Analysis of Two ELISA Formats and Antigen Preparations Using Polyclonal Antibodies Against *Phomopsis longicolla*

L. M. Brill, R. D. McClary, and J. B. Sinclair

Former graduate research assistant, research specialist in agriculture, and professor, respectively, Department of Plant Pathology, University of Illinois at Urbana-Champaign, 1102 South Goodwin Avenue, Urbana 61801-4709. First author's present address: Department of Biology, University of California, 405 Hilgard Avenue, Los Angeles 90024-1606.

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ABSTRACT

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Polyclonal antibodies (PAbs) were produced in New Zealand white rabbits with culture filtrate and mycelial extract immunogen preparations from the soybean (*Glycine max*) fungal pathogen *Phomopsis longicolla*. The PAbs were purified to the immunoglobin fraction and tested in indirect enzyme-linked immunosorbent assay (ELISA) and in double antibody sandwich-ELISA (DAS-ELISA). The PAbs raised to culture filtrate were more specific but less active in binding to members of the *Diaporthe-Phomopsis* complex than were those raised to the mycelial extract immunogen preparation. DAS-ELISA was more specific and 100-fold more sensitive in detecting members of the complex than was indirect ELISA. Variability in specificity between different PAbs was lower in DAS-ELISA compared with indirect ELISA. Immunization of one rabbit with culture filtrate over an extended time resulted in maximum anti-*P. longicolla* activity after three immunizations, and the activity became constant against most members of the complex at the same time. Reactivity to some cultures of *P. longicolla* was undetectable following the fourth and fifth immunizations, whereas reactivity to all of our other cultures of the complex remained high.

Additional keywords: immunochemistry, Phomopsis seed decay, pod and stem blight, stem canker.

The *Diaporthe-Phomopsis* fungal disease complex of soybeans (*Glycine max* (L.) Merr.) is endemic wherever soybeans are grown (27,32). The complex consists of four fungi: *Phomopsis longicolla* T. W. Hobbs, the primary cause of Phomopsis seed decay; *Diaporthe phaseolorum* (Cooke & Ellis) Sacc. var. *sojae* (S. G. Lehman) Wehmeyer (anamorph *Phomopsis phaseoli* (Desmaz.) Sacc.), primary cause of pod and stem blight; *D. phaseolorum* (Cooke & Ellis) Sacc. var. *caulivora* K. L. Athow & R. M. Caldwell (anamorph rare), primary cause of northern stem canker and top dieback; and *D. phaseolorum* (Cooke & Ellis) Sacc. f. sp. *meridionalis* Morgan-Jones (anamorph unknown), primary cause of southern stem canker (23). Symptoms induced by these fungi are similar and overlap; each fungal pathogen found in a given area is capable of causing each disease.

Latent infection of soybean tissues by these fungi is common (32), but its role in the epidemiology of the complex is poorly understood (31). Latent infection may lead to damaging levels of disease development, so imprecise checklists were developed in an attempt to predict the benefits of fungicide applications (33,37). However, more precise diagnosis of the level of infection may allow increased understanding of the diseases and of when the cost of fungicide application is justified.

Enzyme-linked immunosorbent assay (ELISA) is a rapid and reliable method for early detection and quantification of infection of soybeans by fungi of the *Diaporthe-Phomopsis* complex (39). Such a method could be used for epidemiological studies and to time fungicide sprays for disease control. ELISA also is a useful tool for fungal taxonomy, as we demonstrate below.

Our research examined 1) double antibody sandwich-ELISA (DAS-ELISA) and indirect ELISA for sensitivity and specificity of detection of fungi of the complex, 2) the polyclonal antibody (PAb) response against a culture filtrate and a mycelial extract of *P. longicolla,* and 3) the long-term immune response of one rabbit to *P. longicolla* immunization. We show that DAS-ELISA resulted in greater sensitivity and specificity with our PAbs than did indirect ELISA. PAb responses to culture filtrate and mycelial extract immunogens were similar, but the response to culture filtrate was more specific for fungi of the *Diaporthe-Phomopsis* complex and weaker than the response to mycelial extract. Maximum PAb activity against fungi of the *Diaporthe-Phomopsis* complex was obtained after three culture filtrate immunizations. Additional immunizations resulted in a loss of PAb reactivity to some cultures of *Diaporthe* and *Phomopsis,* especially to *P. longicolla.* A preliminary report of this research was published (7) as was the use of PAb 902-1 for detection and quantification of infection of soybean seedlings by *P. phaseoli* and its use in immunoblot analysis (39).

MATERIALS AND METHODS

Fungal materials. Fungi of the *Diaporthe-Phomopsis* complex were isolated from diseased soybean seeds and stems on acidified (pH 4.5) potato-dextrose agar (PDA) (Difco, Detroit, MI) and obtained in pure culture by transferring young hyphal tips to fresh PDA culture plates. Fungi were identified on the basis of descriptions by Hobbs et al (17), Morgan-Jones (23), and Sinclair and Backman (32). Other fungi used, their culturing, and antigen production were described previously (39).

Antibodies. Preimmune serum was collected from each New Zealand white rabbit and designated NRS. Rabbit PAbs to *P. longicolla* were prepared as described previously; culture filtrate and mycelial extract immunogens were used separately. PAbs were purified from sera as in Velicheti et al (39), including DEAE but excluding protein A affinity-chromatography. PAbs raised to culture filtrate immunogen and purified from sera were designated 901-1 and -2, 902-1 to -4, and 903-1 and -2 for rabbits 901–903 with two, four, and two bleedings, respectively. PAbs

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raised to mycelial extract immunogen and purified from sera were designated 902-A and 902-B for first and second bleedings, respectively.

