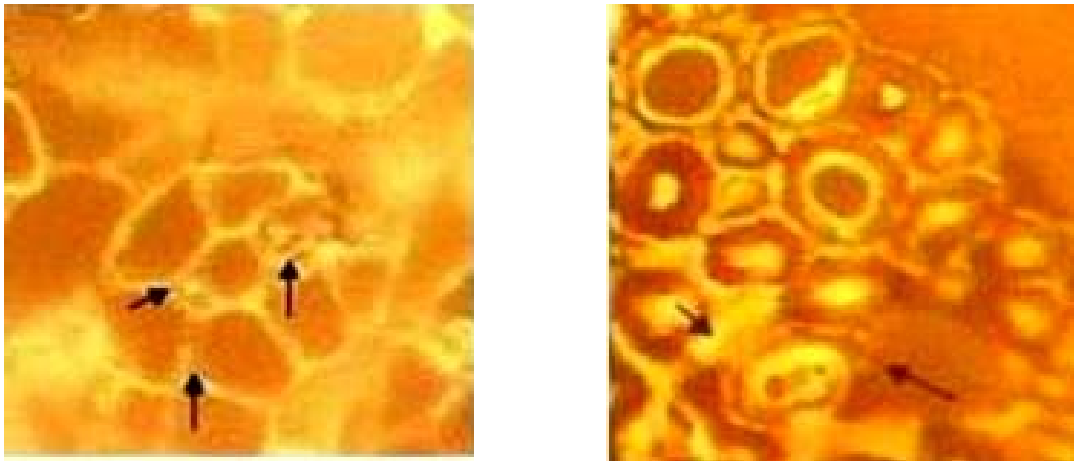
After infection of C-4727 cotton seedlings with both 4-T and AN-3 the mycelia of *V. dahliae* isolates had more thoroughly permeated the root parenchyma. Hypocotyl microscopic sections of AN-Bayaut-2 and C-4727 cotton seedlings had clear differences in pathogen penetration. Hyphae penetrated both the inter- and intracellular C-4727 cotton seedlings. The pathogen freely penetrated the vascular tissues of susceptible C-4727 and can be easily visualized. In the fourth day after infection of C-4727 seedlings we observed colonization of intercellular spaces with AN-3 fungal hyphae (**Figure 3(a)**). In comparison, vascular cells of AN-Bayaut-2 cotton seedlings were thickened and separated from this pathogen (**Figure 3(b)**), and pathogen penetration was not observed.

Tissues of C-4727 vascular system also exhibited cell plasmolysis, *i.e.* plasmolemma discharge from vascular cell wall (**Figure 4(a)**). A histochemical analysis of a non fixed preparation of AN-Bayaut-2 vascular cells shows that POX activity is localized, in external and internal surfaces of cell wall and in the zones of necrosis (**Figures 3(b) and 4(b)**).



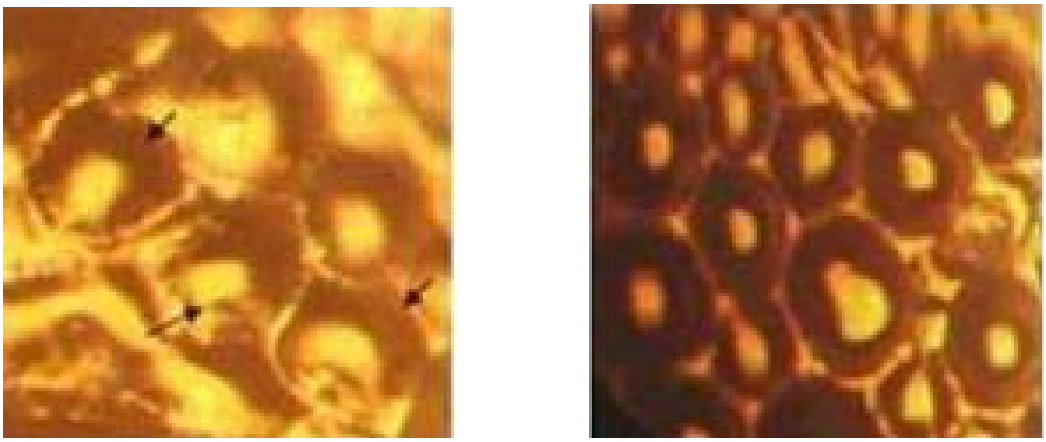
(a) (b)

Figure 2. *V. dahliae* (4-T, a and AN-3, b isolates) penetration of the root rhizoderma of AN-Bayaut-2 cotton seedlings. Arrows indicate nodules in cell wall and necrosis.



