Production and Characterization of Monoclonal Antibodies to *Verticillium dahliae* and Development of a Quantitative Immunoassay for Fungal Biomass

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ABSTRACT

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Monoclonal antibodies (MAbs) were prepared against a purified mycelial protein from *Verticillium dahliae*, a predominant fungus species in the potato early dying complex. The fungal protein was rendered immunogenic by conjugating it to keyhole limpet hemocyanin. Three hybridomas were cloned from a mouse immunized with the conjugated protein. All three cell lines produced immunoglobulin G1-type MAbs that recognized a 60-kDa protein. The specificity of the MAbs was tested using indirect enzyme-linked immunosorbent assay (ELISA), immunoblots, and indirect competitive ELISA. For two of the MAbs, no cross-reactivity was observed when tested against several isolates representing six species of fungi associated with potato. Cross-reactivity was observed to some extent with *V. albo-atrum*. A quantitative immunoassay was developed using an indirect competitive ELISA format that could detect as little as 4 ng of total protein of the fungus and showed a linear relationship between total protein (4 to 500 ng) and absorbance measured at 405 nm. The assay was used to detect and quantify *V. dahliae* colonization in greenhouse-grown potato plants. The immunoassay accurately distinguished colonized from noncolonized plants and quantitatively differentiated the susceptible cultivar (Kennebec) from the resistant cultivar (Reddale).

Additional keyword: soilborne diseases, wilt.

Verticillium wilt is a common vascular disease of potatoes that occurs wherever potatoes are grown. The causal organisms, *Verticillium dahliae* Kleb. and *V. albo-atrum* Reinke & Berthier, can be distinguished by the capacity of the former to produce true microsclerotia and the latter to produce melanized hyphae on potato dextrose or malt agar (6). The symptoms in plants infected with *Verticillium* spp. are similar to those produced by other wilt pathogens, such as *Fusarium oxysporum* and *Pseudomonas solanacearum*. In general, there is loss of turgor in leaflets or leaves followed by chlorosis and necrosis, sometimes localized in only one side of the plant. When irreversible wilt is observed, it cannot be distinguished from natural senescence (28).

A further complication in the diagnosis of this disease comes from interactions between *V. dahliae* and other pathogens. *Pratylenchus penetrans*, the root lesion nematode, is able to interact synergistically with *V. dahliae*, requiring lower inoculum densities to produce a yield reduction than when only a single pathogen is present (23). The role of the soft rot bacterium, *Erwinia carotovora* pv. *carotovora*, and the blackleg bacterium, *E. carotovora* pv. *atroseptica*, in potato early dying also has been suggested, since both are associated with systemic vascular infection resulting in wilt symptoms (29).

One of the main effects of *V. dahliae* infection is the reduction of photosynthetic efficiency of plant tissues (4). Yield losses caused by Verticillium wilt may not be obvious because various factors, such as the use of resistant cultivars, use of fertilizers, irrigation, and pest control strategies, may confound the conclusions (33).

Yield losses in potato due to early dying are highly variable and depend on many factors, but a 30% yield reduction has been documented in Idaho, New York, and Ohio (30,33), and up to 50% losses have occurred under irrigation in Oregon (29).

The nature and the economic importance of this disease has prompted the development of programs to find resistant germ plasm. This necessitated development of fast, specific, sensitive, and inexpensive methods to detect and quantify *Verticillium* spp. in potato tissue. Immunoassays fulfill most of these requirements, but polyclonal antisera raised against this fungus (11,14,35) do not guarantee a successful immunoassay. The main problem arising from the use of polyclonal antisera is their lack of specificity, because cross-reactivity is commonly observed with other species, other genera, and even with host plant proteins (7). A monoclonal antibody (MAb) prepared against surface washings and culture supernatant of *V. dahliae* has been obtained by Van de Koppel and Schots (37) to detect *Verticillium* spp. in roses. However, no cross-reaction tests with other fungi in roses have been reported.

This paper describes the production and characterization of MAbs prepared against a protein isolated from *V. dahliae* and the development of a quantitative immunoassay to detect and quantify *V. dahliae* biomass in potato stem tissue.

MATERIALS AND METHODS

Fungal isolates. *V. dahliae* and *V. albo-atrum* isolates used in this study, as well as other fungal strains, were isolated from potato stems or provided by various faculty members of the Depart-

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ment of Plant Pathology, University of Minnesota, St. Paul. Fungal isolates were maintained in pure culture on potato dextrose–Czapek-Dox agar and placed in vials containing sterile soil for long-term storage. Cultures were grown at room temperature for 7 to 10 days, and 1-cm^2 agar plugs with mycelium were used to inoculate flasks containing Czapek-Dox broth amended with 0.75% sucrose. Flasks were shaken for 16 to 24 h after inoculation and incubated on a bench top for 12 to 14 days at room temperature.

Protein extraction and purification. The mycelia from the flasks were collected on four layers of cheesecloth and rinsed thoroughly with distilled water. The mycelia were ground in a chilled mortar containing 200 mg of acid-washed sand and 3 to 5 ml of Tris extraction buffer. The fungal tissue was homogenized with a pestle and transferred to a glass test tube (2 × 20 cm) and further homogenized for 20 s using a Polytron homogenizer (Brinkman Instruments, Westbury, NY). The suspension was centrifuged at 12,000 × *g* for 15 min at 4°C and treated as described by Sundaram et al. (35). Protein was concentrated by lyophilization, and the powder was redissolved in 1 to 2 ml of 0.05 M Tris, pH 8.00. Protein concentration was estimated using the Bio-Rad protein assay (5) (Bio-Rad Laboratories, Hercules, CA) with bovine serum albumin (BSA) as standard.

Polyacrylamide gel electrophoresis (PAGE) was used for separation and analysis of proteins. Slab gels containing 12% acrylamide, pH 8.8, and 5% acrylamide, pH 6.7, as stacking gel were used (3). Lanes were loaded with 1 to 50 µg of soluble mycelial protein in sample buffer, and electrophoresis was run at 15 mA for 8 h. Gels were fixed and stained with either Coomassie brilliant blue or silver nitrate (12).