Indirect ELISA with culture filtrate antigens and PAbs. Indirect ELISA (Fig. 1A) consisted of the reagent incubations listed below. Well contents were emptied after each step (except substrate), and plates were washed three times with phosphate buffered saline (PBS), pH 7.4, plus Tween 20 (0.05% [v/v]; Sigma Chemical Co., St. Louis, MO). Microtiter plates with reagents were incubated under moist conditions for 2 h at room temperature (22–26C); the substrate was incubated under the same conditions for 0.5–1 h in the dark. The microtiter plates used were Nunc Maxisorb F96 Certified (A/S Nunc, Roskilde, Denmark). Each reagent combination (the PAb reacted with different antigen dilutions) was repeated in two or three wells per plate. No reagent combination was tested exclusively in the outside wells of a plate.

Fifty microliters of the fungal culture filtrate antigen was added to each well. Antigens were diluted $\log_2$ in carbonate buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, 3 mM NaN_3, pH 9.6). Antigen dilutions differed because PAbs differed in anti-*P. longicolla* activity. Dilution ranges were 1:8–1:64 for antigen reacted with PAb 901-1; 1:200–1:1600 for 901-2; 1:200–1:1,600 for 902-1; 1:400–1:3,200 for 902-2, -3, and -4; 1:50–1:400 for 903-1; and 1:400–1:3,200 for 903-2. Antigen controls were potato-dextrose broth (PDB) diluted in carbonate buffer and were 1:16 for 901-1; 1:200 for 901-2; 1:200 for 902-1; 1:400 for 902-2, -3, and -4; 1:50 for 903-1; and 1:400 for 903-2. Wells were blocked with 100 μl of blocking buffer (PBS with 3 mM NaN_3 and 1.0% [w/v] bovine serum albumin [BSA]). Each PAb raised to *P. longicolla* culture filtrate was reacted with each culture filtrate dilution, and NRS from the same rabbit served as negative controls. PAbs were at 5.0 μg/ml in PBS at 50 μl per well. Secondary (2^0) PAbs were affinity purified goat-anti-rabbit whole molecule immunoglobin G alkaline phosphatase conjugate at 0.6 μg/ml in PBS at 50 μl per well (Sigma). Conjugate controls were PBS alone.

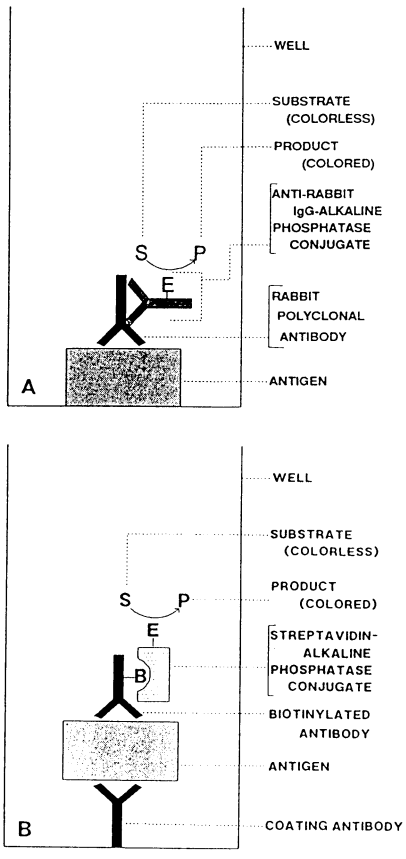

Fig. 1. Schematic diagrams representing a series of bindings leading to a positive reaction in **A,** indirect enzyme-linked immunosorbent assay (ELISA) and **B,** double antibody sandwich-ELISA. Not to scale.

The substrate *p*-nitrophenyl phosphate at 1.0 mg/ml in substrate buffer (1.0% [w/v] diethanolamine, 3 mM NaN_3, pH 9.8) was added at 50 μl per well. Absorbance at 405 nm (A_{405}) was recorded for each well, in the order of substrate addition, with an MR700 ELISA plate reader (Dynatech Laboratories, Alexandria, VA). Substrate reactions were not stopped but proceeded for equal times on individual plates. A positive-negative threshold was set for each plate by calculating the mean A_{405} of antigen controls plus four standard deviations (35). Mean A_{405} values greater than the threshold were considered positive. Assays were done twice.

Maximum sensitivity was determined in the indirect ELISA described above. Antigen was at 10^{-1} to 10^{-4}, $\log_{10}$ μg of protein per well, and control wells contained PDB diluted 1:400 in carbonate buffer. Blocking, PAbs, 2^0 PAbs, substrate, recording of data, and positive-negative thresholds were as described above. Assays were done twice.

Indirect ELISA with mycelial extract antigens and culture filtrate PAbs. The indirect ELISA described above was used on mycelial extract antigens. All reagents were identical (other than antigen), as were incubations and washing of plates. Antigens were diluted 10- to 10,000-fold, $\log_{10}$ in carbonate buffer. Wells containing NRS were used to calculate positive-negative thresholds as described above. Assays were done twice.

