Serological Detection of *Phomopsis longicolla* in Soybean Seeds

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ABSTRACT

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Antiserum to freeze-dried powdered mycelium of *Phomopsis longicolla*, a soybean seed decay pathogen, was used in an indirect ELISA and a modified immunoblot assay for detecting seedborne infections. Antigen of *P. longicolla* was detected by indirect ELISA in as little as 250 ng of freeze-dried mycelium per milliliter of extract. The antiserum reacted strongly with mycelial preparations of *Diaporthe phaseolorum* var. *sojae* and *D. p.* var. *caulivora* but showed comparatively little or no reaction when tested against seven other seedborne fungi. Extracts of whole seeds, but not of seed coats, produced a nonspecific background reaction that obscured the specific reaction. *P. longicolla* could be detected in individual seed coats of symptomless infected seeds. A single infected seed coat in 20 could be readily detected by indirect ELISA of extracts of seed coats. A modified immunoblot assay, designated the seed immunoblot assay (SIBA), was developed to overcome problems with nonspecific interference in indirect ELISA. Mycelium of *P. longicolla* growing onto nitrocellulose paper from infected soybean seeds produced a conspicuous colored blotch after the paper was assayed. Results of SIBA for incidence of *P. longicolla* and *D. p.* var. *sojae* in halved seeds from 10 seed lots correlated ($P<0.001$) with agar plate bioassays of the corresponding seed halves but not with incidence of symptomatic seeds. Indirect ELISA absorbance values for bulked samples of seed coat halves from the same 10 seed lots correlated weakly ($0.10>P>0.05$) with agar plate bioassays but strongly ($P<0.01$) with incidence of symptomatic seeds. Because SIBA detects only viable *P. longicolla* and ELISA does not discriminate between live and dead fungus, SIBA should be a better indicator of pathogen activity on seeds after planting. The two types of serological assays apparently measure different aspects of the disease, however, and both may be useful for evaluating soybean seed lot quality.

Phomopsis seed decay (PSD) is a major cause of poor quality of soybean (*Glycine max* (L.) Merr.) seeds in the United States and other soybean-growing areas of the world (25). The causal fungi, *Phomopsis longicolla* Hobbs, *Diaporthe phaseolorum* (Cke. & Ell.) Sacc. var. *sojae* (Lehm.) Wehm., and *D. p.* var. *caulivora* Athow & Caldwell, invade seeds during or after the maturation period by growing through the pod walls when weather conditions are favorable (12,18,22,26). Infected seeds are subject to damping-off, and seed mortality is often strongly correlated with percent incidence of these fungi in seed lots (19), particularly under favorable soil moisture and temperature conditions (7). *P. longicolla* is more pathogenic to seeds than *D. p.* var. *sojae* or *D. p.* var. *caulivora* and is frequently more abundant in infected seed lots (17,18,23).

Despite the considerable economic impact of losses from PSD, most seed-testing laboratories in the United States do not routinely test soybean seed lots for the causal fungi. One reason may be that procedures for enumerating fungal pathogens on seeds are labor-intensive and time-consuming. In the most widely used procedure, the agar plate bioassay, seeds are incubated for 7–10 days on agar plates, after which the fungi are distinguished from each other on the basis of mycelial appearance, spore type, and other morphological traits.

Immunological methods, particularly enzyme-linked immunosorbent assays (ELISA), have been widely used for detecting seedborne viruses (2,8,13,20) and to a lesser extent for detecting seedborne fungi (16,24). These methods are often more rapid, sensitive, and straightforward to interpret than bioassays. A more recently developed immunological assay, "dot-immunobinding" or "dot-blotting" (9), has not yet been applied to detection of seedborne infections but could offer some of the advantages ELISA has over the agar plate bioassay method, such as rapidity and ease of diagnosis.

The purpose of the present work was to investigate the potential usefulness of ELISA and immunoblotting for determining incidence of infection by *P. longicolla* in soybean seed lots.