(a) (b)

Figure 3. *Verticillium dahliae* (AN-3 isolate) penetration sites of hypocotyls of C-4727 (a) and cotton seedlings (AN-Bayaut-2) (b). Arrows indicate hyphae between cells of C-4727 (a) and cell wall and necrotic zone of AN-Bayaut-2 (b).



(a) (b)

Figure 4. Tissues of C-4727 vascular system after infection with AN-3 isolate of *Verticillium dahliae* (a). Histochemical analysis of POX localization in AN-Bayaut-2 vascular cells infected with AN-3 isolate of *V. dahliae* (b).

3.4. Isozymes with POX Activity in 7-Day-Old Cotton Seedlings

Anionic isozymes of POX accumulate during cell wall lignifications. Zheng [16] and Passardi [17] showed that resistant plants rapidly accumulated lignin after infection with fungal pathogens. The pathogen penetrates through intercellular space by hyphal growth. 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. Isoelectrofocusing shows that 7-days after cotton seedlings of AN-Bayaut-2 had two isozymes that bind to both chitin and *V. dahliae* conidia (**Figure 5, lane 3**) while C-4727 had only one of these types of isozyme (**Figure 5, lane 6**). This data suggest that chitin binding isozymes of POX are signaling molecules in plant defense mechanisms which identify the oligosaccharide containing phytopathogens.

4. Conclusions

Expression of resistance genes to phytopathogens is best observed during the infection process when plant cells are exposed to the pathogen. The riposte reaction presupposes induction of such resistance factors. In our study we examined the dynamics of POX activity in seedlings of two different cotton varieties infected with weak (T-4) and highly virulent (AN-3) isolates of *V. dahliae*. When infected with the highly virulent isolate AN-3, we observed higher POX activity in the infected AN-Bayaut-2 variety as compared to the susceptible C-4727. An increase of POX activity of cotton seedlings infected with *V. dahliae* had a biphasic characteristic during 6 to 8 days.

The first enzyme activation of both varieties was noted 2 h after infection. This activation may be described as

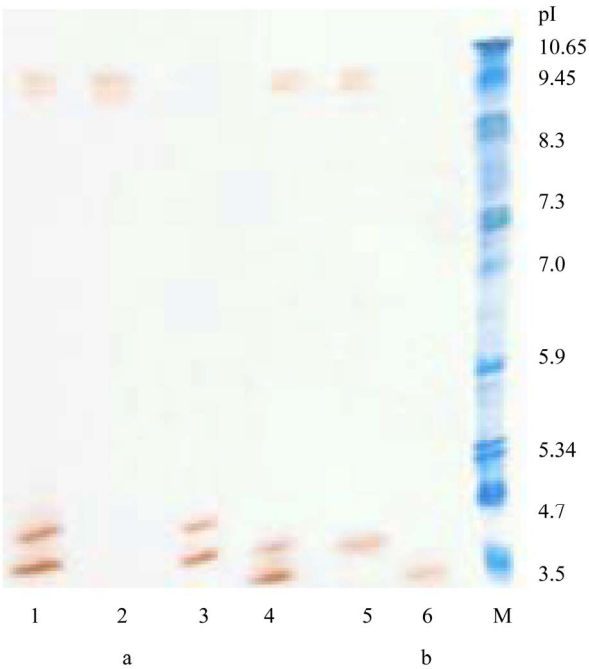


Figure 5. Isozyme spectrum of POX from 7-day-old cotton seedlings (guaiacol/H_2O_2 stained): a. AN-Bayaut-2 cotton seedlings: 1 – total POX fraction; 2 – POX fraction not adsorbed by chitin or *V. dahliae* conidia; 3 – chitin-specific isozymes; b. C-4727 cotton seedlings: 4 – total POX fraction; 5 – POX fraction not adsorbed on chitin and *V. dahliae* conidia; 6 – chitin specific isozyme; M – pI markers (Coomassie BB G-250 stained).