DAS-ELISA with culture filtrate antigens and PAbs. DAS-ELISA (Fig. 1B) was done with reagent incubation conditions as in indirect ELISA. Each reagent combination was repeated in two or three wells per plate, and no reagent combination was exclusively in outer wells of a plate. Assays were repeated once. Plate emptying and washing were done with Tris buffer-Tween 20 as described above. Wells were coated with 50 μl of PAb 902-2 or 902-3 per well at 10 μg/ml in 20 mM Tris-HCl buffer (Sigma), pH 7.4. Blocking was with 100 μl of Tris-BSA per well (Tris buffer containing 1.0% [w/v] BSA and 3 mM NaN_3). Fifty microliters of fungal culture filtrate antigen was added to each well. Dilutions, in Tris buffer, were 1:100–1:100,000, $\log_{10}$. Antigen controls were PDB diluted at 1:1,000 in Tris buffer, 50 μl per well. Biotinylated PAbs were in Tris buffer and were the same PAbs as the coating PAbs (except that coating PAbs were not biotinylated). Biotinylated PAbs were added at a concentration of 10 μg/ml at 50 μl per well. Biotinylation of PAbs was done according to Harlow and Lane (14) with the *N*-hydroxysuccinamide ester of biotin (Zymed Laboratories, South San Francisco, CA). Controls were identical quantities of biotinylated NRS from the same rabbit. Streptavidin-alkaline phosphatase conjugate (Zymed Laboratories), diluted at 1:1,500 in Tris buffer, was added to the wells at 50 μl per well. Conjugate controls were Tris buffer alone. Substrate use was as described above for indirect ELISA.

Maximum sensitivity of *P. longicolla* detection in DAS-ELISA was determined with all reagents, incubations, plate emptying, and washing as described above. *P. longicolla* culture filtrate antigens were at 1–0.0001 μg of protein, $\log_{10}$ per well, in 50 μl. Assays were done twice.

DAS-ELISA with mycelial extract antigens and PAbs. Reactivity to mycelial extracts was tested in DAS-ELISA that was similar to the assay used on culture filtrates. Differences were that 1) PAb 902-A (raised to mycelial extract) was used to coat the wells, 2) mycelial extracts were the antigens, and 3) biotinylated 902-A was used. The concentrations of all reagents were the same as described above. This assay was repeated once and done twice with 902-B as coating PAb and with biotinylated 902-B.

RESULTS

Indirect ELISA with culture filtrate antigens and PAbs. In each ELISA format, controls were negative, repeat results were qualitatively identical in that a given reaction was consistently positive or negative, and repeat wells of a plate varied by less than 5%. Preliminary experiments demonstrated maximum sensitivity with the reagent concentrations we used (data not shown). Maximum sensitivity of 901-1 was 1 μg of antigen protein per well, and 902-1 and 903-1 detected 0.1 μg of antigen protein per

well. PAbs 901-2, 902-2, and 903-2 detected 0.01 μg of antigen protein per well, a sensitivity 10- to 100-fold greater than that of PAbs purified from first-bleeding sera.

The dilution of the culture filtrate of *P. longicolla* 1 that reacted with PAb 901-1 in preliminary experiments was 1:64, and the rest were 1:256. At a dilution of 1:256, mean A_{405} values of three wells for PAbs were 0.404 for 902-1, 0.094 for 903-1, 0.401 for 901-2, 0.944 for 902-2, and 0.859 for 903-2. The response of the animals varied, and three immunizations increased antibody activity over two. Rabbit 901 required three immunizations to have antibody activity against *P. longicolla* equal to that of rabbit 902 after two.

Reactivity of PAbs against culture filtrates of fungi isolated from soybean tissues is shown in Table 1. All were strong with all members of the *Diaporthe-Phomopsis* fungal complex. There were no consistent differences among members of the complex. Reactions produced A_{405} values that steadily decreased with decreasing antigen. When PAbs reacted with the lowest antigen quantity, the mean A_{405} value was marginally above the positive-negative threshold. Fungi other than those of the *Diaporthe-Phomopsis* complex showed reactivity with the PAbs (Table 1) and are referred to as cross-reactive. *Colletotrichum truncatum* and *Fusarium* were the most cross-reactive. *Penicillium, Aspergillus* 2, and *Chaetomium* also were cross-reactive with most PAbs. Specificity of different PAbs varied; 903-1 was the least and 902-1 the most specific. There was no specificity trend between first- and second-bleeding PAbs.

Rabbit 902 was used over a longer period of time for production of PAbs to *P. longicolla* immunogen than were the other rabbits. PAbs from the third (902-3) and fourth (902-4) bleedings raised to culture filtrate immunogen were tested in indirect ELISA. The results are compared with 902-1 and 902-2 in Table 2. PAb activity against fungi of the *Diaporthe-Phomopsis* complex peaked by the second bleeding as judged by A_{405} values (data not shown). Cross-reactivity tended to increase in later bleedings. *C. truncatum* and *Fusarium* had high cross-reactivity, except that 902-4 did not react with *Fusarium* 5-B-8 and had reduced reactivity with *C. truncatum* CS 1. *Chaetomium* was cross-reactive with PAbs of three later bleedings from rabbit 902. Reactivity to culture filtrate of *P. longicolla* 1 decreased with 902-3 and had disappeared with 902-4. Furthermore, 902-4 did not react with four of six cultures of *P. longicolla*. Strong reactivity was maintained to other cultures of the *Diaporthe-Phomopsis* fungal complex.

The A_{405} increased with decreased antigen in the reaction between *Phialophora gregata* C3-15 and PAb 902-3. The reaction was negative at antigen dilutions of 1:400 and 1:800 but positive at 1:1,600 and again negative at 1:3,200. This pattern was repeatable and was different from results presented above.

Indirect ELISA with mycelial extract antigens and culture filtrate PAbs. PAbs to culture filtrate immunogen reacted strongly with mycelial extracts of cultures of the *Diaporthe-Phomopsis* fungal complex (Table 3). Some differences occurred between members of the complex. Reactivity to other mycelial extracts was restricted to 902-2 and 903-2, both of which reacted moderately with *C. truncatum* CS 3. Results were consistent with reactivity to culture filtrates, except that four of six PAbs did not react with mycelial extract of *C. truncatum*. PAb 902-1 was most reactive overall to the selected mycelial extracts of the *Diaporthe-Phomopsis* cultures, in contrast to the reactivity to culture filtrates (Tables 1, 2, and 3).