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MATERIALS AND METHODS

Antigen production. An isolate of *P. longicolla* from an infected soybean seed was positively identified on the basis of colony morphology and spore characteristics (11). Suspensions of pycnidiospores ($1.5–3.0 \times 10^5$ spores per milliliter) produced on Difco potato-dextrose agar (PDA) were germinated in dilute acidified Difco potato-dextrose broth (PDB) at pH 4.4–4.5 in petri dishes. After 24–28 hr at 25 C, nearly 100% of the spores had formed thalli up to 500 μm in length. These germlings were collected on Whatman No. 42 filter paper in a Büchner funnel and washed with 0.02 M phosphate buffered saline, pH 7.4 (PBS). The thalli were then suspended in 50 ml of PBS and washed by centrifugation at 10,000 *g* for 10 min. The pellet was resuspended in PBS, centrifuged two more times, then freeze-dried, ground in a mortar with a pestle, and stored in a desiccator at −5 C. This material was used as both the immunogen and the standard antigen in ELISA tests. Nine other fungi isolated from soybean seeds were used to test the specificity of immunoassays. For ELISA tests, these fungi were cultured in PDB for 2 wk at 25 C. The resulting mycelia were harvested, freeze-dried, and powdered as described for *P. longicolla*.

Antiserum production. Antisera to *P. longicolla* were produced by administering four subcutaneous injections to each of two female New Zealand white rabbits. The first three injections were given at weekly intervals and the fourth injection was given 3 wk after the third injection of one rabbit and 5 wk after the third injection of the other rabbit. The injections consisted of 33 mg of powdered, freeze-dried mycelium of *P. longicolla* emulsified with 1 ml of PBS and 1 ml of adjuvant. Freund's complete adjuvant was used as the emulsifier for the first injection, and Freund's incomplete adjuvant was used for the later injections. The rabbits were bled before the injection regime and 1 wk after the second, third, and fourth injections. The antisera were stored frozen.

Immunoglobulin preparation. Immunoglobulin (Ig) was partially purified by passing 1-ml portions of serum through a

column of protein A-agarose (1) equilibrated with half-strength PBS, then eluting the Ig with a solution of 0.15 M NaCl and 0.58% glacial acetic acid. The eluate was then dialyzed three times against half-strength PBS at 5 C to remove glacial acetic acid. The Ig fractions were adjusted to approximately 1.0 mg/ml with an extinction coefficient of 1.4 $(mg/ml)^{-1}cm^{-1}$ at 278 nm and stored frozen in siliconized glass tubes.

Indirect ELISA. The protocol was similar to those of Crowther and Abu-El Zein (4), Crook and Payne (3), and Lommel et al (21). Polystyrene ELISA plates (Dynatech Laboratories, Alexandria, VA) were coated with standard antigen preparations or test samples in 0.05 M sodium carbonate buffer, pH 9.6, 200 µl per well. After a 2-hr incubation at 30 C, 200 µl per well of the Ig preparation at 10 µg/ml in PBS amended with 0.05% Tween 20 and 2% polyvinylpyrrolidone 40 was added, and the plate was incubated overnight at 4 C. Goat antirabbit Ig conjugated to alkaline phosphatase (Sigma Chemical Co., St. Louis, MO) was then added at a dilution of 1:200, and the plate was incubated for 4 hr at 30 C before substrate addition. The wells were rinsed three times between steps with PBS containing 0.05% Tween 20 and 0.02% sodium azide. The above concentrations of Ig and conjugate were found to be optimum for detection of *P. longicolla* in seed tissue. Absorbance at 405 nm was read on a Titertek Multiskan plate reader. The plates were routinely blanked against wells containing only carbonate coating buffer.

Preparation of seed extracts. Soybean seeds were soaked in the carbonate coating buffer for 2–4 hr, then homogenized with a Polytron homogenizer for 30 sec and centrifuged for 5 min at 10,000 *g*. Appropriate dilutions of supernatant were made using the carbonate buffer. For most indirect ELISA tests, seed coats were removed with forceps after soaking and were assayed separately from cotyledons. In some experiments, seeds were rated for severity of symptoms associated with PSD before processing for immunological assays. Type 1 dry seeds were chalky-colored and apparently mummified, type 2 showed moderate to severe fissuring of the seed coat, and type 3 had only slight fissuring of the seed coat. Asymptomatic seeds that were infected (type 4) and uninfected (type 5) by *P. longicolla* were distinguished by plating. Seeds were surface-sterilized for 30 sec in 0.5% sodium hypochlorite, rinsed three times in sterile deionized water, and cut in half aseptically. One half of each seed was plated on PDA amended with 100 ppm of streptomycin sulfate and 5 ppm of chlortetracycline hydrochloride (amended PDA). The other half of each seed was stored at 5 C. Unplated seed halves that corresponded to plated halves showing infection by selected fungi were used in serological assays.

