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Indirect ELISA Method for Detection of *Verticillium dahliae* in Cottonseeds

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Abstract: Apart from condign climate and lack of resistant varieties, one of the important reasons causing *Verticillium* wilt more and more serious is seed-borne pathogen. To establish an effective method to detect *Verticillium dahliae* in cottonseeds, we have developed an indirect ELISA method. The toxin of *Verticillium dahliae* strain Bp_2 was used as antigen to prepare polyclonal antiserum. The indirect ELISA method was developed by using alkaline phosphatase-conjugated goat antimouse antibody as secondary antibody according to standard ELISA procedures. If the value of P/N (ratio of OD_{405} of a sample to that of negative control)$\geqslant$2, the sample was positive. The titer of the antiserum was 1∶204800 and the toxin could be detected as low as 7.81 $\mu g \cdot L^{-1}$. A11 of *Verticillium dahliae* strains, collected from different areas in Jiangsu Province, performed positive reaction. No cross-reactivity was observed among the isolates representing other species of fungi such as *Rhizoctonia solani*, *Fusarium moniliforme*, *Fusarium graminearum*, *Sclerotinia sclerotiorum*, *Magnaporthe grisea*. After the *Verticillium dahliae* strain Bp_2 or T_9 was cultured for one day, or the toxin could be detected in the culture solution. Two of strains were cultured after three days, the value of P/N was 9.05 and 8.82, respectively, and these high values could remain for 11 days. So *Verticillium dahliae* in cottonseeds could be deduced by detecting the toxin with this developed indirect ELISA. 81 cottonseed samples, randomly chosen from different seed companies, were detected if there was *Verticillium dahliae* with them. Firstly, 100 g cottonseeds from each sample were washed by glide. Coated cotton-seeds were washed till the chemical coating was moved clearly. Then, the cottonseeds were dried at room temperature and separated to five shares each. Each share was put in 150 mL Czapek's medium and then cultured for three days. The culture medium was centrifuged for 30 min at 5000 $r \cdot min^{-1}$, and the supernatant was tested by using indirect ELISA. 12 out of 81 cottonseed samples performed positive reaction. Cotton seedlings were infected by *Verticillium* wilt inoculated with the culture solution of the cottonseed samples that performed positive reaction. These results demonstrated that the method of indirect ELISA could be used for quickly detection of *Verticillium dahliae* in cottonseeds.

Key words: indirect ELISA; cottonseed; *Verticillium dahliae*; toxin

Cotton *Verticillium* wilt is one of the most destructive diseases of cotton. It was firstly found in U.S.A, and then was transmitted to China in the 1930s. This disease diffused to more than 20 Provinces (autonomous region, city) such as Henan, Shandong, Xinjiang and so on and was ranked the plant quarantine object once again in 1995. From the late 1990s, the damage of cotton *Verticillium* wilt in Jiangsu Province was more and more serious year after year, especially in recent years. One of the most

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important reasons of the disease epidemic is that the pathogen is a cottonseed borne pathogen, besides the lack of resistant varieties and the condign climate. Since *Verticillium dahliae* may survive for quite long time in soil, truculence disaster will bring to cotton if the pathogen spreads uncontrolledly. At present, the main detection methods of *Verticillium dahliae* is PCR and immunoassay[1-2]. However, these detection methods are mostly for cultured *Verticillium dahliae*. Untill now, there are no better methods for detection of *Verticillium dahliae* in cottonseeds. Therefore, it is important to establish an effective detection method for detection of *Verticillium dahliae* in cottonseeds especially in coated cottonseeds. This method can not only protect the no disease area, but also generalize the well-bred cottonseeds. This study showed that polyclonal antiserum was prepared against the toxin of *Verticillium dahliae* strain Bp_2 and it also developed the sensitive indirect ELISA, which can be used for quick detection of *Verticillium dahliae* in cottonseeds.

1 Materials and Methods

1.1 Fungi

Verticillium dahliae strains from cotton including Bp_2, T_9, $02SY_3$, $02XZH_3$, $02GY_3$, $02YD_5$, VD_8, $02NJ_2$, V_{151}, $02FN_8$, $02DT_6$, $02XY_3$, $02CS_3$, *Rhizoctonia solani* from cotton, *Fusarium moniliforme* from cotton, *Fusarium graminearum* from wheat, *Sclerotinia sclerotiorum* from rape, and *Piricularia oryzae* from rice were provided by the Institute of Plant Protection, Jiangsu Academy of Agricultural Sciences.

