Techniques

Enzyme-Linked Immunosorbent Assay for Detection of *Verticillium* spp. Using Antisera Produced to *V. dahliae* from Potato

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ABSTRACT

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Polyclonal antisera were prepared against purified mycelial proteins from *Verticillium dahliae*, the predominant fungus species in the potato early dying complex. The antisera tested against crude mycelial preparations of *Verticillium* spp. using indirect enzyme-linked immunosorbent assay (ELISA) reacted positively with 11 of 12 *V. dahliae* isolates from potato, cotton, and soil, but negatively with one isolate from tomato. The antisera did not react with mycelial proteins from *Fusarium* spp. from potato and cotton, with a *Colletotrichum* sp. from potato, or with one isolate of *Rhizoctonia solani* from sugarbeet. Double antibody sandwich ELISA, using polyclonal antisera, detected *V. dahliae* and *V. albo-atrum* in infected roots and stems of potato.

Additional keywords: early dying potatoes, Verticillium wilt of potatoes.

Verticillium wilt of potato (*Solanum tuberosum* L.) may be caused by either *Verticillium albo-atrum* Reinke & Berthier or *V. dahliae* Kleb. In Minnesota, and in other potato-growing areas of central and western United States, this disease is caused primarily by *V. dahliae*. Fungi involved in potato early dying can be identified by cultural isolations from freshly collected stem tissue. Propagule concentrations in infected potato can be determined by plating expressed stem sap on selective medium (3), by counting microsclerotia in stems (19), or by the Anderson sampler-selective medium technique developed by Davis et al (10). Because these methods are laborious, and culturing requires at

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least 1 wk to obtain results, an alternative approach would be to use serological procedures such as enzyme-linked immunosorbent assay (ELISA). Double antibody sandwich (DAS)-ELISA is especially useful for detecting antigens in complex mixtures, such as soil or plant extracts, because the bound antibody specifically captures the antigen(s) of interest, while irrelevant material is removed in the initial wash step.

ELISAs have found widespread application in plant pathology; these assays are routinely used for detection and identification purposes (8,13,19). Their applicability to the detection of fungal pathogens in host tissues has also been reported (1,2,11,16). A number of workers (7,11,12,20) have reported the production of antisera against fungal pathogens but have indicated difficulties with obtaining high specificity. Antisera produced against *V. dahliae* using whole cells, cell walls, soluble homogenates, or conidia as antigenic sources have resulted in various degrees of specificity. Nachmias et al (15) reported the production of an antiserum specific to strains of *V. dahliae* pathogenic to potatoes, using extracellular protein-lipopolysaccharide complexes as an antigen source. Our objective was to determine whether *V. dahliae* could be detected serologically using polyclonal antibodies prepared against the purified *V. dahliae* soluble mycelial proteins. A preliminary report describing a portion of this work has been published (17).

MATERIALS AND METHODS

V. dahliae and *V. albo-atrum* were obtained in pure culture on potato-dextrose agar (PDA) and maintained by hyphal tip transfer. Larger quantities of mycelia were cultured in flasks containing 100 ml of Czapek-Dox broth inoculated with the conidial suspensions. Flasks were incubated at room temperature on a rotary shaker for 7 days. Cultures were harvested by filtering through four layers of cheesecloth and washed thoroughly with distilled water. The soluble mycelial proteins were extracted with 3 vol (w/v) of cold 0.05 M Tris-HCl buffer (pH 8.0) containing 17% sucrose, 0.1% ascorbic acid, and 0.1% cysteine hydrochloride. Each mycelial mat was ground thoroughly with acid-washed sand and Carborundum with a pre-cooled mortar and pestle. The suspension was centrifuged at 12,000 *g* for 15 min at 4 C. The supernatant was collected and centrifuged at 35,000 *g* at 4 C for 30 min. The supernatant was dialyzed for 48 h against cold distilled water at 4 C, and the dialysate was lyophilized. Protein content of the lyophilized powder was determined by using the Bio-Rad protein assay (Bio-Rad Chemical Div., Richmond, CA) (6). Soluble proteins obtained from mycelial extracts were separated by polyacrylamide gel electrophoresis (PAGE) (14% native gel) as described by Benhamou et al (5). Species-specific bands were excised and incubated overnight at 4 C in 1 ml of an extraction solution (0.05 M Tris-HCl buffer, pH 8.0). Eluted protein was dialyzed against distilled water, and the dialysate was lyophilized. The protein content of the lyophilized powder was estimated and used to immunize rabbits.

