Immunodetection of *Phomopsis* Species in Asymptomatic Soybean Plants

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ABSTRACT

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An immunodiagnostic assay was developed for early detection of *Phomopsis phaseoli*, cause of pod and stem blight, and *P. longicolla*, cause of seed decay of soybeans (*Glycine max*), in asymptomatic soybean plant tissue using polyclonal antibodies developed against *P. longicolla*. Immunoblot analysis of the immunogen and cross-reactive fungal antigens showed a broad smear above 50 kDa. Analyses of *P. longicolla* and 17 other common soybean parasitic or saprophytic fungi resulted in strong reactions with all *Phomopsis* spp. and *Colletotrichum truncatum*. The reaction of these fungi to antibodies developed against *P. longicolla* was similar, whether developed against culture filtrates or mycelial extracts. An arbitrary "antigen unit" was devised to measure the amount of antigen in a sample instead of using absorption values. Stem samples from uninoculated (control) and inoculated greenhouse-grown soybean (cv. Hack) plants produced immunoreactions to *P. phaseoli* that agreed with bioassay results and confirmed epiphytic growth of the pathogen.

The *Diaporthe/Phomopsis* complex of soybeans (*Glycine max* (L.) Merr.) is endemic wherever soybeans are grown (21). The complex consists of *D. phaseolorum* (Cooke & Ellis) Sacc. var. *sojae* (S. G. Lehman) Wehmeyer (anamorph *P. phaseoli* (Desmaz.) Sacc.), cause of pod and stem blight of soybeans; *P. longicolla* T. W. Hobbs, cause of Phomopsis seed decay; *D. p.* var. *caulivora* K. L. Athow & R. M. Caldwell (anamorph unknown), cause of northern stem canker; and *D. p.* f. sp. *meridionalis* Morgan-Jones (anamorph unknown), cause of southern stem canker. Members of the complex are all seedborne and latent in soybeans (1,20).

P. phaseoli causes localized infections in soybean stems (3,10). Crop debris is the primary source of inoculum infecting stems, petioles, pods, and, subsequently, seeds during physiological maturation (2,10,13). Plants are exposed to inoculum throughout the growing season, and several *Phomopsis* spp. may be recovered from asymptomatic soybeans at early stages of plant growth (12,13). Disease symptoms and fruiting structures of these pathogens appear on senescing or dead plant surfaces late in the growing season (3,24). A direct relationship between the amount of plant or pod infection and the amount of seed infection was used to determine the potential for the latter (15).

Serological detection of *Phytophthora* sp., *Pythium* sp., *Rhizoctonia solani* Kühn, and *Sclerotinia* sp. in soybean plants (16), of *D. p. caulivora* in soybean stems (19), and of *P. longicolla* in soybean seeds (8) has been reported. This work reports on the immunorelationships among common soybean parasitic and saprophytic fungi with *P. longicolla* and immunodetection of *P. phaseoli* in asymptomatic soybean plant tissues.

MATERIALS AND METHODS

Preparation of fungal antigens. Mycelial plugs (5 mm in diameter) of *P. longicolla* were taken from margins of 4-day-old potato-dextrose agar (PDA) cultures and placed in 250-ml Erlenmeyer flasks containing 100 ml of Difco potato-dextrose broth (PDB) at room temperature (24 ± 2 C) for production of fungal antigens. Culture filtrates were collected daily for 7–14 days, and weekly from the third to sixth week of growth, to obtain antigens of the fungus at different growth stages. The filtrates were combined, dialyzed against deionized distilled water in tubing with a 12,000–14,000 M_r cutoff, lyophilized, and resuspended in phosphate-buffered saline (PBS) at pH 7.4. Mycelial mats obtained from the same cultures were washed with 1.0 L of sterile deionized distilled water and ground with 2 g of acid-washed sand and 10 ml of PBS at 4 C using a mortar and pestle. Extracts were centrifuged at 12,000 *g* for

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10 min at 4 C, and the supernatant was stored at −20 C (5).

