Serological Methods for Detection of *Polymyxa graminis*, an Obligate Root Parasite and Vector of Plant Viruses

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ABSTRACT

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A purification procedure was developed to separate *Polymyxa graminis* resting spores from sorghum root materials. The spores were used as immunogen to produce a polyclonal antiserum. In a direct antigen coating enzyme-linked immunosorbent assay (DAC ELISA), the antiserum could detect one sporosorus per well of the ELISA plate. In spiked root samples, the procedure detected one sporosorus per mg of dried sorghum roots. The majority of isolates of *P. graminis* from Europe, North America, and India reacted strongly with the antiserum. Interestingly, *P. graminis* isolates from the state of Rajasthan (northern India), from Pakistan, and an isolate from Senegal (West Africa) reacted weakly with the antiserum. The cross-reactivity of the serum with *P. betae* isolates from Belgium and Turkey was about 40% of that observed for the homologous isolate. There was no reaction with common fungi infecting roots or with the obligate parasite *Olpidium brassicae*. However, two isolates of *Spongospora subterranea* gave an absorbance similar to that observed with the homologous antigen. The DAC ELISA procedure was successfully used to detect various stages in the life cycle of *P. graminis* and to detect infection that occurred under natural and controlled environments. A simple procedure to conjugate antibodies to fluorescein 5-isothiocyanate (FITC) is described. Resting spores could be detected in root sections by using FITC-labeled antibodies. The potential for application of the two serological techniques for studying the epidemiology of peanut clump disease and for the characterization of *Polymyxa* isolates from various geographical origins is discussed.

Polymyxa spp. are soilborne, obligate intracellular parasites of roots, classified under the order Plasmodiophorales. Currently, two species, *P. graminis* Ledingham and *P. betae* Keskin, are recognized. They transmit at least 12 different plant viruses in the genera *Benyvirus*, *Bymovirus*, *Furovirus*, and *Pecluvirus* (1,30,32). *Peanut clump virus* (PCV) and *Indian peanut clump virus* (IPCV) are members of the genus *Pecluvirus* (40) that affect the production of peanut and monocotyledonous crops in West Africa and in the Indian subcontinent (3,9,40,43). In the absence of cost-effective methods for the management of peanut clump disease (40), it was essential to study its epidemiology with emphasis on identification of the hosts for the virus and *P. graminis* and their influence on the perpetuation and spread of clump disease. Epidemiological studies on an obligate parasite such as *Polymyxa* spp. are comparatively difficult and time consuming, because its detection has traditionally relied on light microscopic inspection of roots. However, results from these studies are expected to aid in the formulation of cost-effective cultural practices to reduce the spread and incidence of clump disease. As one of the steps toward achieving this, we describe in this paper two immunological methods for the detection and quantitative estimation of *P. graminis* in root samples.

MATERIALS AND METHODS

Antigen preparation. Large amounts of sporosori (cystosori or resting spore clusters) of a *P. graminis* isolate (I_1, from the ICRISAT farm) were produced by culturing the intracellular parasite in sorghum roots (*Sorghum bicolor* cv. ICSV 88036) utilizing an automatic immersion system (AIS) (25). The procedure reported by Lange et al. (23) was modified to obtain sporosori of *P. graminis* from sorghum roots that were largely devoid of root material. Approximately 10 g of dried roots, containing over 1×10^7 *P. graminis* sporosori, were homogenized (1 min twice) with a commercial blender (Waring Products, New Hartford, CT) in 100 ml of 0.05 M sodium phosphate-buffered saline, pH 7.2, containing 0.05% Tween-20 (vol/vol) (PBS-Tween). This process resulted in the peeling and fragmentation of the cortical layers. The extract was filtered through a nylon mesh of 22-µ pore size. The filtrate contained mostly sporosori and root cortex fragments. It was pelleted at $3,600 \times g$ for 5 min and washed several times with sterile distilled water by repeated centrifugation. The pellet was resuspended in 2 ml of PBS-Tween, and the sporosori were broken into individual resting spores by trituration in a ground glass tissue grinder. The spore suspension was passed through a 10-µm-pore-size membrane (type TCTP, 47 mm in diameter; Millipore Corp., Bedford, MA) to separate individual spores of approximately 5 µm in diameter (27) from spore clusters and root debris larger than 10 µm. The individual spores were collected on a 3-µm-pore-size membrane (type TSTP, 47 mm in diameter; Millipore Corp.) and washed several times with sterile water to eliminate bacteria and minute root debris. The spores were resuspended in sterile distilled water and then sonicated under ice with a microprobe for 10 min (Fisher sonic dismembrator, model 300; Artek System Co., Farmingdale, NY) in order to break the spore wall to expose internal antigens. The presence of broken resting spores and zoospores was verified by microscopic observation. The spore suspension still contained few bacteria, probably released during sonication. Antibiotics, benzyl penicillin at 1.2 mg/ml, streptomycin sulfate at 0.2 mg/ml, kanamycin monosulfate at 0.2 mg/ml, and gentamicin at 10 µg/ml, were added to the spore suspension to arrest bacterial growth. The spore suspension containing antibiotics was used for immunization.