Immunization technique. For each immunization, 300 g of purified protein was dissolved in 1 ml of 0.01 M phosphate-buffered saline (PBS), pH 7.2, and emulsified with 1 ml of Freund's complete adjuvant (Difco Laboratories, Detroit, MI). The protein + adjuvant were injected intramuscularly into rabbits each week for up to 4 wk. Blood was collected from the middle ear vein, the serum fraction was diluted with 1 vol of glycerol, and 0.01% sodium azide was added before storage at −10 C. The serum used in this study was collected on day 30 after immunizations were started.

Sample preparation. Two types of samples were analyzed by ELISA. Sample A included mycelia of *V. dahliae*, *V. albo-atrum*, and *Fusarium* sp. (1 g of each fungus in 5 ml of 0.05 M Tris-HCl buffer [pH 8.0] containing 17% sucrose, 0.1% ascorbic acid, and 0.1% cysteine hydrochloride) that were homogenized in an ice bath for 5 min with a Brinkman polytron (Brinkman Instruments Co., Westbury, NY). Mycelial extracts were then diluted (1:50) with the carbonate-coating buffer (pH 9.6) (0.06 M Na_2CO_3 + $NaHCO_3$) and used in indirect ELISA. Serum specificity also was checked against tissue homogenates of other fungal species; the fungi used for these assays and their sources are presented in Table 1. Sample B included infected and healthy potato plant extracts that were obtained by grinding and centrifuging 1 g of roots or stem in 5 ml of 0.01 M PBS containing 0.2% sodium sulfite and 0.2% sodium dodecyl sulfate (SDS). Supernatants of these samples were diluted 1:1–1:10 with carbonate-coating buffer.

Infected tubers were obtained from a field with a high population of *V. dahliae* and from infected plants grown in the greenhouse in pasteurized soil to which *Verticillium*-infested ground potato stems had been incorporated. Root and stem fragments from healthy and inoculated plants were subdivided into two parts: 1) root and stem tissues were analyzed for the presence of *V. dahliae* by plating on Czapek-Dox agar medium; 2) root and stem tissues were surface-sterilized, rinsed, weighed, and ground in PBS for analysis by DAS-ELISA.

ELISAs. Two techniques were used: indirect ELISA for fungal homogenates described by Gerik et al (12) and direct DAS-ELISA described by Clark and Adams (9) for plant tissue homogenates. Antigen extracts for the indirect technique were diluted in a carbonate buffer, pH 9.6, and 200 µl per well was added to plates (Immulon 1, Dynatech Corp., Burlington, MA). The plates were incubated for 3 h at 37 C and then were washed three times according to Clark and Adams (9). Rabbit antiserum diluted 1:1,000 with the indirect ELISA buffer (12) was added to wells and incubated for 3 h at 37 C. After washing, conjugate composed of goat-anti-rabbit immunoglobulin coupled with alkaline phosphatase (Sigma Chemical, St. Louis, MO) diluted to 1:1,000 was added to plates and incubated for 1 h at 37 C. p-Nitrophenyl phosphate (1 mg/ml) was then added and incubated 1 h at room temperature. Absorbance A_{405} was recorded 30 min after the addition of substrate using a Bio-Tek Instruments Microplate auto reader (Bio-tek Instruments, Burlington, VT). The reactions were considered positive when their $\bar{x}$ absorbance A_{405} exceeded the $\bar{x}$ absorbance for eight healthy controls plus two times the standard deviation of the $\bar{x}$ of the healthy controls.