Pathogenic and saprophytic fungi frequently isolated from soybeans in Illinois were collected over a 2-yr period and used to test for cross-reactivity: *Alternaria alternata* (Fr.:Fr.) Keissl., *Aspergillus* sp., *Cercospora kikuchii* (Matsumoto & Tomoyasu) M. W. Gardner, *Chaetomium* sp., *Colletotrichum truncatum* (Schwein.) Andrus & W. D. Moore, *D. p. caulivora, D. p. meridionalis, Fusarium* sp., *Macrophomina phaseolina* (Tassi) Goidanich, *Penicillium* sp., *P. phaseoli, Phialophora gregata* (Allington & D. W. Chamberlain) W. Gams, *Phytophthora sojae* M. J. Kaufmann & J. W. Gerdemann, *Rhizopus* sp., *R. solani*, and *Septoria glycines* Hemmi. Isolates of *Phomopsis* spp. were also obtained from A. F. Schmitthenner (Ohio State University), and an isolate of *D. p. meridionalis* was obtained from Alabama (G. Morgan-Jones, Auburn University). Antigens were obtained from combined 15- and 30-day-old fungal cultures by the methods described above.

Production of polyclonal antibodies. Antisera against culture filtrates of *P. longicolla* were developed in New Zealand white rabbits (5). The primary immunogen consisted of 1 mg/ml of soluble protein dissolved in deionized distilled water and emulsified with Freund's complete adjuvant (1:1, v/v) (22). Second (at 21 days) and third (at 60 days) immunizations consisted of the same amount of protein in identical volumes emulsified with Freund's incomplete adjuvant (0.5:1, v/v). Protein content in each sample was estimated by Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA) (4). Antisera were obtained against mycelial extracts of *P. longicolla* as previously described, immunizing each of three rabbits with 0.3 mg/ml of soluble protein. The rabbits were bled 14 days after the second and third immunizations. Antibodies from all antisera were enriched by removal of the blood clot and lipoprotein by sodium dextran sulfate–calcium chloride precipitation. Antisera were purified by precipitation in 50% ammonium sulfate, and then by diethyl amino ethyl agarose (Affi-Gel blue anion exchange resin, Bio-Rad) and protein A affinity chromatography (Pharmacia, Piscataway, NJ), using proprietary methods. Antisera obtained after the second immunization were used in all experiments. Antibodies developed against mycelial extracts and culture filtrates of *P. longicolla* were used to determine cross-reactivity with other fungi from soybeans. Protein A purified immunoglobulin-G (IgG) developed against mycelial extracts of *P. longicolla* was conjugated to alkaline phosphatase (Scripps Laboratories, La Jolla, CA) according to proprietary methods and used as detection reagent in the immunoblot analysis and double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA).

Antigen unit. An arbitrary measurement, or "antigen unit," was devised to provide an approximate relative comparison between samples, since the antigen were not well defined. A 1:100 dilution of *P. longicolla* culture filtrate used for immunization contained 10,000 antigen units per milliliter, and a standard curve was devised for comparison with absorption values of samples (Fig. 1). The detection limit for this parameter was 37 antigen units per milliliter in DAS-ELISA.

Immunoblot analysis of fungal antigens. Culture filtrates of combined 15- and 30-day-old common soybean pathogenic or saprophytic fungi grown on PDB were used to test for common immunodeterminants in culture filtrates of fungi associated with soybeans, uninfected soybean seeds (control), and seeds infected with *P. longicolla*. Culture filtrates used included five isolates of *P. longicolla* (the isolate used as immunogen; isolates 4, 8, 12; and one from Ohio); *D. p. caulivora* (Ohio); *P. phaseoli* isolates 4, 7, and 8; *D. p. meridionalis*; and common soybean pathogenic or saprophytic fungi *A. alternata, Aspergillus* sp., *C. kikuchii, Chaetomium* sp., *C. truncatum, Fusarium* sp., *M. phaseolina, Penicillium* sp., *P. gregata, P. sojae, Rhizopus* sp., *R. solani*, and *S. glycines*. Soybean (cv. Corsoy) pods were harvested from plants grown from surface-disinfested seeds under disease-free conditions in the greenhouse. The pods were surface-disinfested as previously described, and five pods were placed in each of five 150-mm sterile culture plates on two layers of Whatman No. 2 moist filter paper. Pods were uninoculated (control) or inoculated with 9-day-old cultures of *P. longicolla* and incubated for 7 days at 28 C. The seeds were then removed separately, surface-sterilized as previously described, and split in half: one half for biopsy, and the other for immunoassay. Extractions were performed as previously described.