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Production of antiserum. Following an initial preimmune bleed, two New Zealand White inbred rabbits were immunized at weekly intervals with 0.5-ml aliquots containing approximately 1×10^7 individual spores. The first immunization (regarded as day 1) was done subcutaneously at multiple sites below the neck in between the two forelegs, utilizing the spore suspension mixed with 0.5 ml of complete Freund's adjuvant. Subsequent immunizations were given, alternating between intramuscular (spore suspension and incomplete adjuvant at 1:1 ratio) and subcutaneous injections without the adjuvant. Seven injections were given over a period of 2 months. The rabbits were bled to monitor the antiserum titer at weekly intervals starting from 45 days after the first immunization. After the eighth bleed (day 94), the animals were rested for 1 month and a booster injection was given intramuscularly on day 125. Two weeks after this injection (day 139), animals were bled at weekly intervals over a period of 2 months until the titer dropped (day 195). After the addition of sodium azide to give 0.01%, sera were lyophilized in 1-ml aliquots and stored at –20°C.

Enzyme-linked immunosorbent assay (ELISA) procedure. The direct antigen coating (DAC) ELISA procedure was used (19). Five milligrams of root material was triturated using a mortar and pestle in 1 ml of 0.05 M sodium carbonate buffer, pH 9.6. Extract was further diluted in carbonate buffer and added to microtiter plates (Immuno Plate PolySorp F96; Nalge Nunc International, Rochester, NY). Coated plates were incubated in a refrigerator overnight. Subsequent steps, all performed at 37°C for 1 h, sequentially were (i) blocking with low fat milk powder (5% wt/vol) prepared in PBS-Tween, (ii) crude antiserum (1:500 dilution) cross-adsorbed at 37°C for 1 h with an extract prepared by trituration of dried sorghum roots diluted to 0.4% (wt/vol) in conjugate buffer (PBS-Tween containing 2% polyvinyl pyrollidone [40,000 molecular weight] and 0.2% ovalbumin) and further diluted to 1:2,000 prior to adding to the plates, and (iii) goat anti-rabbit immunoglobulin G (GAR-IgG) conjugated to alkaline phosphatase at a 1:1,000 dilution. The substrate used was *p*-nitrophenyl phosphate at 1 mg/ml. Absorbance was recorded at 405 nm (A_{405}) after 1 to 3 h of substrate reaction time at room temperature (25 to 30°C). The measurement was done with an ELISA reader (Titertek Multiskan; Labsystems, Helsinki, Finland) blanked against wells coated with carbonate buffer. Values that were at least threefold higher than those for healthy (*P. graminis*-free) sorghum root extracts were considered to be positive. After testing the various bleeds, the antiserum from bleed 15 (collected on day 181) was used in all subsequent tests.

To serve as negative controls and for serum cross-adsorption, sorghum roots free of *P. graminis* were produced by growing plants

TABLE 1. Soil-inhabiting organisms tested for cross-reactivity with a polyclonal antiserum raised against *Polymyxa graminis* from tropical India (isolate I_1)

Culture[a]	Code no.[b]	Place of origin	Host	Mean % of cross-reactivity[c]
Lower fungi				
Polymyxa betae	A2641*[d]	Opprebais, Belgium	Sugar beet	41.1 ± 3.6
	T17*[d]	Turkey	Sugar beet	42.8 ± 5.1
Polymyxa graminis				
Sporosori	B1*[d]	Loupoigne, Belgium	Barley	99.1 ± 2.0
	C1*[d]	Ottawa, Canada	Barley	81.1 ± 2.7
	F11*[d]	Carcassonne, France	Barley	88.0 ± 1.6
	I1[d]	Patancheru, Andhra Pradesh, India	Sorghum	100.0 ± 5.8
	I1-1*[d]	Patancheru, Andhra Pradesh, India	Sorghum	63.3 ± 2.4
	I1-20*[d]	Patancheru, Andhra Pradesh, India	Sorghum	97.7 ± 5.2
	I1-229*[d]	Patancheru, Andhra Pradesh, India	Sorghum	73.9 ± 3.3
	I9[d]	Boraj, Rajasthan, India	Sorghum	8.0 ± 0.9
	P1[d]	Dudhial, Punjab, Pakistan	Sorghum	7.3 ± 0.6
	J1[e]	Japan	Wheat	96.6 ± 4.4
	S6[d]	Bambey, Senegal	Pearl millet	23.7 ± 2.7
Zoospores	I1-229*	Patancheru, Andhra Pradesh, India	Sorghum	42.7 ± 4.3
Spongospora subterranea	A[f]		Potato	98.4 ± 2.9
	B[f]		Potato	110.6 ± 3.0
Olpidium brassicae	E1(S24)[g]	Sinthion, Senegal	Sorghum	5.2 ± 1.0
	K51(S21)[g]	Niger	Barley	1.1 ± 0.5
Higher fungi				
Aspergillus flavus[h]		Patancheru, Andhra Pradesh, India	Soil	4.9 ± 1.4
Aspergillus niger[h]		Patancheru, Andhra Pradesh, India	Soil	3.0 ± 0.9
Fusarium solani[d]		Gembloux, Belgium	Potato	3.6 ± 2.3
Fusarium moniliforme[h]		Patancheru, Andhra Pradesh, India	Sorghum	5.7 ± 1.2
Rhizoctonia solani[d]		Belgium	Potato	3.6 ± 2.3
Trichoderma viride[h]		Patancheru, Andhra Pradesh, India	Soil	7.6 ± 1.2
Healthy roots			Barley	0.6 ± 0.3
			Pearl millet	1.5 ± 0.3
			Sorghum	3.4 ± 2.7
			Sugar beet	5.6 ± 1.5
			Wheat	2.0 ± 1.1

[a] Sporosori, resting spores in roots, and zoospores of the obligate parasites of the genera *Polymyxa*, *Spongospora*, and *Olpidium* were tested at a dilution of 1×10^3/ml. Fungi cultured on artificial medium were tested at a dilution of 25 µg/ml and mixed with dried healthy sorghum root (75 µg/ml) to approximate the amount of *P. graminis* sporosori in infected sorghum roots.