Seed immunoblots. Soybean seeds were surface-sterilized for 30 sec in 0.5% sodium hypochlorite, rinsed three times in sterile deionized water, and blotted dry with paper towels. Seeds were then placed 2 cm apart on nitrocellulose paper (Schleicher and Schuell, Keene, NH) in 18 × 29 × 5 cm plastic trays with three layers of moist germination towels below the nitrocellulose and three additional layers above the seeds. Trays were enclosed in aluminum foil to maintain high internal relative humidity. After incubation for 2–3 days at 25 C, seeds were removed with forceps and the antigens adsorbed to the nitrocellulose were assayed in a procedure similar to that of Hawkes et al (9). The nitrocellulose was agitated for 1 hr at room temperature in a blocking solution of Tris buffered saline (50 mM Tris-HCl, 200 mM NaCl, pH 7.4) and 5% nonfat dry milk (TBS-milk) (14). The blocking solution was then replaced with Ig preparation (10 µg/ml) in TBS-milk for 1.5 hr with agitation. After three 10-min rinses with TBS-milk, the nitrocellulose was incubated with goat antirabbit Ig (5 µg/ml) conjugated to horseradish peroxidase (Sigma) in TBS-milk for 1.5 hr with shaking. The blots were rinsed twice with TBS-milk, then twice (10 min each time) with TBS, after which a chloronaphthol-hydrogen peroxide substrate was added (9). Positive reactions were complete within 15–20 min. The nitrocellulose was then examined and rated visually for positive reactions, rinsed in deionized water, dried under a heat lamp, and stored in the dark. For some immunoblot assays, seeds were halved aseptically after surface sterilization and corresponding halves were assayed by the immunoblot method and by plating on amended PDA.

TABLE 1. Reactivity in indirect ELISA of antiserum to *Phomopsis longicolla* against other fungi

	Absorbance (405 nm)[b]			
	10 µg powdered mycelium/ml		1 µg powdered mycelium/ml	
Fungus[a]	Exp. 1[c]	Exp. 2[d]	Exp. 3[c]	Exp. 4[d]
Phomopsis longicolla	0.757	0.526	0.068	0.120
Diaporthe phaseolorum var. *sojae*	0.380	0.339	0.029	0.058
D. phaseolorum var. *caulivora*	0.198	0.381	0.025	0.071
Aspergillus flavus	0.047	0.0	0.0	0.0
Alternaria sp.	0.0	0.016	0.0	0.0
Cercospora kikuchii	0.0	0.0	0.0	0.0
Fusarium oxysporum	0.135	0.073	0.0	0.0
F. solani	0.081	0.023	0.0	0.0
Pythium ultimum	0.0	0.0	0.0	0.0
Penicillium sp.	0.055	0.027	0.0	0.014

[a] Mycelium of each species was cultured in PDB, washed, freeze-dried, ground in a mortar and pestle, and homogenized in carbonate coating buffer. After centrifugation, the supernatant was diluted with appropriate amounts of coating buffer.
[b] Values are averages of those from two wells of a microtiter plate.
[c] Antiserum to *P. longicolla* and goat antirabbit immunoglobulin/alkaline phosphatase conjugate were used at concentrations of 10 and 5 µg/ml, respectively. Absorbance readings were made 15 min after substrate addition.
[d] Antiserum to *P. longicolla* and goat antirabbit immunoglobulin/alkaline phosphatase conjugate were both used at a concentration of 1 µg/ml. Absorbance readings were made 60 min after substrate addition.

RESULTS

Antisera. Antisera obtained 1 wk after the final injections had higher reactivity in indirect ELISA than antisera from earlier collections. The antiserum with the highest reactivity, taken 9 wk after the beginning of the injection schedule, was used in all trials.