TABLE 1. Indirect-ELISA of mycelial triturates of fungi using antiserum developed to *Verticillium dahliae*[a]

Fungi	Isolate	Host	Absorbance[a]
V. dahliae	Vd1[b]	Potato	2.579
V. dahliae	70-21[c]	Pepper	0.972
V. dahliae	P-103[c]	Potato	0.707
V. dahliae	V-44[c]	Cotton	0.795
V. dahliae	318[c]	Potato	0.624
V. dahliae	P-297[c]	Potato	0.545
V. dahliae	20-B[c]	Tomato	0.076
V. dahliae	P-10[c]	Potato	0.985
V. dahliae	S-228[c]	Soil	0.494
V. dahliae	BB[c]	Potato	0.711
V. dahliae	V-10-86[c]	Potato	0.992
V. dahliae	P-283[c]	Potato	0.446
V. albo-atrum	Val[b]	Potato	0.570
V. nigrescens	[b]	Potato	0.062
V. tricorpus	S-137[c]	Soil	0.050
Fusarium oxysporum			
f. sp. *vasinfectum*	51-3[d]	Potato	0.046
f. sp. *vasinfectum*	57-1[d]	Potato	0.067
f. sp. *vasinfectum*	58-2[d]	Potato	0.065
f. sp. *vasinfectum*	59-2[d]	Potato	0.102
f. sp. *vasinfectum*	62-1[d]	Potato	0.078
f. sp. *vasinfectum*	65-1[d]	Potato	0.140
f. sp. *vasinfectum*	67-1[d]	Potato	0.080
f. sp. *vasinfectum*	99[d]	Cotton	0.085
f. sp. *vasinfectum*	113[d]	Cotton	0.075
Colletotrichum lindemuthianum	[b]	Potato	0.005
Rhizoctonia solani AG-4	[b]	Sugar beet	0.122

[a] Mean of two assays, four replicates per assay.
[b] Isolates obtained from N. Anderson, Dept. of Plant Pathology, University of Minnesota, St. Paul.
[c] Isolates obtained from R. Rowe, Dept. of Plant Pathology, Ohio State University, Wooster.
[d] Isolates obtained from T. Kommedahl, Dept. of Plant Pathology, University of Minnesota, St. Paul.

Plates for direct DAS-ELISA tests were coated with *V. dahliae*-specific IgG diluted 3 μg/ml in carbonate buffer and incubated for 4 h at 37 C. After washing the plates three times, plant extracts were prepared as in the sample B procedure, diluted in 0.01 M PBS containing 0.2% Na_2SO_3, and 0.2% SDS, added to plates, and incubated for 3 h at 37 C. The plates were washed three times, and 200 μl of 1:500 dilution of enzyme conjugate prepared by coupling *V. dahliae*-specific IgG with alkaline phosphatase in the presence of glutaraldehyde (4) was added to each well and incubated for 4 h at 37 C. The plates were washed three times, 1.0 mg/ml of p-nitrophenyl phosphate was added, and absorbance readings were taken as described above.