Protein profiles of *F. oxysporum* and *Colletotrichum* sp. isolated from potato were run in parallel to those of *V. dahliae*. Analysis by banding patterns was done on silver-stained gels, and a band with a relative mobility (R_f = 0.40) was consistently visualized in *V. dahliae* preparations but not in the other fungal protein profiles. This protein (Vd40) was produced in large amounts and was selected as antigen. Nondenaturing PAGE was carried out for its enrichment, and the protein was eluted from the gel (15).

Immunization. For use as an immunogen, 400 µg of the purified protein was conjugated with 2 mg of keyhole limpet hemocyanin as described by Jemmerson (19). Two BALB/c female mice, 6 weeks old (≈20 g), were used for immunization, and a third mouse was used to collect preimmune sera. For immunization, 300 µl of the protein conjugate (40 to 50 µg) was mixed with 300 µl of complete Freund's adjuvant to form an emulsion that was injected intraperitoneally (i.p.). The mice were immunized two more times with the same dose at 2-week intervals but using incomplete Freund's adjuvant. The titer of the antisera was checked after the second boost using indirect enzyme-linked immunosorbent assay (ELISA), and the mouse with the higher titer (1:5,000) was immunized with 50 µg of soluble protein in one of the tail veins. Three days after the intravenous injection the mouse was euthanized by cervical dislocation, and its spleen was removed for hybridoma preparation.

Cell fusion and cloning. Splenocytes were fused with the P3-X63 mouse myeloma cell line (22) using polyethylene glycol 1500 (Sigma Chemical Co., St. Louis). Fused cells were washed and resuspended in HT media, which consisted of Dulbecco's modified Eagle medium (DMEM, GIBCO Laboratories, Gaithersburg, MD) supplemented with 0.1 mM hypoxanthine, 16 µM thymidine, and 15% fetal bovine serum (Whittaker MA Bioproducts, Walkersville, MD). Cells were distributed in 96-well tissue culture plates, and aminopterin (0.4 µM) was added 24 h later. Cells were regularly fed with HAT medium, and the growth of HAT-resistant cells was observed 10 to 12 days after fusion. Hybridomas were tested for anti-*V. dahliae* antibody production using indirect ELISA as described below. Cells testing positive were cloned by limiting dilution using splenocytes as feeder cells, and clones with stable antibody production were expanded further to produce ascites fluid. For this purpose, BALB/c mice were primed by injecting 0.5 ml of pristane (Aldrich Chemical Co., Milwaukee, WI) i.p., and 10 days later 5 × 10^6 hybridoma cells were injected i.p. in a volume of 0.5 ml of DMEM. Mice were euthanized 10 to 14 days after injecting the cells. The ascites fluid was removed from the peritoneal cavity, centrifuged at 3,000 × *g* for 10 min, and filtered through a 0.45-µm filter into a sterile tube. Sodium azide at 0.2% final concentration was added, and aliquots of 40 µl were dispensed into 0.6-ml sterile tubes and stored at –20°C.

Characterization of MAbs. The isotypes of heavy and light chains of each MAb produced were determined by indirect ELISA using horseradish peroxidase-conjugated goat anti-mouse IgG (immunoglobulin G) antibodies specific for the different mouse heavy- and light-chain polypeptides (Miles Scientific, Naperville, IL).

Indirect ELISA. Indirect ELISA was used to detect anti-*V. dahliae* MAbs in tissue culture supernatants and to test the specificity of the MAbs produced. Total soluble mycelial protein extracted from *V. dahliae* and other fungi was obtained as described above, and a stock solution containing 10 µg of protein per ml of phosphate buffered saline (PBS) was prepared. ELISA microtiter plates (Costar, Cambridge, MA) were coated with 100 ng of protein in 100 µl of PBS and incubated overnight at room temperature. The plates were washed three times with PBS-Tween 20 and blocked with 50 µl of 3% BSA in PBS for 1 h. The plates were washed three times, and the culture supernatant was added and incubated for 1 h before washing again. Fifty microliters of rabbit anti-mouse IgG alkaline phosphatase conjugate (Sigma) was added to each well, and the plates were incubated overnight at 4°C. The plates were washed three times, and 200 µl of 0.75 mg of *p*-nitrophenyl phosphate per ml was dissolved in 10% diethanolamine (pH 9.8) and added. Absorbance at 405 nm was read at various times to detect positive antibody-producing hybridomas. This assay was used to test the specificity of the MAbs against a wide spectrum of fungi associated with potatoes.

Indirect competitive ELISA. An indirect competitive ELISA was developed, adapted, and optimized to quantify *V. dahliae* antigen in potato. For this purpose, a stock solution of 200 µg of protein per ml was prepared in 0.05 M Tris, pH 8.0, and diluted to 1 ng/ml with PBS, pH 7.4. Wells of a microtiter plate were coated with 100 µl of this solution (100 ng of protein per well). An optimum concentration of adsorbed antigen was obtained by testing various coating levels of protein (5 to 200 ng of protein per well). Plates were covered and incubated overnight at room temperature. The plates were washed three times with PBS-Tween 20 and blocked with 50 µl of 3% BSA in PBS added to each well. After incubating the plates for 1 h at room temperature, the plates were used or stored at 4°C for later use. The initial antigen-antibody reaction was done in a nonbinding 96-well plate (Serocluster, Costar). Competing antigen in the form of soluble protein, *V. dahliae* spores, or sap extracted from potato stems was deposited in each well. Various concentrations of each antigen were tested (0 to 512 ng of soluble protein, 0 to 107 spores, and sap diluted 1:2, 1:4, 1:6, and 1:24 [vol/vol] with ELISA buffer). For each antigen, every testing dilution was added in a constant volume (20, 40, or 100 µl). The MAbs were tested at several dilutions (1:2,000, 1:4,000, 1:10,000, 1:20,000, 1:40,000, and 1:64,000). One hundred microliters of MAb solution was added to each well, and the contents were mixed.