1.2 Antigen preparation

According to Koppel's method[3], *Verticillium dahliae* strain Bp_2 was cultured in Czapek's medium at 25℃ for 16 days. Liquid culture was centrifuged for 30 min at 5000 $r \cdot min^{-1}$, and the remaining particles were removed from the supernatant using a 0.45 μm filter. Then the liquid culture was concentrated in a ultrafiltration cell(Millipore corporation) with a 10-kDa cut off until a protein concentration of 0.1 $g \cdot L^{-1}$ was reached. The toxin protein was dialyzed against physiological salt solution at 4℃, and the dialysate was used as antigen.

1.3 Antiserum preparation

BALB/C mice were received four intraperitoneal injections at 3-week intervals. The injection consisted of 200 μL of a 1∶1 emulsified mixture of partially purified toxin protein of *Verticillium dahliae* strain Bp_2 (0.38 $g \cdot L^{-1}$) and Freund's incomplete adjuvant. Blood was collected by extirpating the eyeball and antiserum was obtained.

1.4 ELISA

According to Bouterige's method[4], a little change had been made. Ninety-six-well flat-bottom microtiter plates were coated with antigen diluted in carbonate buffer (100 μL per well). After a 2 h incubation at 37℃ or overnight at 4℃, followed by three washes in PBST, 300 μL of a 1% degrease milk powder solution in PBST were added in each well. The plates were incubated for 1 h at 37℃, washed three times in PBST, and incubated for 1 h at 37℃ with the pooled mouse antiserum diluted in 1% degrease milk powder PBST (100 μL per well). After three additional washes in PBST, 100 μL of alkaline phosphates-conjugated goat anti-mouse immunoglobulin G antibodies diluted 1∶6000 in 1% degrease milk powder PBST was added in each well. The plates were incubated for 1 h at 37℃ and washed three times in PBST. Fresh p-Nitrophenyl phosphate was used as a chromogen (100 μL per well), and incubated at 37℃. The reaction was stopped by the addition of 50 μL 0.2 $mol \cdot L^{-1}$ EDTA, and the absorbance at 405 nm was determined on a multiscan. All tests were performed in triplicate. P/N (positive/negative) value equals the average of samples' OD_{405}/ the average of negative control's OD_{405}.

1.5 Identification of the toxin antiserum

The titers of antiserum against toxin were determined as described above with the antiserum diluted from 1∶6400 to 1∶819200 in 1% degrease milk powder PBST while the normal serum was control. The reactions were considered positive when P/N>

2. The antiserum sensitivity, i. e. the maximum antigen dilution of positive reaction, was assayed with the antigen diluted from 2 mg • L^{-1} to 0.0039 mg • L^{-1} in carbonate buffer. The reactions of the antiserum and the culture solution of *Verticillium dahliae* strains from cotton including Bp_2, T_9, $02SY_3$, $02XZH_3$, $02GY_3$, $02YD_6$, VD_8, $02NJ_2$, V_{151}, $02FN_8$, $02DT_6$, $02XY_3$, $02CS_8$, *Rhizoctonia solani* from cotton, *Fusarium moniliforme* from cotton, *Fusarium graminearum* from wheat, *Sclerotinia sclerotiorum* from rape and *Piricularia oryzae* from rice were tested respectively for the antiserum specificity.

1.6 Assay of toxin in culture filtrate

Verticillium dahliae strain Bp_2 and T_9 were cultured in Czapek's medium at 25℃ respectively. Toxin in culture filtrate taken after cultured 1, 3, 5, 7, 9 and 11 days was assayed using this indirect ELISA as described above, while blank culture medium was negative control.