Western blots. For western blot analysis, electrophoresis of the soluble mycelial proteins from *V. dahliae* and *F. oxysporum* f. sp. *vasinfectum* was performed in nondenaturing gels as described above. After electrophoresis, the gel was transferred to a tray containing transfer buffer (25 mM Tris-HCl, 192 mM glycine, 20% methanol, v/v, pH 8.3) (18) for equilibration for 30 min. The proteins were blotted by electrophoretic transfer onto 0.45 μm of nitrocellulose sheets (Millipore, Bedford, MA) using a Transphor chamber (Hoefer Scientific Instruments, San Francisco, CA), for 45 minutes. The nitrocellulose membrane was then incubated with blocking buffer (PBS-Tween 20, pH 7.4, containing 2% PVP-10 and 3% BSA) for 3 h at 37 C. After washing four times with washing solution, the blotted membranes were incubated overnight with rabbit antiserum diluted 1:300 in indirect ELISA buffer. The membranes were washed four times with washing solution and then incubated for 2 h at 37 C with goat-anti-rabbit immunoglobulin (1:300) coupled with alkaline phosphatase (Sigma Chemical Co.). The membranes were washed four times with washing solution and one more time with distilled water before the naphthol AS-TR phosphate/Fast-Blue RR salt substrate (Sigma Chemical Co.) in 0.2 M Tris-HCl buffer, pH 9.1, was added. After staining for 10 min., the membranes were rinsed with distilled water and air dried.

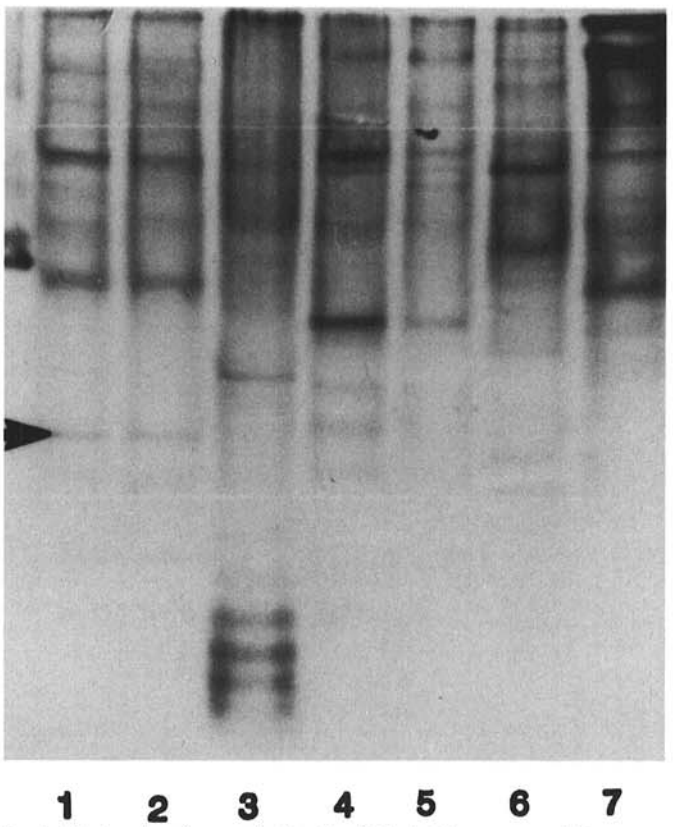

Fig. 1. Electrophoretic protein bands of *Verticillium* spp. and *Fusarium* sp. on a polyacrylamide native gel (14%) and stained with Coomassie blue. 1 and 2, *V. dahliae* (Vdl); 3, *V. dahliae* (pepper isolate); 4 and 5, *V. albo-atrum* (Val); 6, *V. nigrescens*; 7, *Fusarium*. Arrow points to the band that was selected as immunogen.

RESULTS

Electrophoresis of four mycelial protein extracts of *Verticillium* spp. and an extract of *Fusarium* sp. showed that the band patterns obtained from mycelial extracts could vary with culture age. The best protein band separations were obtained with 7-day-old cultures using 14% polyacrylamide gels. Results obtained with Coomassie blue staining showed that *V. dahliae* and *V. albo-atrum* differed from *Fusarium* in the low molecular weight proteins (Fig. 1). One of the most pronounced bands (shown by arrow), which was constantly associated with the *V. dahliae*, was eluted and used as immunogen.