At this point, the Serocluster plate was either incubated for 3 h to overnight (nonequilibrium reaction), or 100 µl from each well was transferred immediately to its replicated well in the ELISA plate (equilibrium reaction). The ELISA plate was incubated at room temperature for 1 h, and the plates were washed three times with PBS-Tween 20. Alkaline phosphatase conjugated rabbit anti-mouse IgG (50 µl) diluted 1:2,000 in ELISA buffer was added to each well, and the plates were incubated overnight. The plates were washed, and 200 µl of substrate (0.75 mg of *p*-nitrophenyl-phosphate per ml in 10% diethanolamine, pH 9.8) was added to

each well, and the absorbances were read at 405 nm. In most experiments, absorbance readings were stopped when A_{405} was close to 2.000 for the wells without competing soluble antigen (100% absorbance or 0% inhibition), and data for the rest of the wells were recorded. The specificity of the MAbs also was tested using this assay in a quantitative manner as 25 to 100 ng of mycelial protein from various fungi were tested. Percent inhibition was calculated as: percent inhibition = $(1 - At/Ab) \times 100\%$, where $At = A_{405}$ for the isolate tested and $Ab = A_{405}$ of the wells without any competing antigen.

Immunoblots. Western blots were done by electrophoretic transfer of fungal proteins (1 to 4 µg) from polyacrylamide gels onto nitrocellulose membranes (NCM) according to Towbin et al. (36). NCMs were blocked overnight with 3% BSA in PBS, washed with PBS-Tween 20, and incubated with different MAbs for 3 h at room temperature. Detection was accomplished using rabbit anti-mouse IgG coupled to alkaline phosphatase and naphtol AS-MX phosphate/fast red TR salt (Sigma) as substrates.

Detection and quantitation of *V. dahliae* in infected potato tissue. Two potato cultivars, Reddale and Kennebec, were used in greenhouse experiments to assess the ability of the immunoassay to detect and quantify *V. dahliae* in infected potato tissue and to compare with the culture plate method developed by Hoyos et al. (17). Seed tubers were cut and allowed to suberize before planting. A 1:1 (vol/vol) mixture of pasteurized soil and vermiculite was used to fill 28-cm-diameter plastic pots. The seed tuber was placed on top together with 20 or 40 cm^3 of *V. dahliae*-infested grain (6×10^5 microsclerotia per g of rice). The tuber and inoculum were covered with a 3- to 4-cm-deep layer of soil-vermiculite. Control tubers were planted with autoclaved grain. Plants were grown at 20 to 24°C under fluorescent lamps using a 15-h photoperiod for 70 to 100 days. Plants grown in the greenhouse were used to test the immunoassay during its development and optimization and to compare it with the culture plate method.

RESULTS

An abundant protein with an $R_f = 0.4$ was identified in PAGE protein profiles (Fig. 1) from extracts of *V. dahliae* mycelia (lane 1) and conidia (lane 2) but was not observed in extracts of *F. oxysporum* (lane 3) or *Colletotrichum* sp. (lane 4). The molecular mass of the purified protein, which was determined in a denaturing 12% acrylamide gel, was 60 kDa, using bovine albumin, egg albumin, carbonic anhydrase, and lactalbumin as molecular weight markers.

Characterization of MAbs. The three stable cell lines (5E7.G9, 7C2.A11, and 3B12.E12) that were cloned produced antibodies of the class IgG1 (heavy-chain gamma-1). The light chain was of the isokappa type. To determine whether the antigen recognized by the MAb was indeed the 60-kDa protein isolated from *V. dahliae* mycelium, Western blots were done. Strips of NCM were probed with each of the MAbs (Fig. 2), revealing that all three MAbs

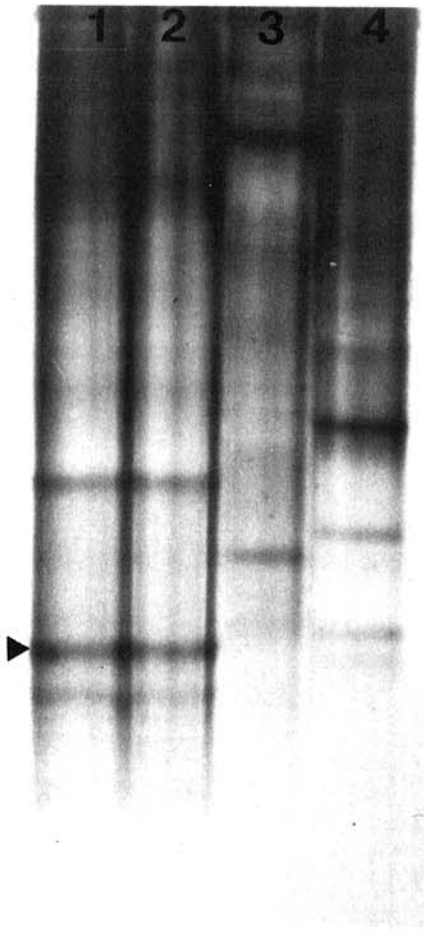

Fig. 1. Protein profiles of *Verticillium dahliae* (mycelium, lane 1), *V. dahliae* (conidia, lane 2), *Fusarium oxysporum* (lane 3), and *Colletotrichum* sp. (mycelium, lane 4). Proteins (5 µg per lane) were separated in a 12% acrylamide gel under nondenaturing conditions and stained with silver nitrate. An abundant protein migrating with an $R_f = 0.40$ (arrow) was observed in *V. dahliae* extracts but not in extracts of the other two genera and was selected to be used as immunogen.