[b] * = Single-sporosorus cultures $I_{1\text{-}1}$, $I_{1\text{-}20}$, and $I_{1\text{-}229}$ derived from the isolate I_1.

[c] Mean absorbance readings at 405 nm as the percentage of that observed for the homologous antigen *P. graminis* from India isolate I_1. Absorbance values were typically 0.087 ± 0.069 (mean ± standard deviation) for the negative control (healthy sorghum roots) and 2.554 ± 0.477 for the positive control (isolate I_1, 1×10^3 sporosori per ml). Means were calculated from five replicates.

[d] Fungus provided by A. Legrève and H. Maraite, Unité de Phytopathologie, UCL, 1348 Louvain-la-Neuve, Belgium (25).

[e] Fungus provided by Y. Ohto, Tohoku National Agricultural Experiment Station, Shimokuriyagawa, Morioka, Iwate, 020-0123, Japan.

[f] Fungus provided by K. Bell and J. Roberts, Department of Fungal and Bacterial Plant Pathology, Scottish Crop Research Institute, Invergowrie, Dundee DD2 5DA, United Kingdom.

[g] Fungus provided by S. Gharbi and M. Verhoyen, Unité de Phytopathologie, UCL.

[h] Fungus provided by the Cereals and Legumes Pathology Units, ICRISAT.

in AIS under conditions identical to those used for *P. graminis* culture. After harvesting, roots were dried and stored at room temperature. Samples of highly infected sorghum roots from the AIS culture, dried at room temperature and ground into a fine powder using a commercial blender, served as positive controls. To quantify the number of sporosori in infected roots, three aliquots of 5 mg were individually homogenized with a tissue mizer (Tekmar Inc., Cincinnati, OH) in 5 ml of distilled water until the majority of sporosori were released from the roots. The number of sporosori was estimated by using a Fuchs-Rosenthal's haemocytometer. The positive control used in the ELISA test contained 1×10^4 to 1.2×10^4 sporosori per mg of dried roots.

Sensitivity of detection of *Polymyxa* sp. The following experiment was done to assess whether the presence of healthy plant material influenced the sensitivity of detection of *P. graminis*. Various concentrations of sporosori ranging from 1×10^2 to 1×10^4 per ml were added to dried healthy sorghum roots to give a final concentration of 5,000, 2,500, 500, 100, 10, and 1 sporosorus per mg of dried roots and triturated in carbonate buffer. Extracts from dried healthy roots served as controls.

The ability of the antiserum to detect different developmental stages of *P. graminis* was studied. A *P. graminis* infection was initiated by inoculation of each sorghum seedling with 4,500 sporosori, and the seedlings were maintained under an AIS. One seedling was harvested each day, starting from the day after inoculation. Roots were washed free of sand and thoroughly rinsed under running deionized water to remove any sporosori or zoospores that may be on the root surface. Half of the fresh root (approximately six to eight rootlets) was analyzed for the presence of *P. graminis* by light microscopy after staining with cotton blue in lactophenol. The other half was tested by ELISA at a 1:20 dilution.

Specificity of antiserum. The antiserum was tested by DAC ELISA for cross-reactivity against common soilborne fungi infecting roots (Table 1). Healthy sorghum root extracts were used as a negative control and extracts containing *P. graminis* (isolate I_1) sporosori as a positive control. Various fungi were grown in potato dextrose broth. After they became confluent, the mycelium was collected on a filter paper and washed three times with sterile distilled water. The material was dried, weighed, lyophilized, and stored at 5°C before use. Since *P. graminis*, *Olpidium brassicae*, and *Spongospora subterranea* cannot be cultured on artificial media, the ELISA tests were done with host tissue infected by these obligate parasites by adjusting the concentration of resting spores (*O. brassicae*) or spore balls (*S. subterranea*) to that of *P. graminis* sporosori (1×10^3/ml). Fungi grown on artificial medium were triturated at a dilution of 25 µg/ml of carbonate buffer containing healthy sorghum root extract (75 µg/ml) to give a ratio equivalent to the concentration of *P. graminis* sporosori in the highly infected sorghum roots.

The ability of the antiserum to detect *Polymyxa* sporosori from various geographical origins that include *P. betae* and *P. graminis* was also assessed by DAC ELISA (Table 1). Resting spores from root homogenate of the various isolates were used at a dilution of 1×10^3 sporosori per ml. Healthy root extracts from respective hosts were used as negative controls.