Indirect ELISA. Antigen of *P. longicolla* was detected by indirect ELISA in as little as 250 ng of freeze-dried mycelium per milliliter. The relationship between ELISA absorbance values and antigen concentration was linear in the range of 250 ng to 10 µg/ml. Direct ELISA was evaluated in preliminary experiments, but the method was abandoned because of low sensitivity (data not shown). Of the eight genera of fungi tested, only members of the *Diaporthe/Phomopsis* complex (*P. longicolla, D. p.* var. *sojae*, and *D. p.* var. *caulivora*) reacted strongly with antisera to *P. longicolla* (Table 1). Freeze-dried mycelia of *Fusarium oxysporum* and *F. solani* at 10 µg/ml gave absorbances between 10 and 20% of that of an equivalent concentration of *P. longicolla*.

TABLE 2. Detection of *Phomopsis longicolla* in whole seeds and seed components using indirect ELISA

Experiment	Source of extract	Absorbance (405 nm)[a]
1[b]	Infected whole seeds[c]	0.136
	Infected seed coats	0.135
	Uninfected whole seeds[d]	0.071
	Uninfected seed coats	0.007
2[e]	Uninfected embryos + *P. longicolla*	0.037
	Uninfected embryos	0.020
	P. longicolla alone	0.605

[a] Values are averages of those from two wells of a microtiter plate.
[b] Seed tissue was ground in a Polytron homogenizer and centrifuged. The supernatant was diluted 1:100 (w/v) with carbonate coating buffer.
[c] *P. longicolla* was recovered from seed halves plated on amended PDA.
[d] No fungi were recovered from seed halves plated on amended PDA.
[e] Seed coats were removed and the remaining seed tissue was homogenized and centrifuged. The supernatant was diluted 1:25 (w/v) with carbonate coating buffer. Powdered mycelium was added to a final concentration of 10 µg/ml before some samples were homogenized.

Extracts of healthy seeds or separated embryos gave much higher background readings than extracts of seed coats, although absorbance values for infected seeds were about the same as those for infected seed coats (Table 2). Addition of an embryo extract to powdered mycelium of *P. longicolla* markedly reduced the absorbance value in indirect ELISA. Attempts to reduce this background by addition of D-galactose to extracts (6) were unsuccessful. Because of this interference, most seed assays by indirect ELISA were made with seed coats. Individual seed coats infected by *P. longicolla* gave higher absorbance values ($P<0.05$) than seed coats of uninfected control seeds, even when the infections were asymptomatic (Fig. 1). Absorbance was directly related to symptom severity rating. Assays of extracts from coats of seeds naturally, but asymptomatically, infected by each of five common seedborne fungi indicated that only extracts of seed coats infected by *P. longicolla* had significantly ($P<0.05$) higher absorbance in the ELISA test than extracts of seed coats with no fungal infections (Fig. 2). As in the specificity assay with freeze-dried mycelium, *Fusarium* was relatively more reactive than other genera; absorbance by extracts of coats asymptomatically infected by *P. longicolla* exceeded, but not significantly ($P>0.05$), absorbance for coats infected by *Fusarium* spp. When coats of seeds showing moderate to severe symptoms of infection by *P. longicolla* (type 2) were mixed with seed coats from a lot having a 1% incidence of *P. longicolla*, the relationship between absorbance and the proportion of infected coats was nearly stoichiometric in the 10–30% range (Fig. 3, 30-min reading). One infected seed coat mixed with 19 healthy coats (5% infected) could be detected by the assay.