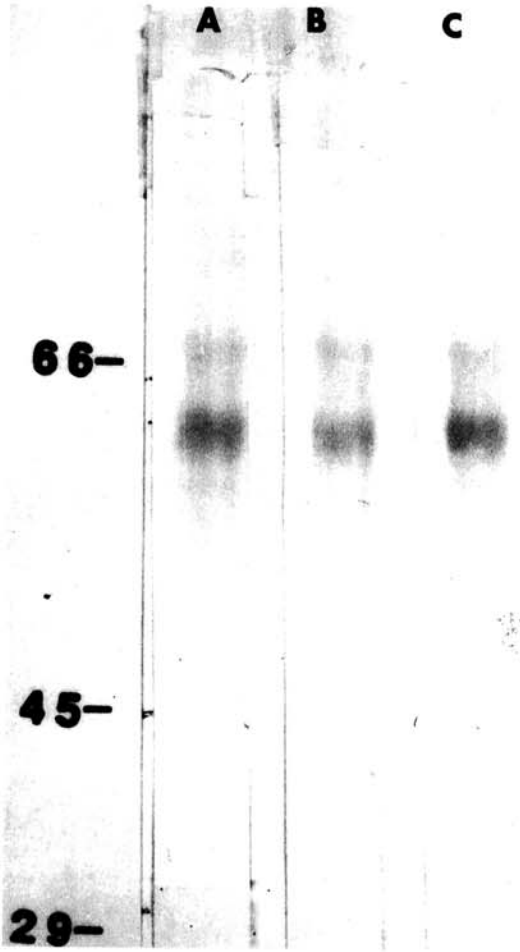

Fig. 2. Immunoblot of sodium dodecyl sulfate-polyacrylamide gel electrophoresis–fractionated mycelial proteins from *Verticillium dahliae* probed with the three monoclonal antibodies used at 1:4,000 dilution for 7C2.A11 (lane A) and 5E7.G9 (lane C) and 1:400 dilution for 3B12.E12 (lane B). Protein (1 µg) was loaded in each lane; molecular weight markers are indicated on the left in kilodaltons.

reacted strongly with a 60-kDa protein and less intensely with another protein of higher molecular weight.

Immunoblots of one representative isolate of each of five other species showed no cross-reaction with the MAbs (Fig. 3), although the amount of protein loaded for each heterologous species was three- to four-times higher than for *V. dahliae*. *V. albo-atrum*, which showed cross-reactivity in ELISA, presented a low molecular mass, 30 kDa, immunoreactive protein in the Western blot.

The three MAbs were tested by indirect ELISA against proteins from other fungi that commonly infect potato (Table 1). Among the fungi tested, *V. albo-atrum* showed the strongest cross-reaction with the MAbs. Only one isolate representing a different genus, *F. solani* SB38, showed some degree of cross-reactivity, (A_{405} = 0.142 for MAb 5E7.69 and A_{405} = 0.179 for MAb 7C2.A11). The isolate *F. oxysporum* #162 showed cross-reactivity with MAb 3B12.E12 (A_{405} = 0.229).

The specificity of the MAbs also was tested with indirect competitive ELISA (Table 2). The *V. dahliae* antigen was tested using 25 ng of protein (1.25 ng/µl) that gave approximately 50% inhibition for MAb 5E7.G9 and 42% inhibition for MAb 7C2.A11. The rest of the fungal antigens were tested in a range from 50 to 100 ng (2 to 4 ng/µl). For the *V. dahliae* isolates tested, inhibition ranged from 26 to 83%, whereas for *V. albo-atrum* inhibition was about 30%. None of the heterologous genera tested gave a percent inhibition larger than 11% for MAb 5E7.G9 or 8% for MAb 7C2.A11.

Once the optimal antigen concentration for plate coating and antibody dilution was determined, indirect competitive ELISA was tested as a quantitative assay. When soluble antigen was quantified as total protein and plotted against absorbance, the inhibition curve obtained fit a logarithmic equation. Thus, when Log nanograms of protein was plotted against absorbance, a linear relationship was observed through the full concentration range with MAbs 5E7.G9 and 7C2.A11 (Fig. 4). The assay was able to detect as little as 4 ng of *V. dahliae* total protein.

Detection of *V. dahliae* in potato plants grown in the greenhouse was possible as early as 80 days after planting. Stems were harvested at this time because wilt symptoms were severe in cv. Kennebec. Using indirect competitive ELISA, *V. dahliae* was detected in the sap of infected Kennebec plants, giving a difference of approximately 1 absorbance unit when compared with noninoculated plants (Fig. 5). In cv. Reddale, detection of *V. dahliae* also was possible, but the difference in absorbance between infected and check plants was small and only significant (P = 0.05) when MAb 5E7.G9 was used in the immunoassay. Vascular col-

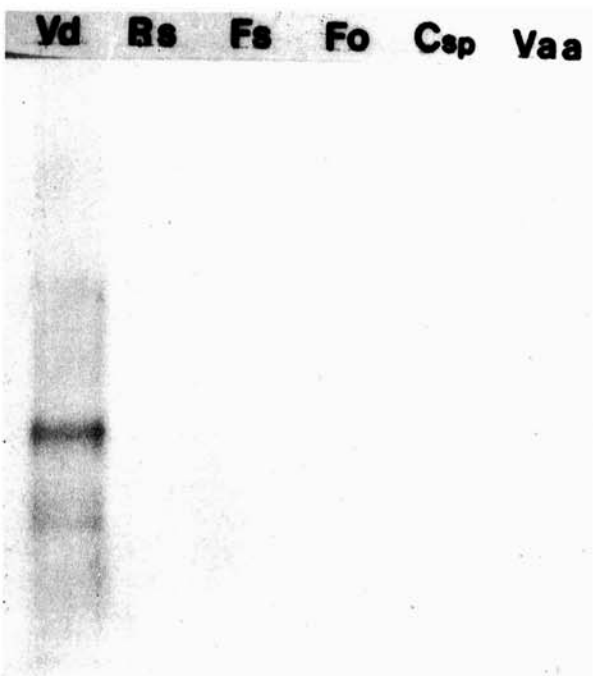

Fig. 3. Immunoblot of sodium dodecyl sulfate-polyacrylamide gel electrophoresis–fractionated mycelial proteins from *Verticillium dahliae* (Vd, 1 µg), *Rhizoctonia solani* (Rs, 4 µg), *Fusarium solani* SB38 (Fs, 4 µg), *F. oxysporum* 162 (Fo, 4 µg), *Colletotrichum* sp. (Csp, 4 µg), and *V. albo-atrum* (Vaa, 2 µg) and probed with monoclonal antibody 5E7.G9 diluted 1:4,000.