The loading samples consisted of 200-μl culture filtrates or mycelial or seed extracts added to an equal amount of sample buffer (1:1) containing sodium dodecyl sulfate (SDS) and 2-mercaptoethanol (14). The mixture was placed in a boiling water bath for 4 min, cooled, and centrifuged at 14,000 *g* for 2 min. A 30-μl sample was loaded into each well of Humo Maxisorb F96 certified plates. Prestained molecular weight markers (RPN 756, Amersham, Arlington Heights, IL) were used as standards. Precast SDS 3–17% polyacrylamide minigels (Jule Biotechnologies, Inc., New Haven, CT) were used throughout the study. Electrophoresis was performed at a constant 200 V in a Bio-Rad Protean II electrophoresis unit in an SDS-polyacrylamide gel electrophoresis tank buffer containing 0.025 M Tris-HCl, 0.192 M glycine, and 0.1% (w/v) SDS, pH 8.3 (EC-870, National Diagnostics, Manville, NJ).

After electrophoresis, gels containing fractionated proteins or glycoproteins were transferred to a 0.45-μm nitrocellulose (NC) membrane (Schleicher & Schuell, Keene, NH) using modified transfer buffer (0.048 M Tris-HCl, 0.039 M glycine, and 0.1% [w/v] SDS [pH 9.0])

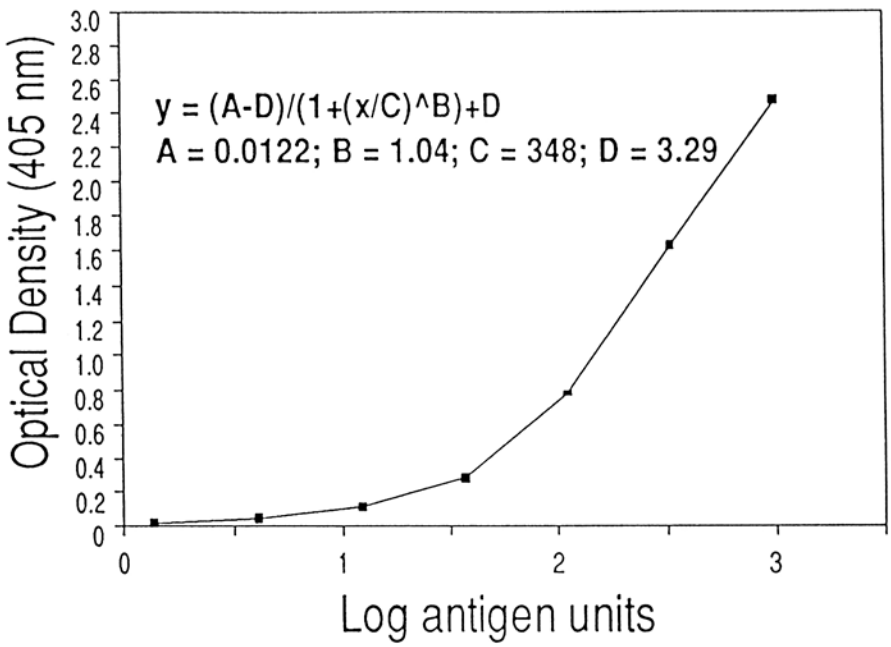

Fig. 1. Relationship between $\log_{10}$ antigen units (1:100 dilution of immunogen [*Phomopsis longicolla* culture filtrates]) and absorption values at 405 nm, using anti-*P. longicolla* immunoglobulin-G in double-antibody sandwich enzyme-linked immunosorbent assay. The soybean samples were diluted to fit the absorption values of this curve.

(23) on a Milliblot SDE semidry apparatus (Millipore Corp., Bedford, MA) for 1 hr at constant voltage of 15 V, then air-dried for 15 min. The NC membrane was blocked for 45 min in BLOTTO (11), consisting of 5% (w/v) nonfat dry milk in TTBS (Tris-HCl with 0.1% Tween 20 [v/v], pH 7.4) with a few drops per liter of antifoam agent A (A-5758, Sigma Chemical Co., St. Louis, MO), with gentle rocking. The NC membrane was incubated with 20 ml of a 1:200 dilution of alkaline phosphatase–conjugated, protein A affinity–purified *P. longicolla* antibodies (IgG) in BLOTTO for 1 hr with gentle rocking, followed by five rinses with TTBS buffer. The NC membrane was incubated in 10 ml of 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium substrates (Kirkgaard and Perry Laboratories, Gaithersburg, MD) according to Sambrook et al (18) for 30 min, washed twice with deionized distilled water, and air-dried.