1.7 Assay of toxin in cottonseed samples

81 suspected cottonseed samples from the Jiangsu Plant Protection Unit were determined whether *Verticillium dahliae* existed in them. The procedure was as follows: each cottonseed sample (100 g) in tuck net was rinsed respectively. Samples of cottonseed without down were rinsed for 23 times by glide while coated cottonseed samples were washed through and through until coating chemical was removed clearly, and then the cottonseed samples were dried in the shade respectively. Each sample was divided to five shares and each share was cultured in 150 mL Czapek's medium contained chloromycetin at 25℃ for three days respectively. Liquid culture was centrifuged for 30 min at 5000 r • min^{-1}, and the culture solution was assayed using this indirect ELISA as described above, while toxin of *Verticillium dahliae* strain Bp_2 was positive control and blank culture medium was negative control.

1.8 Cotton inoculated with culture solution of cottonseed borne *Verticillium dahliae* by needle

Cottonseed samples with positive result of the indirect ELISA were washed and dried as descried above. Each cottonseed sample (100 g) was cultured in 600 mL Czapek's medium contained chloromycetin at 25℃, 150 r • min^{-1} for 6 d respectively, while *Verticillium dahliae* strain Bp_2 was positive control. Liquid culture was centrifuged for 10 min at 2000 r • min^{-1}, and the supernatant was inoculated to the basal stem of cotton variety Simian 3 seedlings in the second euphytta period by needle[15]. The colors of vascular bundle were investigated after 20 days and the disease incidence was calculated.

2 Results and Analysis

2.1 Antiserum sensitivity and specificity

An effective antiserum was produced against the toxin protein *Verticillium dahliae* strain Bp_2. Dilutions of antiserum to 1∶204800 gave $OD_{405} > 2$ times of the OD_{405} of control (P/N>2), while dilutions to 1∶409600 gave P/N < 2 (Fig. 1). So the titre of the prepared antiserum was 1∶204800.

The specificity of the antiserum was tested by using indirect ELISA. The results (Table 1) showed that the reactions of the antiserum and the culture solution of *Verticillium dahliae* standard stain T_9 from U. S. A. or strains from here and there in Jiangsu Province were positive, while the reactions of the antiserum and the culture solution of other species of fungi were negative. Thus, we thinked the prepared polyclonal antiserum were high specificity and could be used for detection the toxin of *Verticillium dahliae*.

The antiserum sensitivity was assayed. The OD_{405} of antigen diluted to 7.81 μg • L^{-1} was more than 2 times of the OD_{405} of control (P/N>2), while diluted to 3.91 μg • L^{-1} gave P/N < 2 (Fig. 2). Therefore, the toxin could be detected as low as 7.81 μg • L^{-1} using the prepared antiserum.

2.2 Assay of toxin in culture filtrate

The toxin in culture solution could be detected after *Verticillium dahliae* strain Bp_2 and T_9 were cultured for one day. The OD_{405} of culture solution of strain Bp_2 and T_9 cultured for three days reached 1.407, 1.371, and the P/N values were 9.05, 8.82,

respectively. And OD_{405} kept high level till cultured for 11 days (Fig. 3). The result indicated that the developed indirect ELISA could be used for quickly detection of toxin in culture solution of *Verticillium dahliae*.