TABLE 1. Cross-reactivity test of monoclonal antibodies (MAbs) against antigens from fungal pathogens of potato[a]

		MAb[b]		
Fungal isolate	Source	5E7.G9 (32,000)	7C2.A11 (16,000)	3B12.E12 (1,500)
Verticillium dahliae	Potato	1.084	1.110	0.903
Fusarium oxysporum 42	Soil	0.006	0.005	0.033
F. oxysporum 158	Soil	0.000	0.003	0.029
F. oxysporum 160	Soil	0.020	0.025	0.050
F. oxysporum 162	Soil	0.103	0.119	0.229
F. oxysporum 4	Potato	0.057	0.060	0.061
Colletotrichum sp. 50	Potato	0.007	0.007	0.042
Colletotrichum sp. STP	Potato	0.010	0.011	0.043
Rhizoctonia solani 3-007	Potato	0.002	0.001	0.037
R. solani 3-005	Potato	0.000	0.002	0.034
R. solani 3-004	Potato	0.001	0.000	0.004
R. solani 3-003	Potato	0.000	0.002	0.038
F. solani SB44	Sugar beet	0.023	0.023	0.039
F. solani SB38	Sugar beet	0.142	0.179	0.114
F. solani SB10	Sugar beet	0.036	0.041	0.051
F. solani PN1	Potato	0.052	0.045	0.054
F. solani PN2	Potato	0.052	0.059	0.069
F. sambucinum N46A	Soil	0.102	0.127	0.092
F. sambucinum N45C	Soil	0.007	0.007	0.028
Alternaria solani	Potato	0.007	0.007	0.031
V. albo-atrum 1	Potato	0.789	0.852	0.638
V. albo-atrum 2	Potato	0.372	0.427	0.315

[a] The test was done using the indirect enzyme-linked immunosorbent assay format, and absorbance was recorded at 405 nm.
[b] The reciprocal of the dilution used for each antibody is indicated in parentheses.

TABLE 2. Cross-reactivity test of monoclonal antibodies (MAbs) against antigens from fungal pathogens of potato, using indirect competitive enzyme-linked immunosorbent

	MAb			
	5E7.G9		7C2.A11	
Fungal isolate	A_{405}	% Inhibition	A_{405}	% Inhibition
Verticillium dahliae Kr3	1.132	44	1.283	46
V. dahliae 218	0.329	84	0.631	73
V. dahliae # 2	1.491	27	1.817	23
V. dahliae # 9	1.419	30	1.726	27
V. dahliae K3	0.522	74	0.852	64
V. albo-atrum 1	1.333	34	1.528	36
V. albo-atrum 2	1.445	29	1.726	26
Fusarium solani SB44	1.915	6	2.283	4
F. solani SB38	1.947	4	2.314	2
F. solani PN1	1.872	8	2.226	6
F. solani PN2	1.816	11	2.289	3
F. oxysporum 42	1.830	10	2.277	4
F. oxysporum 160	1.917	6	2.333	2
F. oxysporum 162	1.905	6	2.274	4
F. sambucinum N45C	1.830	10	2.327	2
Rhizoctonia solani 3-007	1.802	11	2.291	3
R. solani 3-004	1.854	9	2.259	5
Colletotrichum sp. 50	1.880	8	2.286	4
Colletotrichum sp. STP	1.870	8	2.175	8
Alternaria solani	1.992	2	2.303	3
Buffer[a]	2.034	0	2.369	0

[a] Buffer blank: percent absorbance inhibition was calculated using this value as 100%.

onization was evaluated using the culture plate technique, yielding mean values of 2,000 and 300 CFU per ml of sap in cvs. Kennebec and Reddale, respectively.

DISCUSSION

Many types of immunogens have been used to prepare MAbs to fungal pathogens, including intact spores (38), spore extracts (2), hyphal fragments (9,25), culture filtrates (39), surface washings (8), purified preparations of secreted proteins (24), and mycelial protein (31). In this study, a gel-purified protein from *V. dahliae* was selected on the basis of its abundance and mobility in nondenaturing PAGE when compared to the protein profiles of *F. oxysporum* and *Colletotrichum* sp. These two fungi are commonly isolated from potato stems. *F. oxysporum*, like *V. dahliae*, produces vascular wilt symptoms (32) and competes with *Verticillium* spp. for root colonization (13). *C. coccodes* causes black dot of potato, which affects tubers, stolons, roots, and stems, and may be involved in the potato early dying complex (21). Although the use of a purified protein for immunization increases the likelihood of obtaining specific antibodies, it is not guaranteed, since fungal proteins appear to share many antigenic determinants. Similar results were observed by Matthew and Brooker (24) who generated MAbs to *Rhizoctonia solani* using a 40-kDa Sephacryl-fractionated protein that reacted against a series of proteins ranging from 15 to 45 kDa. As shown in this study, all three characterized hybridomas produced MAbs that reacted with more than one protein, suggesting that the antigenic determinant is shared by more than one *V. dahliae* protein.

However, generation of MAbs to a specific antigen may result in the production of strain-specific antibodies of the pathogen that express such antigen in detectable amounts. For example, isolate-specific antibodies have been obtained for zoospores of *Phytophthora cinnamomi* (16). Zoospores lack cell walls, which may contain most of the common antigens responsible for cross-reactivity. In this study, a potato isolate of *V. dahliae* was used to prepare the immunogen, and isolates from potato, ash, and tomato were tested for cross-reactivity using indirect ELISA and indirect competitive ELISA. Although these represent a narrow range of isolates, genetic diversity among *V. dahliae* isolates is also narrow, as tested by classical genetic methods (20) or by molecular techniques (27). In general, strain specificity has not been a problem in immunoassays for fungi, and if it arises, it can be solved by mixing two or more MAbs to obtain a wider range of specificities, and a combination of monoclonal and polyclonal antibodies may be used (31).