TABLE 1. Maximum dilutions of fungal culture filtrates that yielded positive reactions in indirect enzyme-linked immunsorbent assay with polyclonal antibodies (PAbs) developed in three rabbits[a] to *Phomopsis longicolla* culture filtrate

	PAb					
Fungal isolate	901-1	901-2	902-1	902-2	903-1	903-2
Phomopsis longicolla 1	64	1,600	1,600	1,600	100	3,200
P. longicolla 4	64	1,600	1,600	3,200	400	3,200
P. longicolla 8	64	1,600	1,600	3,200	400	3,200
P. longicolla 10	64	1,600	1,600	3,200	400	3,200
P. longicolla 12	64	1,600	800	3,200	400	3,200
P. longicolla (Ohio)	64	1,600	800	3,200	400	3,200
Diaporthe phaseolorum var. *sojae* L3	64	1,600	1,600	3,200	400	3,200
D. p. sojae 4	64	1,600	1,600	3,200	400	3,200
D. p. sojae 5	64	1,600	1,600	3,200	400	3,200
D. p. sojae 7	64	1,600	1,600	3,200	400	3,200
D. p. sojae 8	64	1,600	1,600	3,200	400	3,200
D. p. caulivora	64	1,600	1,600	3,200	400	3,200
D. p. meridionalis	64	1,600	1,600	3,200	400	3,200
Rhizoctonia solani 65L-2	NR[b]	NR	NR	NR	NR	NR
R. solani 67D-3	NR	NR	NR	NR	NR	NR
Macrophomina phaseolina 1	NR	NR	NR	NR	50	NR
M. phaseolina 2	NR	NR	NR	NR	NR	NR
Septoria glycines	NR	NR	NR	NR	100	NR
Colletotrichum truncatum CS 1	32	800	800	3,200	200	3,200
C. truncatum CS 3	32	800	200	3,200	400	3,200
Alternaria sp. 1	NR	NR	NR	NR	NR	NR
Alternaria sp. 2	NR	NR	NR	NR	NR	NR
Penicillium sp.	64	800	NR	400	200	400
Aspergillus sp. 2	64	1,600	NR	800	400	400
Aspergillus sp. 3	NR	NR	NR	NR	NR	NR
Cercospora kikuchii 1	NR	NR	NR	NR	NR	NR
C. kikuchii 2	8	NR	NR	NR	NR	NR
Chaetomium sp.	64	200	NR	3,200	400	1,600
Phytophthora sojae 1	64	NR	NR	NR	100	NR
P. sojae 22	32	400	NR	NR	NR	NR
Fusarium sp. 5-B-8	64	1,600	1,600	3,200	400	1,600
Fusarium sp. 5-C-9	64	1,600	1,600	3,200	200	1,600
Rhizopus sp. 1	NR	NR	NR	NR	NR	NR
Rhizopus sp. 2	NR	NR	NR	NR	NR	NR
Phialophora gregata C3-15	NR	NR	NR	NR	50	NR

[a] New Zealand white rabbits 901–903 were used to produce PAbs to *P. longicolla* culture filtrate immunogen. PAb-1 from each rabbit was purified from the first-bleeding serum and PAb-2 from the second-bleeding serum.
[b] No reaction.

DAS-ELISA with culture filtrate antigens and PAbs. Preliminary experiments demonstrated maximum sensitivity with the reagent concentrations we used (data not shown). Maximum sensitivity was 0.0001 μg of antigen protein per well, which was 100-fold more sensitive than indirect ELISA. All culture filtrates of the *Diaporthe-Phomopsis* complex reacted strongly with 902-3 (Table 4). Reactivity was not higher with any member of the complex. Reactivity of all culture filtrates with 902-2 was qualitatively identical to those with 902-3 but was in some cases lower. Lower reactivity was due to higher positive-negative thresholds with 902-2, since PAbs were subjected to additional freeze-thaw cycles caused by storage equipment problems. Reactivity of 902-2 was higher to *D. p. sojae* than to *P. longicolla*. Some isolates had lower A_{405} values when reacted to 902-3 compared with indirect ELISA. Isolates with lower reactions were *P. longicolla* 1, *D. p. sojae* 4, *D. p. sojae* 8, and *D. p. caulivora*. Lower reactivity did not relate to taxonomic divisions and was consistent with lower reactivity of 902-3 to *P. longicolla* 1 in indirect ELISA. However, other cultures that showed less reactivity in DAS-ELISA reacted strongly to 902-3 in indirect ELISA. DAS-ELISA was affected more by lower PAb reactivity to *Diaporthe-Phomopsis* cultures than was indirect ELISA.

TABLE 2. Maximum dilutions of fungal culture filtrates that yielded positive reactions in indirect enzyme-linked immunsorbent assay with polyclonal antibodies (PAbs) developed in rabbit 902[a] to *Phomopsis longicolla* culture filtrate