Serological detection of *Verticillium sp.* An antigen dilution of 1:50 of *V. dahliae* mycelial extract reacted with an antiserum titer of 1:50 by double diffusion in agarose and 1:1,000 with indirect ELISA. Reactivity of homogenates of 7-day-old mycelial extracts of *V. dahliae* was the same as 30-day-old mycelial homogenates. The *V. dahliae* antiserum also reacted less intensely with dilutions up to 1:100 of homogenized conidia in buffer (Fig. 2).

Antiserum specificity. To determine the possibility of non-specific cross reactions with other fungal isolates from potato, antigens from several *Verticillium* spp. and other fungi often found in potato tubers and roots were tested by indirect ELISA. Of the 12 *V. dahliae* isolates tested, all except isolate 20-B (tomato isolate) reacted positively (Table 1). Among other fungi evaluated, reactions were negative for *Fusarium* (seven potato isolates and two cotton isolates), *Colletotrichum lindemuthianum*, *Rhizoctonia solani*, *V. nigrescens*, and *V. tricorpus*. Our antiserum

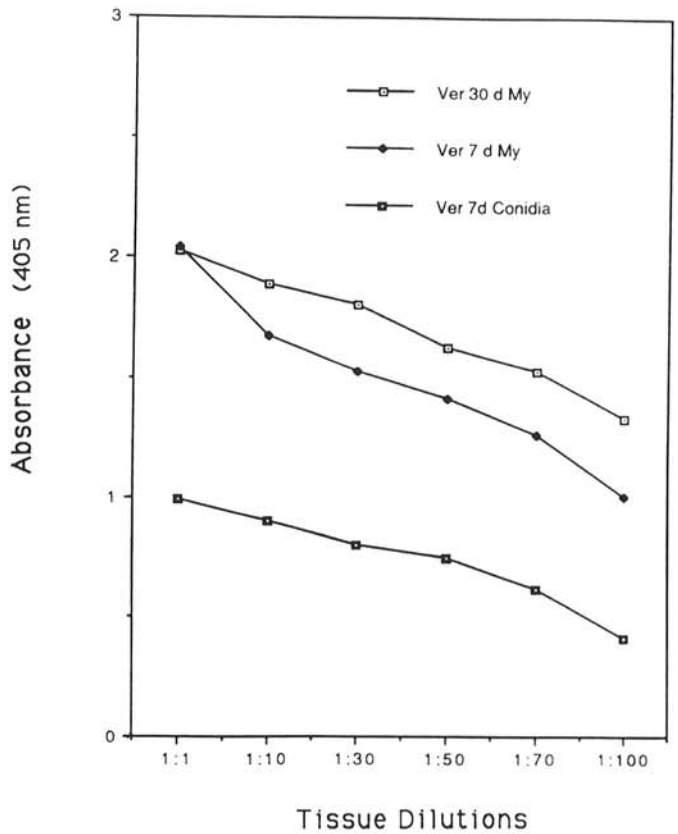

Fig. 2. Reactivity of day 30 antiserum (Vdl) with the mycelial and conidial extracts of *V. dahliae* in indirect enzyme-linked immunosorbent assay. Conidia of *V. dahliae* were separated from hyphae obtained from Czapek-Dox cultures with a 38-μm sieve. One gram of tissue (mycelial and conidia) homogenized separately in an ice bath for 5 min with a Brinkman polytron (Brinkman Instruments, Westbury, NY) after addition of 1 vol of Tris-HCl buffer. Preparations were then diluted 1:1–1:100 with the carbonate-coating buffer and 200 μl of sample used for coating the plates. Antiserum was added at 1:1,000 dilution. Ver 3 d My = *Verticillium dahliae* 30-day-old mycelium; Ver 7 d My = *V. dahliae* 7-day-old mycelium; Ver 7 d conidia = *V. dahliae* 7-day-old conidia.

reacted strongly ($A_{405} = 2.7$) with *V. dahliae* and less intensely ($A_{405} = 0.5$) with *V. albo-atrum* (Fig. 3).