super sensitivity to the pathogen. The second activation of POX was observed during the next few days, and exhibited significant differences in activity. That is, POX activity of AN-Bayaut-2 rapidly increased and maximized 3 days after infection. This was followed by a gradual decreased in activity. POX activity in the C-4727 variety after infection showed a slow increase that continued to increase during the remaining days. The control samples showed no changes in POX activity during the experiment period, however, the POX activity was higher in the resistant AN-Bayaut-2 plant compared to the susceptible C-4727. The increase of POX activity in the C-4727 variety after infection with *V. dahliae* may be explained by progressive fungal colonization of cotton tissues leading to irreversible senescence. Microscopic examination supports this hypothesis.

Thus, our data correlates well with the level of resistance of cotton varieties to fungi pathogens. In addition, the amount of tissue exhibiting necrosis also correlated with the dynamics of POX activity in different parts of the seedlings. The difference of these parameters was dependent on the isolate of *V. dahliae* which was used for infection. That is, the AN-3 isolate, which is the more virulent, caused more necrosis and significantly more POX activity than the 4-T strain.

The hypersensitivity plant reaction to pathogen attack causes POX gene expression and a biphasic accumulation of mRNA transcripts which encode biosynthesis of anionic POX [18]. The first phase occurs 2 to 9 hours after infection with the pathogen. The second phase of POX transcript accumulation differs for susceptible and resistant genotype. This phase in susceptible plants occurs slower and in resistant plants occurs in 24 to 48 hours after the first phase. Thus, susceptible plants have a slower response allowing greater cell damage. Pathogen penetration and penetration inside the root tissues is observed for both susceptible and resistant cotton varieties, but in the resistant variety the surface symptoms do not appear. However, the depth of pathogen penetration depends on the resistance of cotton variety. Observation of surface symptoms of wilt infection depends on overgrowth of fungal hyphae in plant cells. POX localization around cell wall shows active lignifications which blocks fungal penetration. In this case, chitin, the component of cell walls of *V. dahliae*, may induce lignifications. Free radicals produced by the action of POX are highly reactive and form covalent bounds with proteins and carbohydrates of fungi cell walls. Thus, POX may be considered as a defense factor in wilt pathogenesis [11].

These findings offer the potential utilization of chitin binding POX as a biochemical tool to guide breeding programs to increase resistance to *V. dahliae*.

5. Acknowledgements

This work was supported by a grant from the Department of Science and Technologies Development, Republic of Uzbekistan.

REFERENCES

[1] N. Benhamou, "Elicitor-Induced Plant Defense Pathways," *Trends in Plant Science*, Vol. 1, No. 7, July 1996, pp. 233-240.

[2] C. C. Lin and C. H. Kao, "Abscisic Acid Induced Changes in Cell Wall Peroxidase Activity and Hydrogen Peroxide Level in Roots of Rice Seedlings," *Plant Science*, Vol. 160, No. 2, 2001, pp. 323-329. doi:10.1016/S0168-9452(00)00396-4

[3] L. C. Van Loon, "Induced Resistance in Plants and Role of Pathogenesis-Related Proteins," *European Journal of Plant Pathology*, Vol. 103, 1997, pp. 735-765. doi:10.1023/A:1008638109140

[4] R. Hammerschmidt and J. Kuc, "Lignification as a Mechanism for Induced Systemic Resistance of Cucumber," *Physiological and Molecular Plant Pathology*, Vol. 20, 1982, pp. 61-71. doi:10.1016/0048-4059(82)90024-8

[5] A. A. Aver'yanov, V. P. Lapikova, O. N. Nikolaev and A. I. Stepanov, "Active Oxygen-Associated Control of Rice Blast Disease by Riboflavin and Roseoflavin," *Biochemistry* (Moscow), Vol. 65, No. 11, November 2000, pp. 1292-1298.