Fungal isolate	PAb 902-1	902-2	902-3	902-4
Phomopsis longicolla 1	1,600	1,600	400	NR[b]
P. longicolla 4	1,600	3,200	3,200	3,200
P. longicolla 8	1,600	3,200	3,200	3,200
P. longicolla 10	1,600	3,200	3,200	NR
P. longicolla 12	800	3,200	3,200	NR
P. longicolla (Ohio)	800	3,200	3,200	NR
Diaporthe phaseolorum var. *sojae* L3	1,600	3,200	3,200	3,200
D. p. sojae 4	1,600	3,200	3,200	3,200
D. p. sojae 5	1,600	3,200	3,200	3,200
D. p. sojae 7	1,600	3,200	3,200	3,200
D. p. sojae 8	1,600	3,200	3,200	3,200
D. p. caulivora	1,600	3,200	800	1,600
D. p. meridionalis	1,600	3,200	3,200	1,600
Rhizoctonia solani 65L-2	NR	NR	NR	NR
R. solani 67D-3	NR	NR	NR	NR
Macrophomina phaseolina 1	NR	NR	400	NR
M. phaseolina 2	NR	NR	NR	NR
Septoria glycines	NR	NR	NR	NR
Colletotrichum truncatum CS 1	800	3,200	1,600	400
C. truncatum CS 3	200	3,200	3,200	3,200
Alternaria sp. 1	NR	NR	NR	NR
Alternaria sp. 2	NR	NR	NR	NR
Penicillium sp.	NR	400	NR	NR
Aspergillus sp. 2	NR	800	NR	NR
Aspergillus sp. 3	NR	NR	NR	NR
Cercospora kikuchii 1	NR	NR	NR	NR
C. kikuchii 2	NR	NR	NR	NR
Chaetomium sp.	NR	3,200	3,200	400
Phytophthora sojae 1	NR	NR	800	1,600
P. sojae 22	NR	NR	NR	1,600
Fusarium sp. 5-B-8	1,600	3,200	1,600	NR
Fusarium sp. 5-C-9	1,600	3,200	1,600	3,200
Rhizopus sp. 1	NR	NR	NR	NR
Rhizopus sp. 2	NR	NR	NR	NR
Phialophora gregata C3-15	NR	NR	1,600	NR

[a] New Zealand white rabbit 902 was used to produce PAbs to *P. longicolla* culture filtrate immunogen. PAb-1 was purified from the first-bleeding serum and PAb-2, -3, and -4 from the second-, third-, and fourth-bleeding sera, respectively.
[b] No reaction.

Specificity and consistency with different PAbs in DAS-ELISA were better than those in indirect ELISA (Table 4). The only fungus outside the *Diaporthe-Phomopsis* complex that reacted was *C. truncatum*. The *C. truncatum* isolates also were strongly cross-reactive in indirect ELISA. Isolates *Macrophomina phaseolina* 1, *Penicillium, Aspergillus* 2, *Chaetomium, Phytophthora sojae* 1, *Fusarium* 5-B-8 and 5-C-9, and *P. gregata* C3-15 were not cross-reactive in DAS but were in indirect ELISA.

DAS-ELISA with mycelial extract antigens and PAbs. PAbs 902-A and 902-B were from the first- and second-bleeding sera raised to mycelial extract immunogen, respectively. Reactivity of PAbs 902-A and 902-B to mycelial extract antigens is shown in Table 5. Both PAbs were highly reactive with all members of the *Diaporthe-Phomopsis* fungal complex. Mean A_{405} values for 902-A and 902-B to *P. longicolla* were 0.676 and 1.249 and to *D. p. sojae* were 0.546 and 1.257, respectively. PAb 902-A had a significantly higher mean reaction ($P < 0.01$) to *P. longicolla* than to *D. p. sojae*. Maximum mycelial extract dilutions that gave a positive reaction were comparable between the two PAbs. The A_{405} values were higher for 902-B than for 902-A (Table 5). Background was higher for 902-B. Positive-negative thresholds for 902-A were 0.014–0.073 for ELISA plates (four of five below 0.027), whereas those for 902-B were 0.094–0.238 (four of five below 0.121). Differences between the A_{405} to mycelial extracts of the *Diaporthe-Phomopsis* fungal complex and the positive-negative threshold were greater for 902-B than for 902-A.

Cross-reactivity for 902-A and 902-B (raised to mycelial extract) was higher than for 902-2 and 902-3 (raised to culture filtrate) in DAS-ELISA (Tables 4 and 5). Ten mycelial extracts cross-reacted with 902-A and nine with 902-B. Cross-reactive mycelial extracts were different for 902-A and 902-B, except that both mycelial extracts of *C. truncatum* cross-reacted with both PAbs. A negative reaction of a fungus with a higher A_{405} value than another fungus with a positive reaction sometimes occurred, since the positive-negative threshold of different microtiter plates varied, and these fungi were assayed on different microtiter plates. The reaction to fungi of the *Diaporthe-Phomopsis* fungal complex was stronger than the reaction to *C. truncatum* for PAbs in each ELISA, except for some reactions with PAbs from later bleedings (Table 2). A_{405} values for the *Diaporthe-Phomopsis* complex were higher with mycelial extracts and PAbs 902-A and 902-B than

TABLE 3. Maximum dilutions of selected mycelial extracts that yielded positive reactions in indirect enzyme-linked immunosorbent assay with polyclonal antibodies (PAbs) developed in three rabbits[a] to *Phomopsis longicolla* culture filtrate

Fungal isolate	PAb 901-1	901-2	902-1	902-2	903-1	903-2
Phomopsis longicolla 1	100	1,000	10,000	1,000	1,000	1,000
P. longicolla 4	100	1,000	10,000	1,000	1,000	1,000
Diaporthe phaseolorum var. *sojae* 8	100	1,000	10,000	10,000	1,000	10,000
D. p. caulivora	100	1,000	10,000	1,000	1,000	1,000
D. p. meridionalis	100	1,000	1,000	1,000	100	1,000
Rhizoctonia solani 65L-2	NR[b]	NR	NR	NR	NR	NR
Alternaria sp. 2	NR	NR	NR	NR	NR	NR
Colletotrichum truncatum CS 3	NR	NR	NR	100	NR	1,000

[a] New Zealand white rabbits 901–903 were used to produce PAbs to *P. longicolla* culture filtrate immunogen. PAb-1 from each rabbit was purified from the first-bleeding serum and PAb-2 from the second-bleeding serum.
[b] No reaction.

with culture filtrates in DAS-ELISA with PAbs 902-2 and 902-3 (data not shown), and many mycelial extracts were detectable at higher dilutions than were the culture filtrates (Tables 4 and 5). Thus, PAbs raised to mycelial extract exhibited higher activity but lower specificity than those raised to culture filtrate.