Comparison between ELISA and light microscopy. A soil sample was collected from IPCV-H- (38) and *P. graminis* isolate I_1-infested areas. The soil was dried at room temperature and crushed in a commercial blender. Serial dilutions were prepared using sterile sand and then planted with sorghum (14). The plants were maintained under glasshouse conditions using Hoagland's nutrient solution. For comparison of ELISA tests with light microscopy, one-third of each root sample was stained by boiling fresh sample in lactophenol with cotton blue. The remaining portion of the root sample was dried, cut into small fragments, and

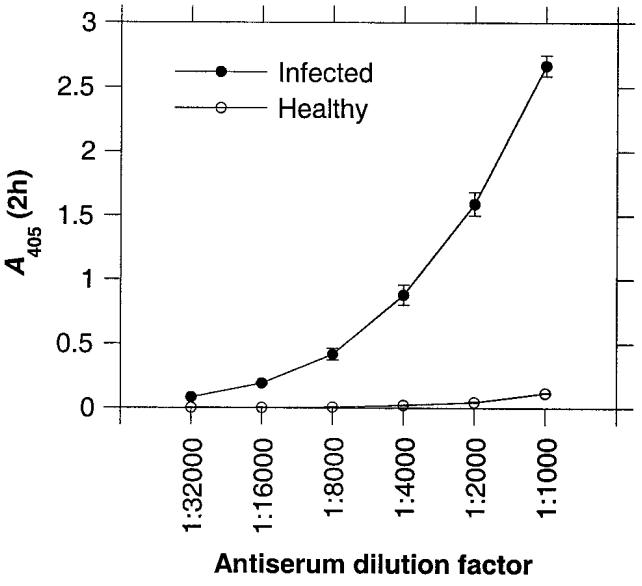

Fig. 2. Relationship between antiserum dilution factor (bleed 15) and absorbance values at 405 nm (A_{405}) measured after 2 h of substrate reaction time in direct antigen coating enzyme-linked immunosorbent assay. Wells were coated with sorghum root extract containing resting spores (●) (2×10^3 sporosori per ml) and the equivalent weight of healthy root extract (○). The intervals represent the standard deviation ($n = 3$).

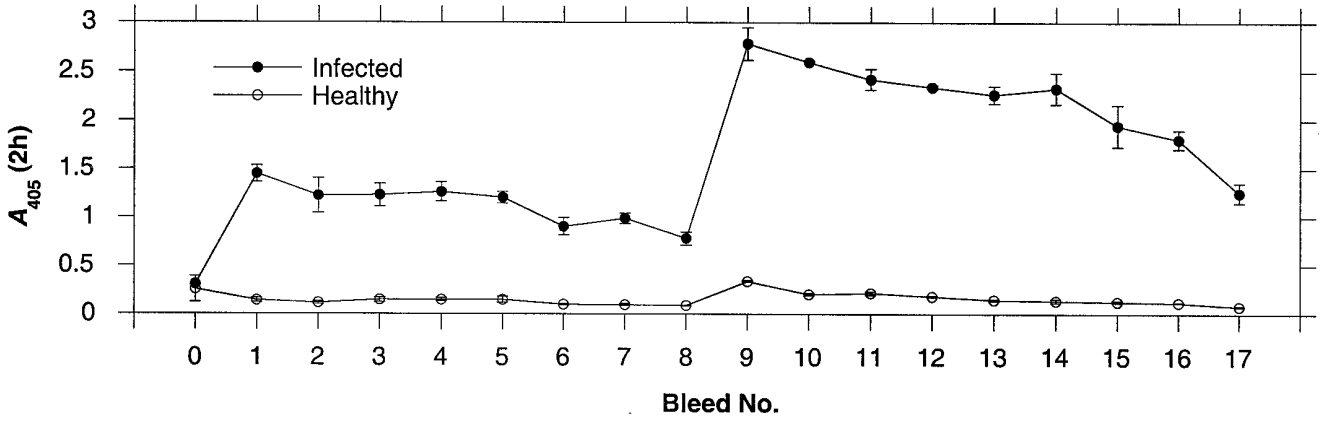

Fig. 1. Progress of *Polymyxa graminis* antiserum titer for bleeds collected over time. Bleed 0 is the preimmune serum. Bleed 1 was collected on day 45. A booster injection was given on day 124, 1 month after collecting bleed 8, and bleed 9 was collected 15 days later (day 139). The last bleed, no. 17, was collected on day 195. Wells were coated with sorghum root extracts containing *P. graminis* (●) (2×10^3 sporosori per ml) and the equivalent weight of healthy roots (○). Mean absorbance values at 405 nm (A_{405}) were recorded after 2 h of substrate reaction time. The intervals represent the standard deviation ($n = 3$).

thoroughly mixed, and aliquots of 1.25 to 5 mg dry weight (approximately one-third of the root) were triturated in carbonate buffer (1 mg in 5 ml) and processed by ELISA. Healthy and infected roots were included for controls. The most probable number (MPN) of infective unit of *P. graminis* per liter of soil was calculated from the proportion of infected plants in each soil-sand dilution (7,14) and compared by the two techniques.

Roots of peanut and *Cyperus rotundus* (nut grass or purple nutsedge) collected from an IPCV-H-infested field were also included for ELISA tests to assess feasibility of detection in naturally infected roots.