[6] G. P. Bolwell, K. A. Blee, V. S. Butt, P. R. Davies, S. L. Gardner, C. Gerrich, F. Minilaeva, E. G. Rowntree and P. Woitaszek, "Recent Advances in Understanding the Origin of the Apoplastic Oxidative Burst in Plant Cells," *Free Radical Research*, Vol. 31, December 1999, pp. 137-145. doi:10.1080/10715769900301431

[7] C. S. Bestwick, I. R. Brown, M. H. R. Bennett and J. M. Mansfield, "Localization of Hydrogen Peroxidase Accumulation during the Hypersensitive Reaction of Lettuce Cells to *Pseudomonas syringae* pv. phaseolicola," *The Plant Cell*, Vol. 9, No. 2, February 1997, pp. 209-221.

[8] K. E. Hammond-Kosak and J. D. Jones, "Resistant Gene-Dependent Plant Defense Responses," *The Plant Cell*, Vol. 8, No. 10, October 1996, pp. 1773-1791.

[9] I. V. Maksimov, E. A. Cherepanova, I. E. Akhmetova and R. M. Khairullin, "Contribution of Chitin and Its Oligomers to the Induced Resistance of Plants against Phytopathogens," *Agricultural Chemistry*, Vol. 8, 2004, pp. 77-89.

[10] C. Schweikert, A. Liszkay and P. Schopher, "Polysaccharide Degradation by Fenton Reaction- or Peroxidase-Gene-Rated Hydroxyl Radicals in Isolated Plant Cell Walls," *Phytochemistry*, Vol. 61, No. 1, September 2002, pp. 31-35. doi:10.1016/S0031-9422(02)00183-8

[11] C. Dunand, M. Tognolli, M. Meyer, P. Simon and C. Penel, "Identification and Characterization of Ca^{2}+ —

Pectate Binding Peroxidase in *Arabidopsis thailiana*," *Journal of Plant Physiology*, Vol. 159, No. 11, November 2002, pp. 1165-1171. doi:10.1078/0176-1617-00768

[12] P. Bonatti, P. Medeghini, G. Lorenzini, R. Formasiero, R. Boroni, C. Nali and E. Sgarbi, "Cytochemical Detection of Cell Wall Bound Peroxidase in Rust Infected Broad Bean Leaves," *Journal of Phytopathology*, Vol. 140, No. 4, April 1994, pp. 319-325. doi:10.1007/BF02980704

[13] Sh. I. Ibragimov, "Catalogue of Cotton Varieties. Volume 2," Tashkent, Uzbekistan, 1993.

[14] Z. Golubenko, A. Akhunov, N. Khashimova, Yu. Beresneva, E. Mustakimova, F. Ibragimov, N. Abdurashidova and R. Stipanovic, "Induction of Peroxidase as a Disease Resistance Response in Resistant (*Hibiscus trionum*) and Susceptible (*Althea armeniaca*) in the Family Malvaceae," *Phytoparasitica*, Vol. 35, No. 4, August 2007, pp. 401-413. doi:10.1007/BF02980704

[15] O. N. Lowry, N. Rosebrouhg and R. I. Randall, "Protein Measurement with the Folin Phenol Reagent," *Biolgical Chemistry*, Vol. 193, November 1951, pp. 265-275.

[16] H. Z. Zheng, C. L. Cui, Yu. Zhang, D. Wang, Yu. Jing and K. Y. Kim, "Active Changes of Lignification-Related Enzymes in Pepper Response to Glomus Introduces and/or Phytophthoracapsici," *Journal of Zhejiang University Science*, Vol. 6B, No. 8, July 2005, pp. 778-786. doi:10.1631/jzus.2005.B0778

[17] F. Passardi, C. Penel and C. Dunand, "Performing the Paradoxical: How Plant Peroxidase Modify the Cell Wall," *Trends in Plant Science*, Vol. 9, No. 11, November 2004, pp. 534-540.

[18] P. Kawalleck, E. Schmelzer, K. Hahlbrock and I. E. Somssich, "Two Pathogen-Responsive Genes in Parsley Encode a Tyrosine-Rich Hydroxyproline Glycoprotein (HRGP) and Anionic Peroxidase," *Molecular and General Genetics*, Vol. 247, No. 4, 1995, pp. 444-452. doi:10.1007/BF00293146