With some weakly cross-reactive mycelial extracts, decreasing antigen produced increasing A_{405} values. The reaction between 902-B and mycelial extract of *Penicillium* was positive at dilutions of 1:10,000 and 1:100,000 but negative at 1:100 and 1:1,000; this was also true for the same antigen dilutions in the reaction between 902-A and mycelial extract of *P. sojae* 22. Again, this pattern was repeatable in these reactions.

DISCUSSION

Comparison of first and second bleedings. PAbs had increased activity against *P. longicolla* from second bleedings. The response to an immunogen is often relatively constant after two immunizations. However, the PAbs probably were largely carbohydrate specific, if consistent with results reported by others (5,6,10, 15,16,24,28,42). Affinity of anticarbohydrate antibodies should not increase, but the quantity of these antibodies could increase with additional immunizations (E. W. Voss, Jr., *personal communication*). Background increased for second-bleeding PAbs with both culture filtrate and mycelial extract immunogens. A population of antiprotein epitope PAbs may have had increased affinity between the first and second bleedings, leading to increased cross-reactivity. Antibodies with higher affinity are more cross-reactive (38).

Individual animal responses. Rabbits were the same strain, but their PAb responses varied. Differences between rabbits in PAb binding to *P. longicolla* from first and second bleedings suggested there was more variability in the speed than in the strength of the response. Large differences between and within rabbits at different stages of immunization have been documented (38).

Maximum sensitivity. The higher sensitivity of PAbs from second bleedings was consistent with their higher activity as measured by A_{405} values. We do not know whether higher anti-*P. longicolla* antibody levels, higher affinity, or both accounted for the higher sensitivity. Ricker et al (29) found lower activity but higher specificity of early-bleed antisera with *Botrytis cinerea* as the immunogen, which was similar to our results. The 100-fold increase in sensitivity of DAS-ELISA may be due to antigen concentration on wells by capture antibodies, compared with antigen bound directly to plastic in indirect ELISA. Concentrated sites would bind more biotinylated antibody. Monoclonal antibody (MAb) titers were 10^5- to 10^6-fold higher in DAS-ELISA than in indirect ELISA in barley yellow dwarf virus systems (8).

TABLE 4. Maximum dilutions of fungal culture filtrates that yielded positive reactions in double antibody sandwich–enzyme-linked immunosorbent assay (DAS-ELISA) with polyclonal antibodies (PAbs) developed in rabbit 902[a] to *Phomopsis longicolla* culture filtrate and qualitative consistency (+) or inconsistency (−) with indirect ELISA

Fungal isolate	PAb 902-2	PAb 902-3	ELISA comparison
Phomopsis longicolla 1	1,000	10,000	+
P. longicolla 4	100	10,000	+
P. longicolla 8	100	10,000	+
P. longicolla 10	1,000	10,000	+
P. longicolla 12	1,000	10,000	+
P. longicolla (Ohio)	1,000	10,000	+
Diaporthe phaseolorum var. *sojae* L3	1,000	10,000	+
D. p. sojae 4	10,000	10,000	+
D. p. sojae 5	10,000	10,000	+
D. p. sojae 7	10,000	10,000	+
D. p. sojae 8	10,000	10,000	+
D. p. caulivora (Ohio)	1,000	10,000	+
D. p. meridionalis (Florida)	1,000	10,000	+
Rhizoctonia solani 65L-2	NR[b]	NR	+
R. solani 67D-3	NR	NR	+
Macrophomina phaseolina 1	NR	NR	−
M. phaseolina 2	NR	NR	+
Septoria glycines	NR	NR	+
Colletotrichum truncatum CS 1	1,000	10,000	+
C. truncatum CS 3	100	1,000	+
Alternaria sp. 1	NR	NR	+
Alternaria sp. 2	NR	NR	+
Penicillium sp.	NR	NR	−
Aspergillus sp. 2	NR	NR	−
Aspergillus sp. 3	NR	NR	+
Cercospora kikuchii 1	NR	NR	+
C. kikuchii 2	NR	NR	+
Chaetomium sp.	NR	NR	−
Phytophthora sojae 1	NR	NR	−
P. sojae 22	NR	NR	+
Fusarium sp. 5-B-8	NR	NR	−
Fusarium sp. 5-C-9	NR	NR	−
Rhizopus sp. 1	NR	NR	+
Rhizopus sp. 2	NR	NR	+
Phialophora gregata C3-15	NR	NR	−

[a] New Zealand white rabbit 902 was used to produce PAbs to *P. longicolla* culture filtrate immunogen. PAb-2 was purified from the second-bleeding serum and PAb-3 from the third-bleeding serum.
[b] No reaction.