Detection of sporosori by fluorescent antibody technique (FAT). Root fragments (approximately 5 mm in length) from *P. graminis*-infected and healthy plants were fixed and then processed by FAT. Samples were fixed in 3% glutaraldehyde prepared in 0.1 M phosphate buffer (pH 7.2) under vacuum. Fixed roots were washed with phosphate buffer, dehydrated in a graded series of ethanol, and embedded in Spurr epoxy resin (Ladd Research Industries, Inc., Burlington, VT). Transverse sections of approximately 8-µm thickness were transferred to windows of glass multispot slides and briefly heated on a hot plate at 50°C to adhere to the glass. GAR-IgG (Sigma R-3128; Sigma Chemical Co., St. Louis) was conjugated with fluorescein 5-isothiocyanate (FITC) (Sigma F-7250) by the following procedure. FITC (15 mg) was dissolved in 1 ml of dimethyl sulfoxide (Sigma D-2650) and mixed with 14 ml of 0.1 M sodium carbonate buffer (pH 9.6) (16). One milligram of GAR-IgG was dissolved in 1 ml of 0.1 M carbonate buffer and dialyzed against 15 ml of FITC solution overnight in a cold room. Excess FITC was removed by dialysis against PBS (41). Crude *P. graminis* antiserum was cross-adsorbed for 1 h at 37°C with an equal volume of healthy sorghum root extract (dried roots at 0.4% [wt/vol]) prepared in conjugate buffer. The immunoprecipitate was removed by centrifugation and the cross-adsorption process repeated three times. IgG for *P. graminis* was extracted from the supernatant with neutral ammonium sulfate (16) and used at 100 µg/ml.

The following procedure was adopted for staining thin sections of fresh or dried roots by FAT. The staining was done in glass multispot slides using 20 µl of reagent per window at each step. Sections were first soaked in PBS-Tween containing 10% low fat milk (blocking buffer) for 1 h at 37°C. After washing under a gentle stream of distilled water, they were soaked in *P. graminis* IgG for 3 h at 37°C or overnight at 5°C prepared in blocking buffer. After washing the root sections in distilled water, FITC-labeled GAR-IgG was added at a dilution of 1:20 prepared in blocking buffer and incubated for 3 h at 37°C. After washing in distilled water, sections were mounted in 90% glycerol in 0.1 M PBS and examined under a Olympus microscope (Olympus Optical Co. Ltd., Tokyo), with a provision for epifluorescence. Photographs were taken at ×80 or ×100 final magnification using a Kodak 400 ASA (Eastman Kodak Co., Rochester, NY) color reversal film.

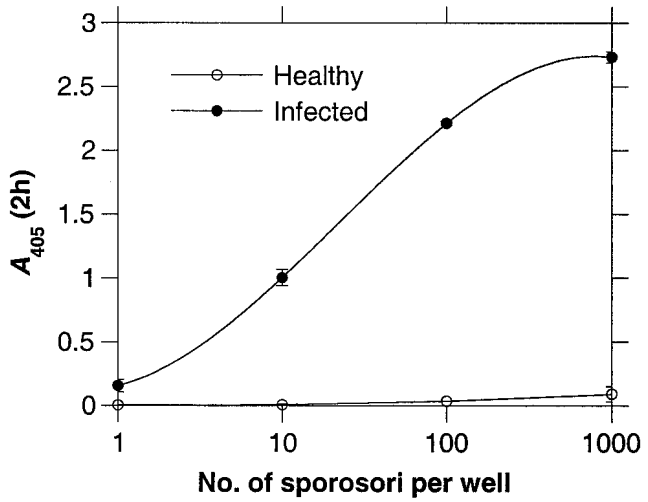

Fig. 3. Relationship of the number of *Polymyxa graminis* sporosori (●; fitted curve: $A_{405} = -0.1747x^3 + 1.2277x^2 - 1.6126x + 0.716$) prepared from highly infected sorghum roots, and the equivalent weights of healthy sorghum roots (○) with A_{405} measured after 2 h of substrate reaction time in direct antigen coating enzyme-linked immunosorbent assay with wells filled with 100 µl of reagent. The intervals represent the standard deviation ($n = 3$).

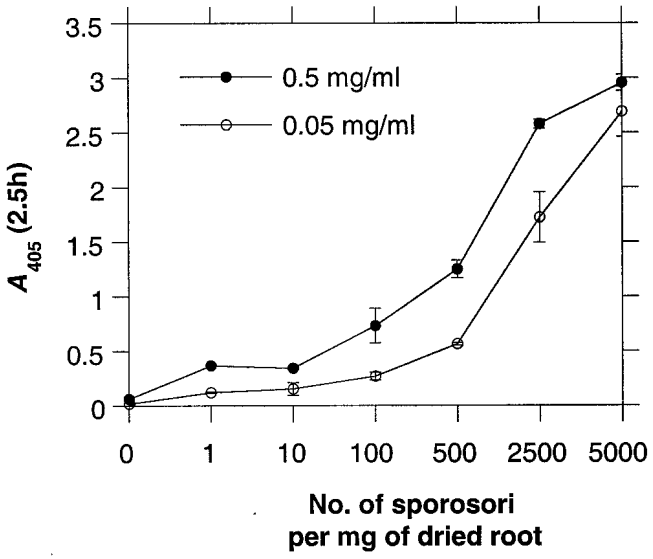

Fig. 4. Detection of *Polymyxa graminis* sporosori in spiked root samples. Relationship between the number of sporosori per milligram of dried sorghum root and absorbance values at 405 nm (A_{405}) measured after 2.5 h of substrate reaction time in direct antigen coating enzyme-linked immunosorbent assay. Two dilutions were tested: 0.5 mg (●) and 0.05 mg (○) of dried root per ml. The intervals represent the standard deviation ($n = 3$).