A major problem in using immunoassays is the lack of specificity of antibodies generated to plant pathogenic fungi. Thus, thorough cross-reaction tests were carried out. The specificity of the MAbs was tested using several types of assays against fungi commonly isolated from potato stem tissue. As a first screening method to eliminate cross-reacting antibodies, indirect ELISA was employed. In this assay, antibodies bind to adsorbed antigen, whereas in indirect competitive ELISA, antibodies first bind to antigen in soluble form. Some differences were observed between these methods: with indirect ELISA, *F. solani* SB 38, showed some cross-reactivity with MAbs 5E7.G9 (A_{405} = 0.142) and 7C2.A11 (A_{405} = 0.179), but in indirect competitive ELISA, this isolate gave only a 4 and 2% inhibition for each antibody, respectively. In contrast, *R. solani* 3-007 tested negative in indirect ELISA (A_{405} = 0.002), but gave an 11% inhibition in indirect competitive ELISA. However, when higher concentrations of antigen were tested, inhibition did

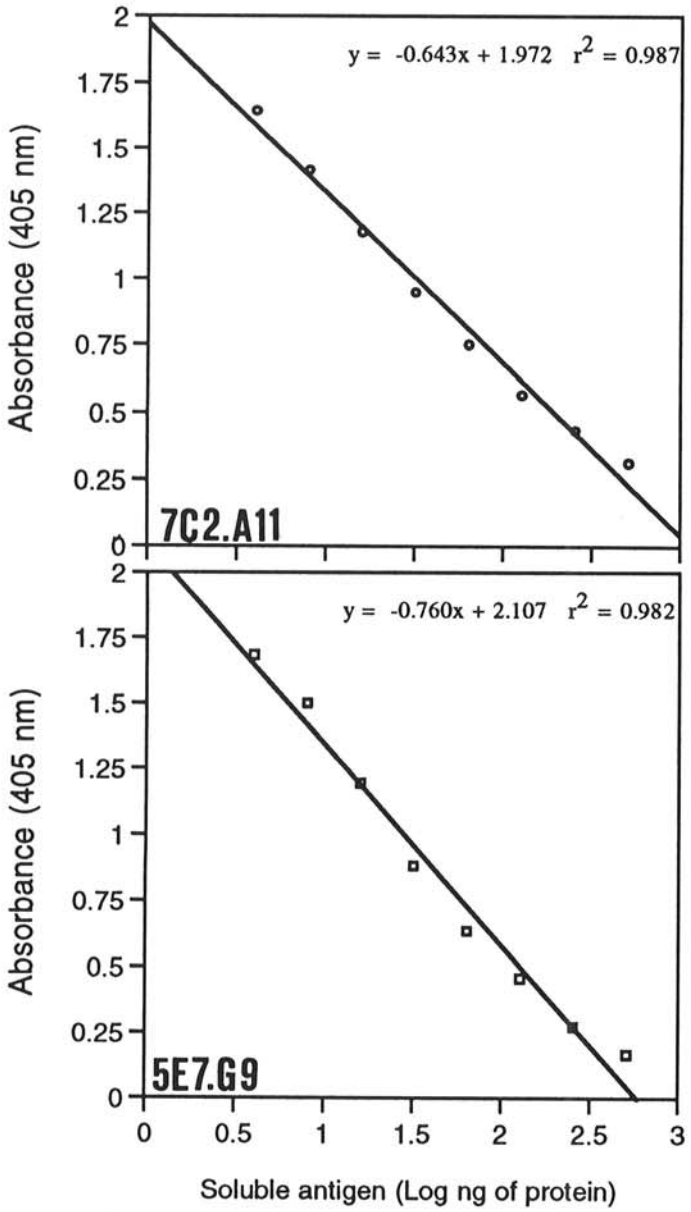

Fig. 4. Inhibition curve to quantify soluble antigen from *Verticillium dahliae* with monoclonal antibodies 5E7.G9 and 7C2.A11 used at 1:40,000 dilution. Antigen quantities range from 4 to 512 ng of total protein.

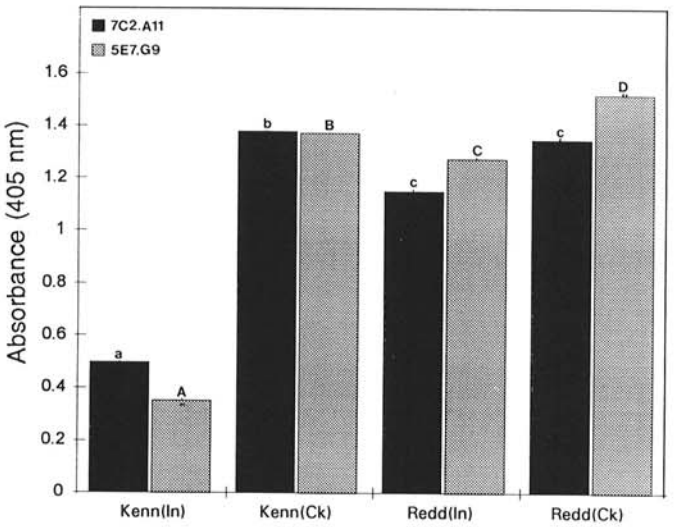

Fig. 5. Indirect competitive enzyme-linked immunosorbent assay to quantify *Verticillium dahliae* colonization in potato plants using monoclonal antibodies (MAbs) 7C2.A11 and 5E7.G9. Individual Student's *t* tests were done for each cultivar (n = 10); in cv. Kennebec (susceptible) it was possible with both antibodies (P = 0.01) to distinguish infected (In) from healthy checks (Ck), whereas in cv. Reddale (resistant) only with MAb 5E7.G9 (P = 0.05) was it possible to distinguish infected from healthy plants. Lowercase letters indicate significantly different values for MAb 7C2.A11, and uppercase letters indicate significantly different values for MAb 5E7.G9.

not increase for these two fungi or for any of the other fungal isolates tested, whereas when the homologous antigen was assayed the percent inhibition increased as a function of antigen concentration before reaching a maximum. When MAbs were tested using immunoblots in which up to five-times more protein was loaded for the heterologous species, no cross-reacting bands were observed, suggesting that the selected *V. dahliae* protein was indeed absent in these fungi.