TABLE 5. Maximum dilution ($\log_{10}$) of mycelial extracts that yielded positive reactions in double antibody sandwich–enzyme-linked immunosorbent assay with polyclonal antibodies (PAbs) developed in rabbit 902[a] to *Phomopsis longicolla* mycelial extract

Fungal isolate	PAb 902-A Maximum dilution	PAb 902-A A_{405}[b]	PAb 902-B Maximum dilution	PAb 902-B A_{405}
Phomopsis longicolla 1	10^{-5}	0.670	10^{-5}	1.299
P. longicolla 4	10^{-5}	0.744	10^{-5}	1.333
P. longicolla 8	10^{-4}	0.610	10^{-4}	1.006
P. longicolla 10	10^{-5}	0.669	10^{-5}	1.342
P. longicolla 12	10^{-4}	0.669	10^{-4}	1.207
P. longicolla (Ohio)	10^{-5}	0.695	10^{-4}	1.305
Diaporthe phaseolorum var. *sojae* L3	10^{-4}	0.481	10^{-5}	1.165
D. p. sojae 4	10^{-5}	0.470	10^{-5}	1.297
D. p. sojae 5	10^{-5}	0.645	10^{-5}	1.402
D. p. sojae 7	10^{-5}	0.559	10^{-4}	1.247
D. p. sojae 8	10^{-5}	0.573	10^{-4}	1.172
D. p. caulivora (Ohio)	10^{-5}	0.607	10^{-5}	1.163
D. p. meridionalis (Florida)	10^{-4}	0.797	10^{-5}	1.169
Rhizoctonia solani 65L-2	10^{-2}	0.034	NR[c]	0.078
R. solani 67D-3	10^{-2}	0.027	NR	0.077
Macrophomina phaseolina 1	NR	0.030	10^{-2}	0.102
M. phaseolina 2	10^{-2}	0.032	NR	0.080
Septoria glycines	10^{-4}	0.021	NR	0.090
Colletotrichum truncatum CS 1	10^{-5}	0.324	10^{-5}	0.555
C. truncatum CS 3	10^{-5}	0.350	10^{-5}	0.804
Alternaria sp. 1	NR	0.007	NR	0.005
Alternaria sp. 2	10^{-5}	0.009	NR	0.052
Penicillium sp.	NR	0.023	10^{-5}	0.072
Aspergillus sp. 2	NR	0.034	10^{-4}	0.122
Aspergillus sp. 3	NR	0.021	NR	0.085
Cercospora kikuchii 1	NR	0.036	10^{-2}	0.145
C. kikuchii 2	NR	0.015	10^{-4}	0.125
Chaetomium sp.	NR	0.041	10^{-4}	0.261
Phytophthora sojae 1	NR	0.010	10^{-3}	0.136
P. sojae 22	10^{-5}	0.011	NR	0.092
Fusarium sp. 5-B-8	NR	0.020	NR	0.075
Fusarium sp. 5-C-9	10^{-3}	0.030	NR	0.089
Rhizopus sp. 1	NR	0.012	NR	0.111
Rhizopus sp. 2	NR	0.013	NR	0.106
Phialophora gregata C3-15	10^{-4}	0.029	NR	0.052

[a] New Zealand white rabbit 902 was used to produce PAbs to *P. longicolla* mycelial extract immunogen. PAb 902-A was purified from the first-bleeding serum and PAb 902-B from the second-bleeding serum.
[b] The mean A_{405} value (absorbance at 405 nm) for each PAb reacted to each mycelial extract at a dilution of 10^{-2}
[c] No reaction.

PAb reactivity to fungal antigens. High reactivity of most PAbs to *Diaporthe-Phomopsis* cultures occurred. Gleason et al (13) reported lower reactivity to both *D. p. sojae* and *D. p. caulivora* than to *P. longicolla* with PAbs produced to 24-h-old mycelium of *P. longicolla.*

Cross-reactivities with *Fusarium* were stronger in our study than in that of Gleason et al (13). They had minimal cross-reactivity to *Aspergillus flavus;* we had none to one isolate of *Aspergillus* and high cross-reactivity to the other. Cross-reaction to *Penicillium* from their study was weak, and most of ours was strong. PAb 902-1 did not cross-react more than PAbs of Gleason et al (13), but their PAbs may be more specific than ours. Culture filtrate antigens were more specific than those from the fungal body (see below), which further suggested that 24-h-old immunogen elicited more specific PAbs than did immunogen from our older cultures.

Fungi in the same genus demonstrate serological similarity (3,4,11,12,19,40), but often not all species are reactive. Some *Pythium* spp. appeared antigenically identical, some cross-reactive, and some antigenically unique (21). PAbs to *Phytophthora fragariae* did not cross-react with 18 fungal species from strawberry roots but did with some isolates of *Phytophthora cactorum* and *Pythium middletonii* (4). The reaction of antiserum against *B. cinerea* was 48% as strong as that to *B. allii,* 10–24% for species of *Monilinia* and *Sclerotinia,* whereas other fungi had a reaction of less than 0.1% compared with that of *B. cinerea* (30). *Sclerotinia* and *Monilinia* are in the Sclerotiniaceae, and *Botrytis* is the anamorph of *Monilinia* (1).

Comparison of our results with those above support a close taxonomic relationship between fungi of the *Diaporthe-Phomopsis* fungal complex of soybeans. This is compatible with taxonomy based on morphology (23).

Isolates of *C. truncatum* showed a close serological relationship with *Diaporthe* and *Phomopsis.* Fungi in the *Diaporthe-Phomopsis* fungal complex and *C. truncatum* are virulent soybean pathogens with similar life cycles (32). It is possible that components of each with functional similarity were recognized by PAbs. A cotton pathogen, *Verticillium albo-atrum* (nondefoliating strain SS4), differed antigenically from a defoliating strain (T9) (43). SS4 was more closely related serologically to mildly virulent *V. nigrescens* than to T9 (43). A highly virulent isolate of *Gremmeniella abietina* in New York appeared immunologically identical to the European counterpart. The common North American race was immunologically different and less virulent than both (9).