Seed immunoblot assay (SIBA). Mycelium growing onto

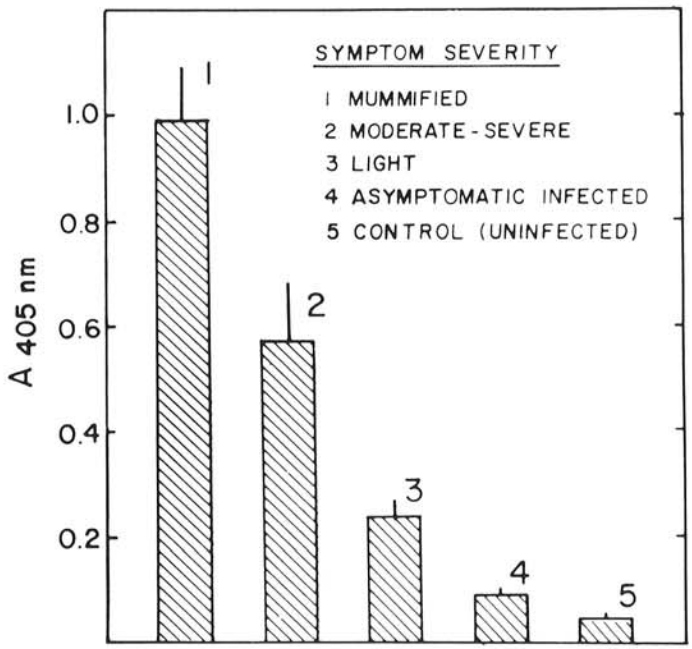

Fig. 1. Relationship between absorbance in indirect ELISA and symptoms of Phomopsis seed decay for individual soybean seeds. Assays were performed on seed coats only. Immunoglobulin (Ig) specific to *P. longicolla* and goat antirabbit Ig/alkaline phosphatase conjugate were used at concentrations of 10 and 5 μg/ml, respectively. Seed coat preparations were tested at a dilution of 1:100 (w/v) in buffer. Absorbance readings were made 15 min after substrate addition. Bars indicate one standard error of the mean for six single-seed replicates per symptom group.

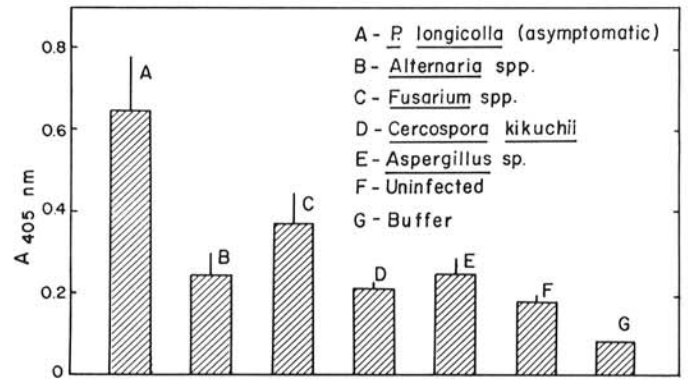

Fig. 2. Mean absorbance for seeds infected by various fungi in indirect ELISA using antiserum to *P. longicolla*. Fungi were identified by plating one half of each seed on amended PDA before ELISA. Assays were performed on the seed coat of the remaining portions of each seed. Immunoglobulin and goat antirabbit/alkaline phosphatase conjugate were used at concentrations of 10 and 5 μg/ml, respectively. Seed coat preparations were tested at a dilution of 1:100 (w/v) in buffer. Absorbance readings were made 30 min after substrate addition. Bars indicate one standard error of the mean for five single-seed replicates per fungus.

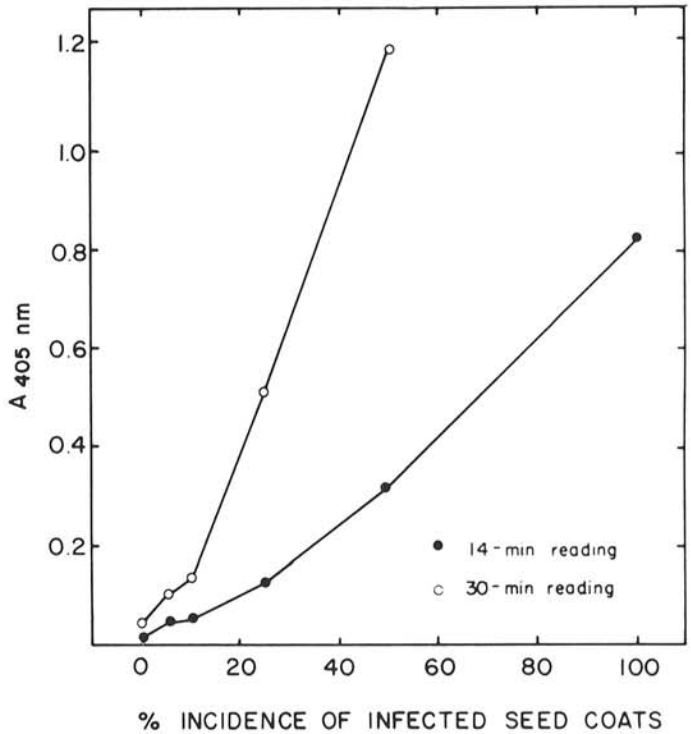

Fig. 3. Effect of dilution of seed coats known to be infected by *P. longicolla* with seed coats known to be uninfected on detection of *P. longicolla* by indirect ELISA. Immunoglobulin and goat antirabbit/alkaline phosphatase conjugate were used at concentrations of 10 and 5 μg/ml, respectively. Seed coat preparations were tested at a dilution of 1:100 (w/v) in buffer. Data points are averages of absorbance for two wells on an ELISA microtiter plate.