TABLE 2. Comparison between enzyme-linked immunosorbent assay (ELISA) and observation under a light microscope for the detection of *Polymyxa graminis* in roots of plants grown on serial dilutions of soil naturally infested with *Indian peanut clump virus* (IPCV)

		Number of plants per category				
		Microscopy degree of infection[b]		ELISA ($r = S_{A405}/H_{A405}$[c])		
Dilution factor[a]	No. of plants tested	0	+	$r < 3$	$3 < r < 5$	$r \geq 5$
1:2	14	8	6	8	3	3
1:4	14	11	3	11	2	1
1:8	14	13	1	13	0	1
1:16	14	12	2	12	2	0
1:32	14	13	1	13	1	0
MPN[d]		18 ± 5		18 ± 5		

[a] The soil was sampled on the ICRISAT farm at Patancheru where *Indian peanut clump virus* Hyderabad isolate (IPCV-H) and *P. graminis* isolate I_1 originated.

[b] Degree of infection based on the presence of sporosori by light microscopy: 0, no infection but some root may present structures resembling the plasmodial stage; and +, presence of sporosori.

[c] Ratio between absorbance value readings at 405 nm (A_{405}) observed for the sample (S) and for healthy sorghum roots (H). Mean absorbance values (± standard deviation [SD]) were typically 0.155 ± 0.106 for the negative control (healthy sorghum roots) and 1.807 ± 0.274 for the positive control (isolate I_1, 1×10^3 sporosori per ml).

[d] Quantification of *P. graminis* in soil by the most probable number (MPN) method was compared by the two techniques. The MPN of infective units (± SD) of *P. graminis* per liter of dry crushed soil was calculated on the proportion of plants containing sporosori or giving a $r \geq 3$ in an ELISA test.

RESULTS

Detection of *P. graminis* resting spores by ELISA. The *P. graminis* antiserum reacted with healthy sorghum root extracts. Therefore, it was necessary to cross-adsorb the serum with healthy root extracts. When antisera collected at different intervals were titrated by ELISA against root extracts containing 2×10^3 sporosori per ml, the bleeds (9 to 17) drawn after administering the booster gave relatively high absorbance values (Fig. 1). The serum detected, without ambiguity, *P. graminis* resting spores in root extracts at a dilution of 1:2,000 (Fig. 2). Therefore, this antiserum dilution was used in all future tests. The DAC ELISA system could readily detect one sporosorus per well of the ELISA plate. A mean absorbance value (± standard deviation) of 0.156 ± 0.050 was observed in wells coated with one sporosorus. Wells coated with an equivalent weight of healthy sorghum roots gave a mean absorbance of 0.004 ± 0.001 (Fig. 3).

Healthy sorghum root extracts used at a dilution of 0.5 mg per ml and spiked to contain 1 and 10 sporosori per mg gave absorbance values exceeding 0.367 ± 0.006 optical density (OD) units, while healthy sorghum root extracts gave a mean absorbance value of 0.061 ± 0.016. Absorbance values were similar for the extracts spiked to contain 1 and 10 sporosori per mg. However, when the sporosori concentration exceeded 10 per mg of dried roots, it was possible to estimate the sporosori concentration in roots on the basis of the intensity of the reaction in ELISA. It is apparent from the results presented in Figure 4 that a 10-fold difference between the dilution of spiked root extracts (0.5 versus 0.05 mg/ml) continued to give differences in OD values that allowed discrimination between various sporosori concentrations in roots.

Reliability of ELISA for estimation of sporosori concentration. Initially, it was essential to cross-adsorb the serum to eliminate nonspecific reactions due to healthy root extracts. Cross-reactivity with commonly occurring root fungi was determined (Table 1). The serum did not react with any of the higher fungi.

The reliability of ELISA was assessed by making numerous comparisons with visual examination of stained roots by light microscopy for the presence of sporosori. A perfect correlation between ELISA results and examination by light microscopy was observed (Table 2). The MPN of infective units per liter of soil estimated by ELISA and by light microscopy were identical (Table 2).

The ELISA procedure was used to estimate sporosori concentration in two naturally infected species (Table 3). *C. rotundus* consistently gave high proportions of infected plants as well as high absorbance values. Interestingly, the majority of peanut plants from IPCV-infested soil failed to give positive results, and only a few plants showed the presence of *P. graminis* as assessed by low absorbance values in ELISA.

Detection of various stages of the *Polymyxa* life cycle. Root samples inoculated with *P. graminis* were analyzed by ELISA, and the same samples were observed under a light microscope after staining. *P. graminis* antigens could be detected 2 days after inocu-

TABLE 3. Detection of *Polymyxa graminis* in field samples by enzyme-linked immunosorbent assay

		Number of plants in each class and A_{405}[b]						
		Positive			Negative			Total number
Date of sampling	Species[a]	*n*	A_{405}	Range	*n*	A_{405}	Range	of plants
9 July	Peanut	0			16	0.045	0.027–0.096	16
	Sorghum		1.367	1.258–1.449		0.051	0.044–0.057	
16 July	Peanut	3	0.197	0.108–0.300	27	0.037	0.000–0.078	30
	Cyperus rotundus	22	0.954	0.309–1.564	8	0.017	0.010–0.023	30
	Sorghum		1.132	1.090–1.182		0.017	0.008–0.029	
21 July	Peanut	5	0.306	0.240–0.397	25	0.020	0.000–0.146	30
	Cyperus rotundus	17	0.845	0.206–2.489	13	0.090	0.010–0.157	30
	Sorghum		2.388	2.342–2.428		0.065	0.049–0.082	

[a] Roots of peanut and *Cyperus rotundus* (nut grass or purple nutsedge) plants were sampled at three occasions during July 1998 in the field RCW17 situated on the ICRISAT farm at Patancheru. Sorghum roots with and without infection by *P. graminis* were included as controls in three replicates at each test. Peanut was sown on 25 June, and only plants exhibiting clump disease symptoms were included in this test.