The specificity of the antibodies prepared for *V. dahliae* mycelial extracts fractionated by PAGE was confirmed by western immunoblotting. As shown in Figure 4, the antiserum reacted with the purified protein of *V. dahliae* antigens, but not with the *F. oxysporum* proteins.

Comparison of ELISA for detecting *V. dahliae* and *V. albo-atrum* in potato plants. Two immunoenzymatic methods enabled the identification of *V. dahliae* and *V. albo-atrum* in pure culture and from infected plants (roots and stems 2 mo after inoculation in the greenhouse). Indirect ELISA was not sufficiently specific for detecting *V. dahliae* and *V. albo-atrum* in potato plants, because positive reactions also occurred with healthy roots and stems. For this reason, direct DAS-ELISA was compared in later trials. DAS-ELISA was carried out with extracts prepared according to the sample B procedure. In the different batches, the percentage of plants in different groups that tested positive by ELISA varied from 20 to 100. Even if some absorbance values (A_{405}) appeared to be low, the low level of nonspecific reaction (0.075–0.15) permitted differentiation of the positive and negative reactions (Table 2). The recovery of *V. dahliae* from infected stem tissues (obtained by plating) was found to be highly correlated with DAS-ELISA.

DISCUSSION

Antibodies prepared from PAGE-separated proteins obtained from an isolate of *V. dahliae* enabled the detection of 11 different cultured isolates of this pathogen from potato, cotton, and soil (Table 1). Serum obtained at day 30 and diluted 1:1,000 reacted strongly ($A_{405} = 2.7$) with *V. dahliae* and to a lesser extent ($A_{405} = 0.5$) with *V. albo-atrum*. Previous workers have reported various degrees of specificity of antisera produced against fungal antigens (7,11,12,14,15,20). Many of these antisera were prepared using tissue preparations containing fungal cell walls or cell wall materials. Gerik et al (12) produced relatively specific *V. dahliae* antiserum with a soluble protein extract (mycelial crude extract), although their antiserum also reacted with *Fusarium* sp. The lack of specificity of their serum was probably due to the presence of proteins common to *Verticillium* and *Fusarium*. One potentially useful method for circumventing this difficulty was to produce a more highly purified immunogen. A procedure that was used in this work and also used by Ouellette and Benhamou (16), was to isolate specific protein(s) from mycelial extracts fractionated by PAGE.

The results with infected potato plants indicated that using the specific IgG of *V. dahliae* with DAS-ELISA constituted a method of detection of the fungus in the roots and stems of inoculated plants. Isolation of this fungus from potato stems on specific agar medium is possible only about 60–70 days after inoculation in the greenhouse or 100 days or longer in the field. A comparison of assays from DAS-ELISA with isolation of *V. dahliae* from infected tissues (by plating) has provided additional evidence to support the validity of this technique.

Because the antiserum obtained in this study appears to be specific for *V. dahliae* and *V. albo-atrum* and did not cross-react with other species of *Verticillium*, *Fusarium*, and *Colletotrichum*, it may be a valuable aid in the detection and identification of *V. dahliae* and *V. albo-atrum*. Studies are underway to determine