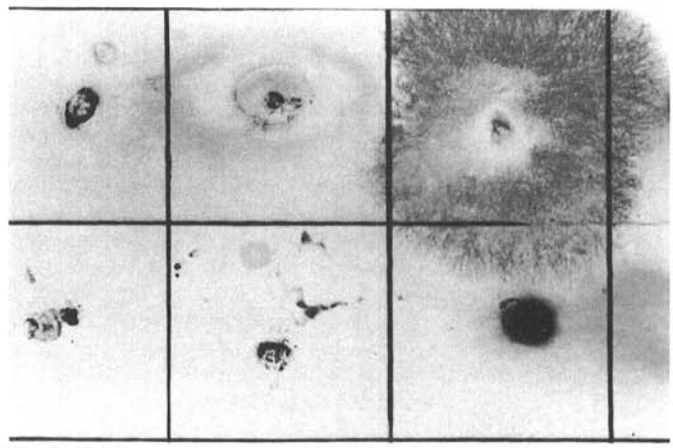

Fig. 4. Seed immunoblot assay (SIBA) for detection of Phomopsis seed decay fungi. One seed was placed on each of the six 2×2 cm squares shown. The blot in the upper right corner is a positive reaction; the other blots are negative reactions.

nitrocellulose paper from seeds infected by *P. longicolla* could be readily identified by development of a blue color within 20 min after addition of substrate (Fig. 4). The color was on both the mycelium and the paper. Spots of blue sometimes appeared where seeds had rested on the paper during incubation. These artifacts were readily distinguishable from mycelial blots by much faster development, intense coloration, and blurred rather than distinct boundaries (Fig. 4). Mycelium of *P. longicolla* also gave a visible reaction when seeds were incubated on Whatman No. 1 filter paper; however, the blots were fainter than on nitrocellulose and most of the color from positive reactions appeared to be in the mycelium rather than on the paper. When mycelium was grown from agar disks and/or infected seeds onto nitrocellulose, positive reactions were obtained by SIBA for *P. longicolla, D. p.* var. *sojae*, and *Chaetomium* sp. but not for *Cercospora kikuchii, Alternaria* sp., *Fusarium* spp., *Trichoderma* sp., *Aspergillus flavus, Penicillium* sp., *Epicoccum* sp., or *Rhizopus* sp. The dark mycelium of *Chaetomium* sp. was clearly distinguishable from the white mycelium of *P. longicolla* and other species.

Evaluation of seed lots. ELISA absorbance values for samples consisting of seed coats from 50 seed halves from each of 10 seed lots correlated relatively weakly ($0.10 > P > 0.05$) with the agar plate bioassay for percent incidence of PSD fungi in the corresponding seed halves (Fig. 5A). Results of SIBA for incidence of PSD fungi on 48 seed halves from each of the same 10 seed lots correlated strongly ($P < 0.001$) with results of agar plate bioassay of the corresponding seed halves (Fig. 5B). Incidence of type 1 and type 2 symptoms in the same 10 seed lots was significantly correlated with ELISA absorbance values ($r = 0.789$, $P < 0.01$) but not with SIBA results ($r = 0.230$, $P > 0.10$).

DISCUSSION

Our results indicate that both indirect ELISA and SIBA could be useful in evaluating soybean seed lots for infection by members of the *Diaporthe/Phomopsis* complex. Results of indirect ELISA were related to symptom severity, whereas results of SIBA were similar to those of the plate bioassay. Thus, the two tests appear to measure different aspects of the disease and are not strictly comparable. However, a preliminary evaluation of each technique is possible.