Conidia production and inoculation. Mycelial plugs (5 mm in diameter) from the margins of 5-day-old cultures of *P. phaseoli* were transferred to 9-cm PDA culture plates and incubated under continuous fluorescent light (160 $\mu E \cdot s^{-1} \cdot m^{-2}$) for 15 days at 25 ± 2 C to stimulate production of α-conidia. The α-conidia in each plate were collected aseptically by adding 10 ml of sterile deionized distilled water and brushing the culture surface. The conidial suspension was then adjusted to 1×10^6 α-conidia per milliliter (3). Soybean seedlings (cv. Hack) at growth stage V1 were spray-inoculated on adaxial and abaxial surfaces until runoff with α-conidial suspension of *P. phaseoli*. Seedlings sprayed with sterile deionized distilled water served as controls. Plants were incubated at temperatures of 25 C during the day and 20 C at night with 20% relative humidity under 12 hr of light.

Collection and processing of greenhouse samples. Soybean stem pieces were cut aseptically between nodes 1 and 2 of greenhouse plants at growth stages V2, V3, and V4 (7). Stem pieces were then split longitudinally in half: one half for ELISA, and the other for plating on PDA after being treated with 0.5% NaOCl and dipped in 1:40 paraquat (1′-dimethyl-4,4′-bipyridinium dichloride) (v/v), followed by five rinses in sterile deionized distilled water (6). For ELISA, stem samples were weighed and then ground in PBS extraction buffer (0.2 g of KH_2PO_4, 2.9 g of Na_2HPO_4, 8 g of NaCl, 0.2 g of KCl, 0.2 g NaN_3, and 0.5 ml of Tween 20 per liter of buffer [pH 7.4]) in the proportion of 1 g per 5 ml, using sterile plastic grinders. The extract was centrifuged at 12,000 *g* for 15 min at 4 C, and the supernatant stored at −20 C.

Recovery of *P. phaseoli* from soybean plants. Stem samples from inoculated and uninoculated greenhouse-grown plants were plated on PDA (pH 7.0) after being either untreated or surface-treated with 0.5% NaOCl, followed by dipping in paraquat as previously described. The presence of *P. phaseoli* was recorded after 10 days. The untreated control was used to determine whether *P. phaseoli* was associated with stem samples, and the disinfested sample to determine whether the pathogen was internally borne.

DAS-ELISA. ELISA plates (Nunc Maxisorb F96 certified, A/S Nunc, Roskilde, Denmark) were coated with 100 µl of purified *P. longicolla* antibodies (10 µg/ml) in carbonate coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, 3 mM NaN_3 [pH 9.6]) and incubated for either 2 hr at room temperature or overnight at 4 C. Thereafter, samples were handled and processed at room temperature. Wells were washed three times with TTBS and blocked with 200 µl of BLOTTO for 1 hr. They were drained, and a 100-µl aliquot of plant extract was added to each well in a dilution series (1:5) in BLOTTO, so that one of the sample concentrations matched on the standard curve. Carbonate coating buffer or PDB diluted in carbonate buffer at 1:20 and 1:200 were used as controls. Plates were incubated for 1 hr. Wells were washed three times with wash buffer; after 100 µl of alkaline phosphatase–conjugated anti-*P. longicolla* IgG in TBS was added, they were incubated for 1 hr. Wells were washed five times with TTBS and incubated in 100 µl of substrate (1 mg/ml of *p*-nitrophenyl phosphate in 1.0% [w/v] diethanolamine buffer with 0.2 g of NaN_3 per liter [pH 9.8]) for 3 hr, and the spectrophotometric absorption was recorded at 405 nm. Absorption values were expressed as antigen units.