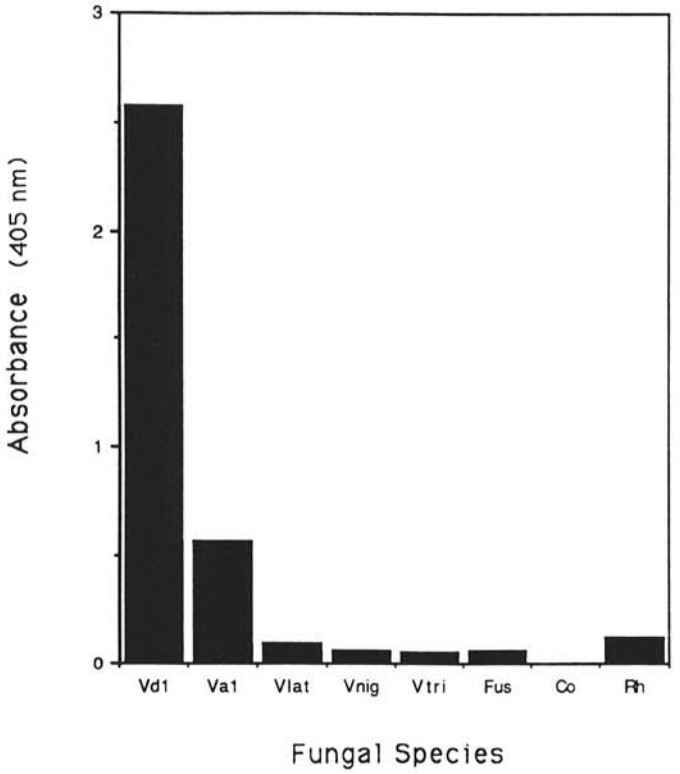

Fig. 3. Reaction of day 30 antiserum with eight fungal isolates in indirect enzyme-linked immunosorbent assay. Fungi included *Verticillium dahliae* (Vdl), *V. albo-atrum* (Val), *V. lateritium* (Vlat), *V. nigrescens* (Vnig), *V. tricorpus* (Vtri), *Fusarium* (Fus), *Colletotrichum* (Co), and *Rhizoctonia* (Rh). Plates were coated with fungal homogenates diluted 1:50, and antiserum was diluted 1:1,000. Each value is the mean of four replicates.

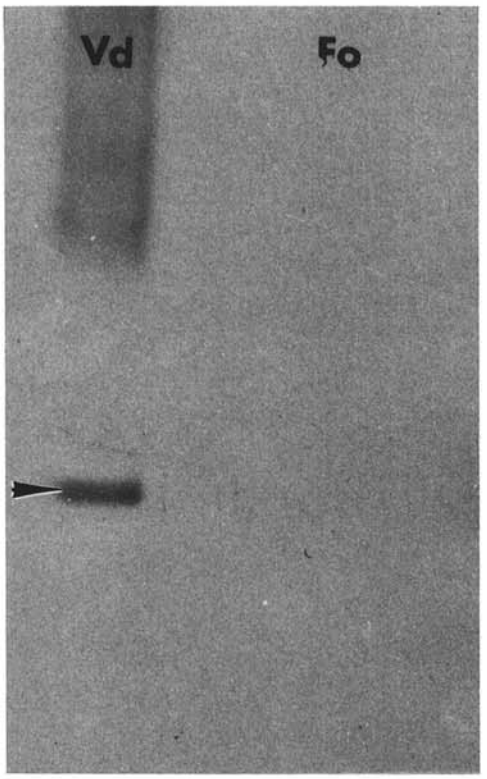

Fig. 4. Western blot analysis of *Verticillium dahliae* (Vd) and *Fusarium oxysporum* (Fo) soluble mycelial proteins stained with whole rabbit antiserum diluted 1:300 and anti-rabbit IgG coupled to alkaline phosphatase. The arrow indicates the protein band in Vd isolates corresponding to the band separated by electrophoresis and used as the immunogen.

TABLE 2. DAS-ELISA for detection of *Verticillium dahliae* in potato[a]

	Absorbance A_{405nm}			
	Healthy		Inoculated	
Technique	Root	Stem	Root	Stem
DAS-ELISA[b]	0.075	0.150	0.505	2.477

[a] Mean of 25 samples in four different assays.
[b] Double antibody sandwich enzyme-linked immunosorbent assay.

if this assay will provide a quantitative measurement of fungus propagules in potato stem tissue that would be comparable to results obtained with methods employed by Anderson and Hoyos (3). The results of this study suggest that a relatively specific fungal antiserum can be produced with specific protein obtained by PAGE fractionation. The antiserum produced in this study appears to be more specific than that of previous workers who used whole cell homogenates or cell wall preparations as immunogens (7,11,12,20).

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