Although the quantitative accuracy of indirect ELISA has been questioned (21), absorbance values from indirect ELISA can be considered to be measures of the amount of antigen in a sample and thus the biomass of the target organism(s). However, such information might be of limited usefulness in the evaluation of seed lots because of uncertainty about the viability of the measured biomass and difficulty in estimating infection incidence. Declines in the incidence of members of the *Diaporthe/Phomopsis* complex in soybean seed in the plate bioassay and associated increases in germination of infected seed lots have been observed during seed storage (10,27). Although it is not known whether the antigenicity of *P. longicolla* also declines, work with other fungi has indicated that ELISA absorbance does not decline along with fungal viability in stored seeds (15). Consequently, ELISA results might not distinguish between nonviable and viable pathogens, thus leading to the assignment of a "false positive" in some instances. Difficulty in estimating infection incidence of *P. longicolla* from indirect ELISA results is due to both the dependence of absorbance on severity of infection that we observed (Fig. 1) and the statistical uncertainty associated with indirect sampling procedures such as those used with ELISA (5). Our observation that a highly symptomatic seed (type 1) can have approximately 10 times the absorbance of an asymptomatically infected seed indicates that estimates of infection incidence cannot be based on quantitative results of ELISA because the distribution of infection severity classes in seed lots is not known. Consequently, estimates must be based on the frequency of occurrence of positive reactions in a number of multiple-seed samples from each seed lot. Although it might be possible to narrow the confidence limits for infection incidence by use of a multiple-stage sampling procedure such as that used for *Epichloë typhina* in tall fescue seed lots (15), a relatively large number of samples would still be required. Overall, although results of indirect ELISA could be of use in estimating the severity of infections, it is unlikely that this test would be useful as the sole measure of activity of *P. longicolla* in soybean seed lots. The technique might be useful in ecological or physiological studies involving the measurement of changes in pathogen activity in plant tissue, however.

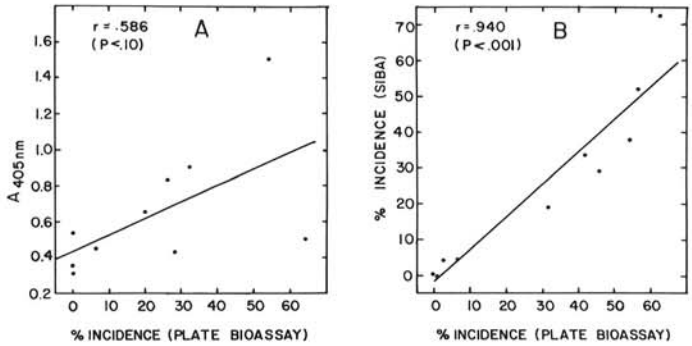

Fig. 5. Regressions of **A,** absorbance in indirect ELISA and **B,** results of the seed immunoblot assay (SIBA) against percent incidence of Phomopsis seed decay fungi in halved seeds from 10 soybean seed lots, as measured by agar plate bioassay. For the agar plate bioassays, seed halves were plated on PDA amended with antibiotics. For indirect ELISA, seed coats from 50 seed halves per seed lot were homogenized in carbonate coating buffer and centrifuged, and the supernatant was tested at a final dilution corresponding to 1:100 (w/v). Absorbance was measured 30 min after addition of substrate. Values are means of two wells on a microtiter plate. For SIBA, halves of 48 seeds per lot were incubated individually on nitrocellulose for 3 days before assay.

The seed immunoblot assay we developed can be considered to be a variant of the plate bioassay method in which identification of pathogens is based on serology rather than on morphology. SIBA is clearly superior to the plate bioassay by requiring fewer media materials and less time, incubator space, and mycological expertise; disadvantages include high cost (for nitrocellulose paper) and inability to identify fungi other than those for which antisera are available. If a suitable paper cheaper than nitrocellulose could be found, SIBA could be a desirable alternative to the plate bioassay for fungi in the *Diaporthe/Phomopsis* complex and other fungi that grow vigorously from plant tissues.

The reactivity of our antiserum to *P. longicolla* with *D. p.* var. *sojae* and *D. p.* var. *caulivora* supports the widely held view that these fungi are closely related (11,17,23) and is similar to the cross-reactivity of antiserum to *D. p.* var. *caulivora* with *Phomopsis* sp. (24). Antisera that could distinguish *P. longicolla* from *D. p.* var. *sojae* and *D. p.* var. *caulivora* might be obtained by production of monoclonal antibodies. The use of such an antiserum might alleviate the problems we encountered with whole-seed interference with ELISA and could allow positive identification of *P. longicolla* in both ELISA and SIBA.

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