Serological relationships differed with different fungal preparations. The cross-reaction to *Colletotrichum* was higher with mycelial extract than with culture filtrate antigen. Additional cultures cross-reacted with PAbs raised to mycelial extract (902-A and 902-B) only. However, only *C. truncatum* mycelial extract strongly cross-reacted. A protein-lipopolysaccharide antigen in culture filtrate of *V. dahliae* was not detectable from extracts of *V. nigrescens, V. nubilum,* or *V. tricorpus* and differed from cross-reactive antigens of mycelia (25). Medically important fungi produce unique and shared culture filtrate antigens, and extracts were more cross-reactive with more shared antigens (18). Sphaerules of *Coccidioides immitis* contained cross-reactive antigens, and culture filtrate reactions were more specific (20). However, the response was stronger to mycelial extract than to culture filtrate in the rabbit we used and in mice (7).

The reaction to *Diaporthe* and *Phomopsis* was stronger than to *C. truncatum,* so rabbit systems recognized epitopes unique to the *Diaporthe-Phomopsis* fungal complex. Populations of antibodies specific for these fungi could probably be prepared by cross-absorbing against cross-reactive antigens, which increases specificity of PAbs (3,11,29,40). A 50% saturated $(NH_4)_2SO_4$ precipitate of a mycelial extract from *V. dahliae* resulted in species-specific antisera (12), and ribosomes from fungi used as immunogen gave rise to PAbs specific at the genus and sometimes at the species level with indirect ELISA (36). Increased antibody reaction specificity to fungi may result from production of monoclonal antibodies and was reviewed recently (41).

Long-term immune response. Less dominant epitopes often are recognized in later immunizations (38). In another study, the highest PAb activity against *P. longicolla* was after the final injections in two protocols; each rabbit had weekly immunizations for the first 3 wk and a final immunization 3 wk later in one protocol and 5 wk later in the other (13). No mention was made of decreased PAb reactivity following later immunizations.

Except for the reaction of *D. p. caulivora* against PAb 902-3, no isolates of *D. phaseolorum* had greatly reduced reactivity to PAbs 902-3 or 902-4. Decreased reactivity to some *P. longicolla* isolates demonstrated small antigenic differences in the *Diaporthe-Phomopsis* fungal complex. Some isolates of *P. longicolla* reacted to PAb 902-4, consistent with microscopic and cultural variability (23). In another report, immunotolerance was induced by a capsular polysaccharide of *Cryptococcus neoformans* that inhibited lymphocyte migration (28). It is possible that lymphocytes of rabbit 902 were affected similarly in our study.

Comparison of indirect ELISA and DAS-ELISA. We propose that a partial explanation for the increased specificity in DAS-ELISA is that individual antigen molecules that cross-reacted in indirect ELISA but not in DAS-ELISA have only one epitope in common with those of *P. longicolla.* Antigen molecules of *P. longicolla* should have at least two epitopes that the population of PAbs recognizes (Fig. 1B). Capture antibody could bind one epitope; then another epitope on the same molecule would be bound by biotinylated antibody. If antigens had only one epitope in common with antigens of *P. longicolla,* these epitopes would bind capture PAbs, and no epitopes would be available for biotinylated PAbs. In contrast, in indirect ELISA, antigens with only one epitope in common with antigens of *P. longicolla* could have the epitope exposed to antibodies, and a different portion could be bound to the well (Fig. 1A). It is also possible that epitope conformations in indirect ELISA vs. DAS-ELISA differed and contributed to these differences. Amouzou-Alladaye et al (4) detected *P. fragariae* more specifically in strawberry roots with DAS-ELISA than with indirect ELISA, and DAS-ELISA, but not indirect ELISA, was specific enough to successfully detect *V. dahliae* and *V. albo-atrum* in potato plants (34). Differences in cultures exhibiting reduced reactivity between assay formats may be a reflection of exposure of different epitopes to detecting antibodies or of different conformations of those epitopes in indirect ELISA vs. DAS-ELISA. A study with MAbs showed epitope conformation can be distorted in indirect ELISA, leading to unreliable results (2).

DAS-ELISA, but not indirect ELISA, was qualitatively identical with two PAbs against culture filtrate immunogen. Thus, DAS-ELISA was superior with our PAbs. However, DAS-ELISA as a screening assay detected only cross-reactive monoclonal antibodies with *P. longicolla* mycelial extract as immunogen and antigen (L. M. Brill and J. B. Sinclair, *unpublished data*), whereas indirect ELISA detected specific monoclonal antibodies against *Sirococcus strobilinus* (22) and *Leptosphaeria korrae* (26).

Different antigen preparations from fungi of the same species. Reactions with fungi of the *Diaporthe-Phomopsis* fungal complex were consistent between culture filtrate and mycelial extract antigens when PAbs raised against culture filtrate were used. Common antigens were in mycelium and growth medium. The reactions of PAbs raised to culture filtrate against mycelial extracts sometimes differed between members of the complex. The concentration of antigens in mycelial extracts was not determined and may explain these differences. Neither fungus with nonreactive culture filtrate contained antigens in the mycelium that cross-reacted when released by extraction. With *C. truncatum,* four of six PAbs raised to culture filtrate did not react with mycelial extract, suggesting cross-reactive components may be modified upon secretion into the medium and are not extractable in reactive form. However, PAbs that did cross-react with mycelial extract of *C. truncatum* recognized an antigen(s) that others did not. Some antigens may be identical in the mycelium and when secreted; some antigens are more loosely associated with mycelium than others; or both of these explanations may be valid. Antisera against cell wall and mycelial extract antigens of *Verticillium* shared common immunoglobulins, but antigens differed (11).

Reactions of cell wall and mycelial extract antigens were comparable against homologous and heterologous antisera; thus, common antigens were identified (18). Two of five MAbs produced to mycelial antigens (apparently from the mycelium surface) of *S. strobilinus* recognized culture filtrate antigens (22). These results are similar to ours, illustrating some antigens are shared between mycelia and culture filtrates.

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