[b] Mean absorbance measured at 405 nm calculated on the basis of the number of plants in each class.

TABLE 4. Detection of the early stages of the *Polymyxa graminis* life cycle by light microscopy and direct antigen coating enzyme-linked immunosorbent assay (DAC ELISA) in roots of sorghum plants each artificially inoculated with 4,500 sporosori and maintained in an automatic immersion system under glasshouse conditions[a]

	Microscopy[b]		ELISA[c]				
Days after inoculation	*ni*/*N*	Life cycle stages	$r < 3$	$3 \le r \le 10$	$r \ge 10$	Total	A_{405}
2	6/7	Plasmodia	0	0	7	7	1.526 ± 0.343
3	9/12	Plasmodia	0	6	5	11	0.814 ± 0.302
4	7/9	Plasmodia	0	1	5	6	1.070 ± 0.368
5	4/5	Plasmodia	0	0	6	6	2.178 ± 0.169
6	4/6	Plasmodia	0	2	6	8	1.193 ± 0.400
7	3/8	Plasmodia	1	3	4	8	0.565 ± 0.340
8	4/8	Plasmodia, zoosporangia	0	5	3	8	1.080 ± 0.738
9	5/8	Plasmodia, zoosporangia	0	2	6	8	1.226 ± 0.445
10	5/8	Zoosporangia, plasmodia	1	2	5	8	0.850 ± 0.375
11	5/9	Zoosporangia, plasmodia	0	4	4	8	0.939 ± 0.398
12	5/9	Zoosporangia, plasmodia	0	6	2	8	1.005 ± 0.161

[a] Each day, half of a fresh sorghum root (approximately six to eight rootlets) was analyzed by light microscopy after staining, and individual rootlets of the remaining root portion were processed by ELISA.

[b] Number of rootlets infected (*ni*) with *P. graminis* on the number of rootlets analyzed (*N*), and life cycle stages identified, presented in the order of importance in the root.

[c] Number of rootlets per category of reaction in ELISA. The samples were classed according to the ratio (*r*) between absorbance readings at 405 nm (A_{405}) observed for the sample and for healthy sorghum root extracts. A_{405} is the mean absorbance values (± standard deviation [SD]) observed for all the rootlets of individual plant and measured after 3 h of substrate reaction time. Mean absorbance values were typically 0.080 ± 0.031 (± SD) for healthy sorghum root extracts and 2.606 ± 0.834 for infected root extracts (1×10^3 sporosori per ml).

lation, when plasmodia were apparent. Subsequently, zoosporangia were observed and their number became higher than that of plasmodia starting 10 days after inoculation. Antigens from zoosporangia could be readily detected by ELISA (Table 4). A zoospore suspension adjusted to 10^3/ml gave a mean absorbance value of 1.025 ± 0.103 in ELISA, equivalent to 42.7% of that observed for an equivalent concentration of sporosori (2.400 ± 0.295) (Table 1).

Detection of *Polymyxa* sporosori in roots by FAT. Staining of sporosori was readily apparent when processed by FAT (Fig. 5). In both dried and fresh roots, sporosori fluoresced with a typical apple green color. Healthy root samples were not stained. The majority of the specific staining was restricted to the outer layers of the resting spores, and the inner part of the spores was orange-brown. The isolates I_9 (Rajasthan, India) and P_1 (Punjab, Pakistan) were stained only at a few scattered points all along the periphery of the resting spore. The root stele showed autofluorescence (blue color) that could be eliminated by exposing the sections to PBS-Tween containing 10% low fat dry milk. *P. graminis* sporosori autofluoresced with an orange-brown color easily distinguished from the specific staining with FITC.

Determination of cross-reactivity of antiserum with a range of *Polymyxa* spp. isolates, *S. subterranea*, and *O. brassicae*. By both ELISA and FAT, *O. brassicae* gave negative reactions. Interestingly, two isolates of *S. subterranea* reacted strongly in both tests. The majority of *Polymyxa* spp. isolates tested from different geographical origins reacted strongly with the antiserum. Two isolates from clump-infested soils in Rajasthan (I_9) and Pakistan (P_1) showed a negligible reaction and an isolate from Senegal (S_6) showed a weak reaction with the antiserum. Absorbance values for two isolates of *P. betae* were consistently lower than those observed for the homologous isolate. The serum did not react with extracts from healthy roots of the various hosts tested (Table 1).