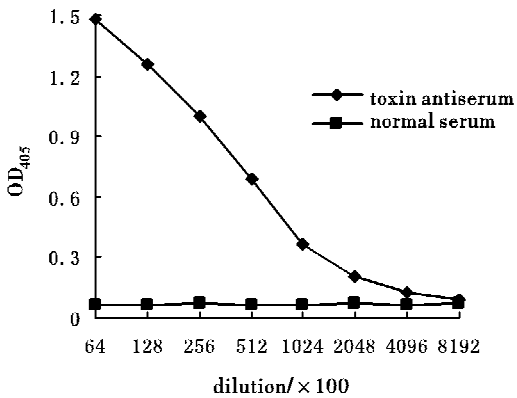

Fig. 1 Titres of antiserum against toxin

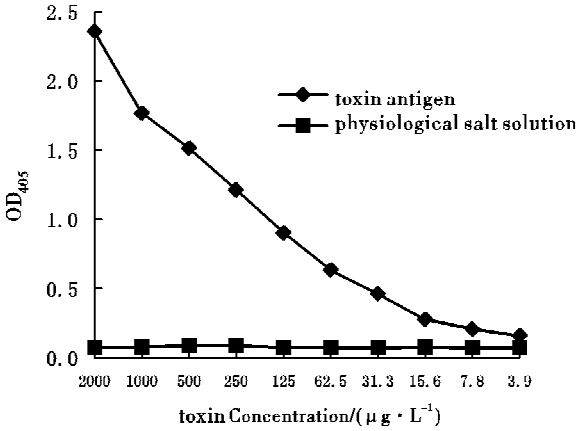

Fig. 2 Determination of minimum amount of the toxin antigen

Table 1 Specialization of antiserum against toxin

	Fungi(collect location)	OD_{405}	P/N value	Positive/negative reaction
Verticilium dahliae from cotton	T_9 (U. S. A)	1.668	21.95	Positive
	$02SY_3$ (Siyang county, Jiangsu Province)	0.658	8.66	Positive
	$02XZH_3$ (Xuzhou city, Jiangsu Province)	1.067	14.04	Positive
	$02GY_3$ (Guanyun county, Jiangsu Province)	0.428	5.63	Positive
	$02YD_5$ (Yandu county-level city, Jiangsu Province)	1.352	17.79	Positive
	VD_8 (Nantong city, Jiangsu Province)	1.465	19.28	Positive
	$02NJ_2$ (Nanjing city, Jiangsu Province)	0.682	8.97	Positive
	V_{151} (Tongzhou county-level city, Jiangsu Province)	0.499	6.57	Positive
	$02FN_8$ (Funin county, Jiangsu Province)	1.148	15.11	Positive
	$02DT_6$ (Dongtai county-level city, Jiangsu Province)	1.098	14.45	Positive
	$02XY_3$ (Xinyang testing farm of agriculture academy in Jiangsu coastal area)	1.192	15.68	Positive
	$02CS_3$ (Changshu county-level city, Jiangsu Province)	1.661	21.86	Positive
Rhizoctonia solani from cotton		0.113	1.49	Negaive
Fusarium monili forme from cotton		0.110	1.45	Negative
Fusarium graminearum from wheat		0.086	1.13	Negative
Sclerotinia sclerotiorum from rape		0.089	1.17	Negative
Piricularia oryzae from rice		0.083	1.09	Negative
Positive control(Antigen)		2.160	28.42	Positive
Negative control		0.076	--	--

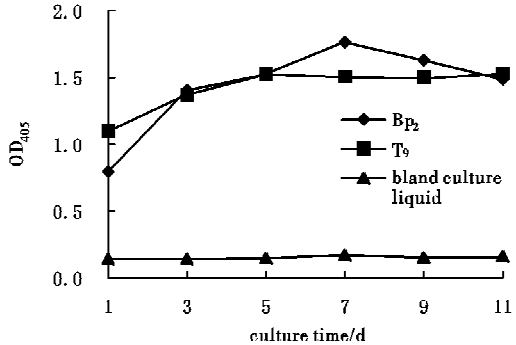

Fig. 3 Detection of toxin in the culture of *Verticillium dahliae* strain Bp_2, T_9

2.3 Application in detection of *Verticillium dahliae* in cottonseed with indirect ELISA

81 suspected cottonseed samples were determined whether *Verticillium dahliae* existed in them with this indirect ELISA. The results (Table 2) showed that there were 4 out of 62 coated cottonseed samples, 8 out of 17 cottonseed samples without down and 0 out of 2 down cottonseed samples were positive reaction. The results also showed that only part of shares (20 g each) of one cottonseed sample

with positive result was positive reaction. The culture solution of 12 cottonseed samples with positive result was inoculated to the basal stem of cotton variety Simian 3 seedlings by needle respectively. 20 days later, the cotton seedlings had been infected by examination the colors of vascular bundle. The disease incidence in details was shown in Table 3.