When fungal antigens are employed for immunization, MAbs of the IgM class are commonly obtained for fungi of medical importance (10) and for plant pathogenic fungi (7). In general, IgG antibodies show greater specificity than IgM antibodies. Xia et al. (40) obtained a IgG1 specific to *Pyricularia grisea*, whereas the MAbs that showed cross-reactivity were of the IgM and IgG2 classes. Dewey et al. (9) found that MAbs with broad specificity to *Ophiostoma ulmi* were of the IgM class, whereas the species-specific antibodies were IgG1 and IgG2a. In our preliminary experiments, hybridomas were generated by immunizing mice with the purified protein (data not shown). Only MAbs of the IgM class were obtained. However, when the *V. dahliae* protein was conjugated with keyhole limpet hemocyanin and used as immunogen, IgG1 was produced by all isolated hybridomas. These MAbs bind with high affinity to the antigen either in solution or in solid phase.

Fungal biomass was accurately quantified using indirect competitive ELISA. Although this method includes an extra microtiter plate-filling step, it is as sensitive as indirect ELISA and is not as likely to yield false positive results. Experience in our laboratory shows that indirect competitive ELISA is more sensitive than double-antibody sandwich–ELISA (DAS-ELISA, data not shown), and quantitation is possible over a wide range of soluble antigen concentrations. Moreover, the amount of reagent needed is 10% of that required for DAS-ELISA.

Chitin (1) and ergosterol (34) contents have been used to estimate fungal biomass in plant tissues, and although both methods are reliable, they are time-consuming because complete hydrolysis of chitin is required for analysis and a complicated extraction procedure is needed for ergosterol estimation. Both are general methods for fungi and cannot distinguish genera or families. The MAb-based immunoassay developed in this study has the advantage of being fast, simple, and genus specific. Recently, polymerase chain reaction (PCR) assays have been developed to differentiate three pathogenic *Verticillium* spp. Primer sets were designed based on small differences in the genomic sequences identified in the internal spacer regions of the ribosomal genes and were used in PCR experiments to distinguish *V. dahliae*, *V. albo-atrum* (18), and *V. tricorpus* (26) from each other. By using an internal control DNA template in such experiments, quantification of the pathogen in diseased field plants is possible (26).

A PCR-based assay appears to be more specific than the MAb-based assay, because cross-reactivity with *V. albo-atrum* was observed. The former assay would be particularly useful for identification and taxonomic purposes. However, for detection and quantitation in a large number of plants, the MAb-based immunoassay offers advantages in terms of speed, ease of use, and costs. Unlike the PCR assay, in which genomic DNA must be extracted from the plant tissue, the immunoassay only requires a small sample of sap that is obtained by squeezing the basal 10-cm segment of the potato stem. Soluble protein is present in plant sap, and differences in vascular tissue colonization have been detected using the immunoassay and corroborated with the culture plate technique. Field tests in which both methods are compared will be reported elsewhere (J. Plasencia and E. E. Banttari, *unpublished data*).

LITERATURE CITED

1. Atlas, R. M., and Bartha, R. 1987. Microbial Ecology: Fundamentals and Applications. 2nd ed. Benjamin Cummings Publishing Co., Menlo Park, CA.
2. Banowetz, G. M., Trione, E. J., and Krygier, B. B. 1984. Immunological comparisons of teliospores of two wheat bunt fungi, *Tilletia* species using monoclonal antibodies and antisera. Mycologia 76:51-52.
3. Benhamou, N., Ouellette, G. B., Asselin, A., and Maicas, E. 1983. The use of polyacrylamide gel electrophoresis for rapid differentiation of *Gremmeniella abietina* isolates. Pages 68-76 in: Proc. Int. Symp. *Scleroderris* Canker Conifers. P. D. Manion, ed. Nijhoff Publishing Co., Boston.
4. Bowden, R. L., and Rouse, D. I. 1987. Effects of *Verticillium dahliae* on photosynthesis and transpiration of potato. (Abstr.) Phytopathology 77: 1703.
5. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248-254.
6. Commonwealth Mycological Institute. 1970. Descriptions of Pathogenic Fungi and Bacteria. No. 256, *Verticillium dahliae*. Commonwealth Mycological Institute, Kew, England.
7. Dewey, F. M. 1992. Detection of plant-invading fungi by monoclonal antibodies. Pages 47-62 in: Techniques for the Rapid Detection of Plant Pathogens. J. M. Duncan and L. Torrance, eds. Blackwell Scientific Publications, Oxford.
8. Dewey, F. M., MacDonald, M. M., and Philips, S. S. 1989. Development of a monoclonal-antibody-ELISA, DOT-BLOT and -DIP-STICK immunoassay for *Humicola lanuginosa* in rice. J. Gen. Microbiol. 135:361-374.
9. Dewey, F. M., Munday, C. J., and Barsier, C. M. 1989. Monoclonal antibodies to specific components of the Dutch elm disease pathogen *Ophiostoma ulmi*. Plant Pathol. 38:9-20.
10. Drouhet, E. 1988. Overview of fungal antigens. Pages 10-35 in: Fungal Antigens: Isolation, Purification and Detection. E. Drouhet, G. T. Cole, L. Repentigny, J. P. Latge, and B. Dupont, eds. Plenum Publishing, New York.
11. Fitzell, R., Fahy, P. C., and Evans, G. 1980. Serological studies on some Australian isolates of *Verticillium* spp. Aust. J. Biol. Sci. 33:115-124.
12. Garfin, D. E. 1990. One dimensional gel electrophoresis. Pages 425-441 in: Guide to Protein Purification. M. P. Deutscher, ed. Academic Press, San Diego, CA.
13. Gerik, J. S., and Huisman, O. C. 1983. Mode of colonization of roots by *Verticillium* and *Fusarium*. Pages 80-83 in: Ecology and Management of Soilborne Plant Pathogens. C. A. Parker, A. D. Rovira, K. J. Moore, P. T. W. Wong, and J. K. Kollmargen, eds. American Phytopathological Society, St. Paul, MN.
14. Gerik, J. S., Lommel, S. A., and Huisman, O. C. 1987. A specific serological staining procedure for *Verticillium dahliae* in cotton root tissue. Phytopathology 77:262-266.
15. Hager, D. A., and Burgess, R. R. 1980. Elution of proteins from sodium dodecyl sulfate-polyacrylamide gels; Removal of sodium dodecyl sulfate and renaturation of enzymatic activity. Results with sigma subunit of *Escherichia coli* RNA polymerase, wheat germ DNA topoisomerase, and other enzymes. Anal. Biochem. 109:76-86.
16. Hardham, A. R., Suzaki, E., and Perkin, J. L. 1986. Monoclonal antibodies to isolate-, species-, and genus-specific components of the surface of zoospores and cysts of the fungus *Phytophthora cinnamomi*. Can. J. Bot. 64:311-321.
17. Hoyos, G. P., Zambino, P. J., and Anderson, N. A. 1991. An assay to quantify vascular colonization of potato by *Verticillium dahliae*. Am. Potato J. 68:727-742.
18. Hu, X., Nazar, R. N., and Robb, J. 1993. Quantification of *Verticillium* biomass in wilt disease development. Physiol. Mol. Plant Pathol. 42:23-26.
19. Jemmerson, R. 1987. Multiple overlapping epitopes in the three antigenic regions of horse cytochrome *c*. J. Immunol. 138:213-219.
20. Joaquim, T. R., and Rowe, R. C. 1990. Reassessment of vegetative compatibility relationships among strains of *Verticillium dahliae* using nitrate-nonutilizing mutants. Phytopathology 80:1160-1166.
21. Johnson, D. A., and Miliczky, E. R. 1993. Distribution and development of black dot, Verticillium wilt, and powdery scab on Russet Burbank potatoes in Washington State. Plant Dis. 77:74-79.
22. Kearney, J. F., Radbruch, A., Liesang, B., and Rajewsky, K. 1979. A new myeloma cell line that has lost immunoglobulin expression but permits the construction of antibody-secreting hybrid cell lines. J. Immunol. 123: 1548-1550.
23. Martin, M. J., Riedel, R. M., and Rowe, R. C. 1982. *Verticillium dahliae* and *Pratylenchus penetrans*: Interactions in the early dying complex of potato in Ohio. Phytopathology 72:640-644.
24. Matthew, J. S., and Brooker, J. D. 1991. The isolation and characterization of polyclonal and monoclonal antibodies to anastomosis groups of *Rhizoctonia solani*. Plant. Pathol. 40:67-77.
25. Mitchell, L. A., and Sutherland, J. R. 1986. Detection of seed-borne *Sirococcusstrobilinus* with monoclonal antibodies in an enzyme-linked immunosorbent assay. Can. J. For. Res. 16:945-948.
26. Moukhamedou, R., Hu, X., Nazar, N., and Robb, J. 1994. Use of poly-