RESULTS AND DISCUSSION

The immunogenic components of *P. longicolla*, whether from mycelial extracts or culture filtrates, appeared as broad smears >50 kDa in immunoblot analysis (Fig. 2). Extracts of *Phomopsis* spp. and *C. truncatum* showed a strong reaction with *P. longicolla* antibodies, whereas extracts from *Aspergillus* sp., *Chaetomium* sp., and *M. phaseolina* showed a weak reaction. Reaction with fungal extracts was similar with antibodies that developed against either mycelial extracts or culture filtrates, except for *M. phaseolina*, which showed a strong reaction with antibodies that developed against mycelial extracts (Velicheti et al, *unpublished*). However, *P. sojae, Rhizopus* sp., *R. solani, S. glycines*, and *C. kikuchii* showed no reaction to anti-*P. longicolla* IgG. All isolates of *Phomopsis* spp. showed strong reactions, with quantitative variations, to *P. longicolla* antibodies that developed against either culture filtrates or mycelial extracts. No reaction was observed for PDB or for extracts from

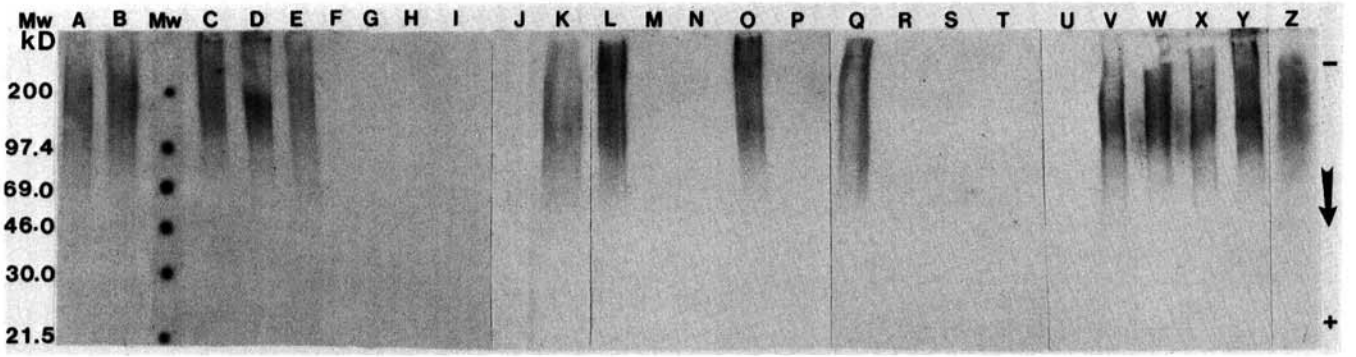

Fig. 2. Immunoblot analysis of culture filtrates from combined 15- and 30-day-old culture filtrates of *Phomopsis longicolla* (immunogen) and common soybean pathogenic or saprophytic fungi grown on potato-dextrose broth, using protein A affinity-purified *P. longicolla* antibodies conjugated to alkaline phosphatase. A 30-µl sample was loaded to each lane: **(A)** *P. longicolla* (immunogen), **(B)** *P. longicolla* (Ohio), **(C)** *P. phaseoli* isolate 5, **(D)** *P. phaseoli* isolate 7, **(E)** *Colletotrichum truncatum* isolate 2, **(F)** *Penicillium* sp., **(G)** *Aspergillus* sp. isolate 2, **(H)** *Chaetomium* sp., **(I)** *Fusarium* sp., **(J)** *Alternaria alternata*, **(K)** *D. phaseolorum* var. *caulivora*, **(L)** *D. phaseolorum* f. sp. *meridionalis*, **(M)** *Rhizoctonia solani*, **(N)** *Cercospora kikuchii*, **(O)** *P. phaseoli* isolate 8, **(P)** *Rhizopus* sp., **(Q)** *D. p. caulivora* (Ohio), **(R)** *Phytophthora sojae*, **(S)** *Septoria glycines*, **(T)** *Phialophora gregata*, **(U)** *Macrophomina phaseolina*, **(V)** *P. longicolla* isolate 12, **(W)** *P. longicolla* isolate 4, **(X)** *P. longicolla* isolate 8, **(Y)** *P. phaseoli* isolate 4, and **(Z)** seeds of soybean pod inoculated with *P. longicolla*.

seeds of uninoculated pods.