Table 2 Illustration of the cottonseed samples of positive reaction in indirect ELISA

Serial number of cottonseed sample		OD_{405}			Average	Reaction
1-21	①	0.384	0.352	0.463	0.400	Negative
(coated	②	0.488	0.502	0.593	0.528	Negative
cottonseed)	③	0.744	0.777	0.932	0.818	Positive
	④	0.813	0.816	0.958	0.862	Positive
	⑤	0.219	0.213	0.224	0.219	Negative
Positive control		1.478	1.447	1.571	1.499	
Negative control		0.353	0.316	0.378	0.349	
2-4	①	0.561	0.574	0.587	0.574	Positive
(cottonseed	②	0.310	0.319	0.318	0.316	Negative
without down)	③	0.482	0.455	0.516	0.484	Negative
	④	0.566	0.571	0.605	0.581	Positive
	⑤	0.247	0.259	0.291	0.266	Negative
2-5	①	0.403	0.411	0.396	0.403	Negative
(cottonseed	②	0.606	0.589	0.567	0.587	Positive
without down)	③	0.607	0.581	0.565	0.584	Positive
	④	0.446	0.437	0.426	0.436	Negative
	⑤	0.485	0.532	0.459	0.492	Negative
Positive control		1.055	1.030	0.974	1.020	
Negative control		0.246	0.255	0.254	0.252	

Table 3 The disease incidence of inoculated simian 3 seedlings

Serial number of cottonseed sample	Disease incidence	Cottonseed sample	Disease incidence
1-8	70.00	2-13	58.33
1-21	50.00	2-14	63.64
2-1	63.64	2-15	30.00
2-4	54.55	2-16	45.45
2-5	41.67	3-13	60.00
2-8	41.67	Positive control(Bp_2)	60.00
2-11	54.55	Negative control(PDB)	0.00

3 Discussion

Cotton *Verticillium* wilt caused by *Verticillium dahliae* kleb is a kind of vascular bundle wilt disease and the plant quarantine object. The routine methods for detection *Verticillium dahliae* are complex manipulation, taking a long time but not high specificity. Immunoassay with antiserum prepared against the mycelium of *Verticillium dahliae* may have the disadvantages of low antiserum titre and cross-reaction with other pathogen of the same antigen component[6]. Toxin protein produced by *Verticillium dahliae* is the major reason of causing the susceptive variety wilt[7]. Therefore, *Verticillium dahliae* can be detected by using the antiserum against the toxin. Liu et al successfully detected *Verticillium dahliae* in the pathogen's cultural filtration, cotton seedlings inoculated pathogen and diseased cottons in the field using the antiserum of toxin

protein[8]. Moreover, Qi's study indicated that the antiserum prepared against severe defoliating virulence strain of *Verticillium dahliae* on cotton could be used to identify severe defoliating virulence strains, and the P/ N value may be used to measure differential types on virulence of vast strains[2]. But there are no reports on detection of *Verticillium dahliae* in cottonseed using this method. In this paper, polyclonal antiserum was prepared against toxin of *Verticillium dahliae* strain Bp_2 and the indirect ELISA method for detection of *Verticillium dahliae* in Cottonseeds was developed. The detection method can be used for quickly detection *Verticillium dahliae* in cottonseed, which has the advantages of simpleness and high reliability at present time.

Chen's study indicated that the percentage of interior matured seed from the dehisced bolls borne *Verticillium dahliae* was 0.025%[9]. In this paper, each cottonseed sample was randomly selected 100 g (about 1200 seeds) for detection, and then the 100 g seeds were divided to five shares and each share was cultured in a flask. That didn't mean every share borne fungus. Hence, the results of indirect ELISA of some shares were positive and the others were negative for the same cottonseed sample. But 100 g seeds were cultured in one flask and the culture solution was inoculated to the cotton seedlings in order to certify the reliability of the indirect ELISA results in bioassay experiment. Moreover, the second antibodies were not peroxides-conjugated but alkaline phosphates-conjugated goat anti-mouse antibodies, which avoided the interferes of peroxides and hydroxybenzene in the plants and increased the reliability of the experiment results.

At present, most coating chemical is not used for *Verticillium* wilt but for seedling diseases. *Verticillium dahliae* detected from the coated cottonseed samples illuminated the coating chemical didn't killed *Verticillium dahliae* completely and coated cottonseeds weren't germfree cottonseeds in this paper. The results also showed that the rate of borne *Verticillium dahliae* from down and coated cottonseeds was much lower than that from the cottonseeds without down. Indirect ELISA for detection of *Verticillium dahliae* in cottonseeds will be more significant if monoclonal antibodies are prepared.

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