merase chain reaction-amplified ribosomal intergenic sequences for the diagnosis of *Verticillium tricorpus*. Phytopathology 84:256-259.
27. Okoli, C. A. N., Carder, J. H., and Barbara, D. J. 1993. Molecular variation and sub-specific groupings within *Verticillium dahliae*. Mycol. Res. 97:233-239.
28. Pegg, G. F. 1981. Biochemistry and physiology of pathogenesis. Pages 193-253 in: Fungal Wilt Diseases of Plants. M. E. Mace, A. A. Bell, and C. H. Beckman, eds. Academic Press, New York.
29. Powelson, M. L. 1985. Potato early dying in the Pacific Northwest caused by *Erwinia carotovora* pv. *carotovora* and *E. carotovora* pv. *atroseptica*. Am. Potato J. 62:173-176.
30. Powelson, M. L., and Rowe, R. C. 1993. Biology and management of early dying of potatoes. Annu. Rev. Phytopathol. 31:111-126.
31. Priestley, R. A., and Dewey, F. M. 1993. Development of a monoclonal antibody immunoassay for the eyespot pathogen *Pseudocercosporella herpotrichoides*. Plant Pathol. 42:403-412.
32. Rich, A. E. 1983. Potato Diseases. Academic Press, New York.
33. Rowe, R. C., Davis, J. R., Powelson, M. L., and Rouse, D. I. 1987. Potato early dying: Causal agents and management strategies. Plant Dis. 71:482-489.
34. Strange, R. N. 1982. Pathogenic interactions of microbes with plants. Pages 472-489 in: Experimental Microbial Ecology. R. G. Burns and J. H. Slater, eds. Blackwell Scientific Publications, Oxford.
35. Sundaram, S., Plasencia, J., and Banttari, E. E. 1991. Enzyme-linked immunosorbent assay for detection of *Verticillium* spp. using antisera produced against *V. dahliae* from potato. Phytopathology 81:1485-1489.
36. Towbin, H., Staehelin, T., and Gordon, J. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc. Natl. Acad. Sci. USA 76:4350-4354.
37. Van de Koppel, M. M., and Schots, A. 1994. A double (monoclonal) antibody sandwich ELISA for the detection of *Verticillium* species in roses. Pages 99-104 in: Modern Assays for Plant Pathogenic Fungi: Identification, Detection and Quantification. A. Schots, F. M. Dewey, and R. P. Oliver, eds. CAB International, Wallingford, England.
38. Wright, S. F., Morton, J. B., and Sworobuk, J. E. 1987. Identification of vesicular-arbuscular mycorrhizal fungus by using monoclonal antibodies in an enzyme-linked immunosorbent assay. Appl. Environ. Microbiol. 53:2222-2225.
39. Wycoff, K. L., Jellison, J., and Ayers, A. R. 1987. Monoclonal antibodies to glycoprotein antigens of a fungal pathogen, *Phytophthora megasperma* f. sp. *megasperma*. Plant Physiol. 85:508-515.
40. Xia, J. Q., Lee, F. N., Scott, H. A., and Raymond, L. R. 1992. Development of monoclonal antibodies specific for *Pyricularia grisea*, the rice blast pathogen. Mycol. Res. 96:867-873.