Extracts of stems from inoculated plants at V2 growth stage showed a weak reaction with *P. longicolla* antibodies (Fig. 3). Reaction between the extracts from inoculated plants at growth stages V3 and V4 progressively increased. The low background reaction to stem extracts of uninoculated plants suggested that antigen units were primarily due to the pathogen. *P. phaseoli* grew from all inoculated stem samples except for those treated with NaOCl plus paraquat and uninoculated controls.

This is the first reported use of ELISA for detection of *Phomopsis* spp. in asymptomatic soybean plants. These results verified latent infection by *P. phaseoli* in soybean seedlings (20). Also, these results confirmed the epiphytic nature of *P. phaseoli* (3,9), since the fungus was detected both by ELISA and by biopsy of stems on PDA. The gradual increase in antigen at V3 and V4 growth stages suggested that the fungus had proliferated over the plant surface after inoculation. The strong immunoreactions between *P. longicolla* and other members of the *Diaporthe/Phomopsis* complex supports the close taxonomic relationship among this group (10,17). Cross-reactivity has been reported among *Phomopsis* spp. for antibodies that developed against *P. longicolla* (8) and those that developed against *D. p. caulivora* (19). The strong reaction of *P. longicolla* antibodies with *C. truncatum* showed the presence of common immunogenic determinants; this reaction could influence results whenever both fungi were present in soybean tissue. *C. truncatum* was not observed in greenhouse-grown plants. *P. longicolla* antibodies showed weak reactions with *Aspergillus* sp., *Chaetomium* sp., and *M. phaseolina*.

Development of antigen units may allow the measurement of an approximate quantity of fungal biomass in plant tissue. The increase in antigen units in inoculated seedlings with time showed that ELISA could detect differences in the quantity of *P. phaseoli* in asymptomatic soybean seedlings. An assay of asymptomatic tissues on PDA would provide information on the presence, but not the level of colonization or quantity, of the fungus. Quantitative information could be useful in monitoring pathogen levels in soybeans, which aids in disease management inputs, such as effective timing of fungicide application (16). The pathogen was detected in greenhouse-inoculated plants by ELISA, and its viability was confirmed through biopsy of plant parts on PDA. However, immunodetection cannot give information regarding viability of the fungus (8). The correlation between detection of *Phomopsis* spp. using immunological and cultural methods showed that ELISA could be used for early detection of *Phomopsis* spp. in soybeans.

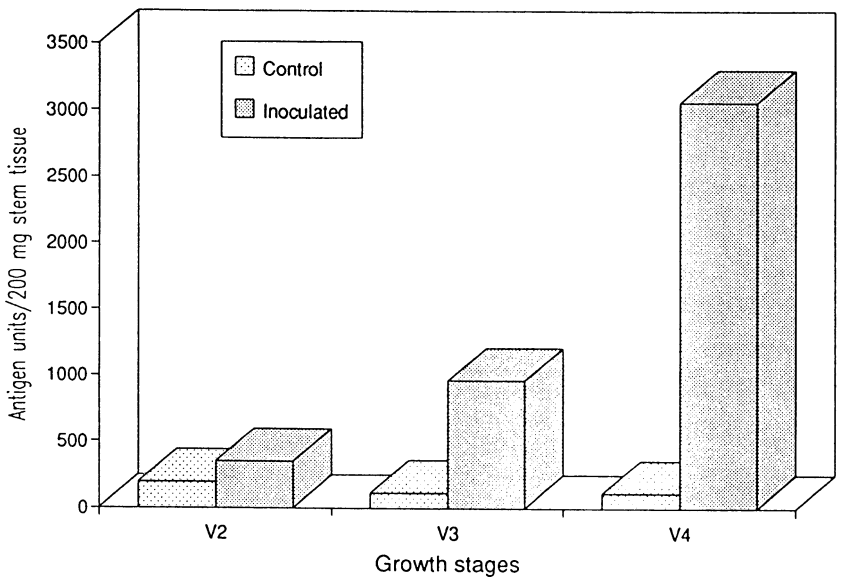

Fig. 3. Antigen units of *Phomopsis phaseoli* in either uninoculated (control) or spray-inoculated greenhouse-grown soybean plants using anti-*P. longicolla* immunoglobulin-G in double-antibody sandwich enzyme-linked immunosorbent assay.

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