DISCUSSION

Traditionally, detection and estimation of *Polymyxa* spp. in roots of infected plants depended on staining with such dyes as cotton blue and examination under a light microscope. This method is laborious and involves use of such hazardous chemicals as phenol. More recently, to facilitate rapid detection of *Polymyxa* spp., molecular techniques have been developed (35,36,47). These techniques, even though very specific, are expensive and require special skills and equipment not readily available in developing countries. Furthermore, they are not useful when a large number of samples are to be tested. Advances have been made in applying immunological methods for the detection of various fungi (10,11,15) and such obligate parasites as *S. subterranea* and *P. brassicae* (12,17, 23,33,46,48). In this study, we present data on the estimation of *P. graminis* sporosori by immunochemical methods. It was essential first to develop a procedure for isolating sporosori from root fragments. The procedure yielded sporosori largely devoid of root material, as ascertained by visual examination of sporosori preparation. Sonication was found to be an important step for disrupting sporosori and the consequent release of antigens. Indeed, previous rabbit immunizations with nonsonicated spores gave sera of very low titer and poor specificity (data not shown). During the sonication process, some of the sporosori germinated, as evidenced by the presence of broken resting spores and zoospores swimming in the preparation. Zoospores are likely to be a better source of antigens than are sporosori. However, sporosori are recommended because they can be easily distinguished from other organisms in roots and they occur in large numbers in preferred hosts (24,39).

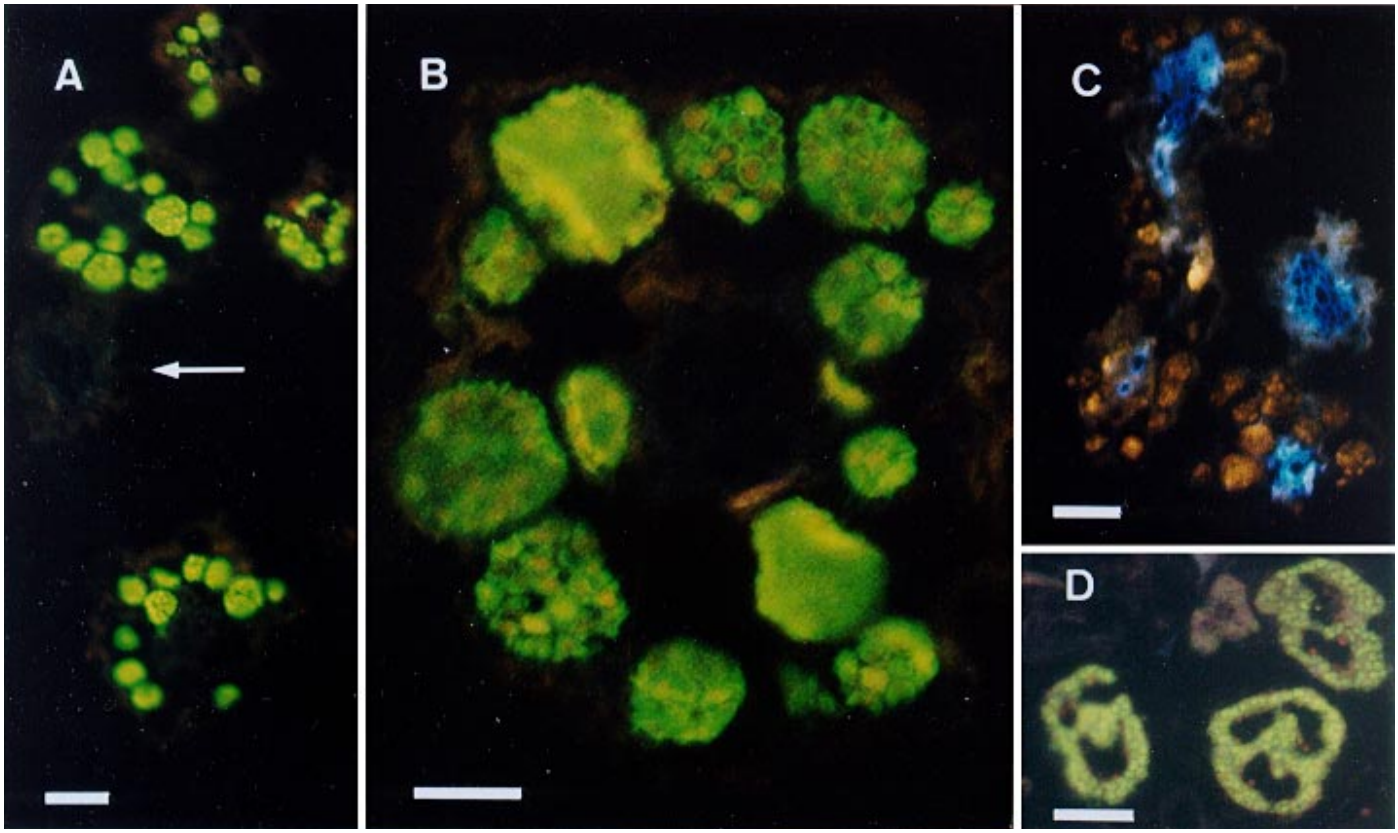

Fig. 5. Detection of sporosori by fluorescent antibody technique using fluorescein 5-isothyocyanate (light micrographs). Homologous reaction with *Polymyxa graminis* isolate I_1 from Patancheru, India. **A**, Transversal sections through four infected and one noninfected (arrow) sorghum root. Scale bar represents 50 µm. **B**, Root with detail of a section through individual resting spores showing the specific staining restricted to the outer layers of the spores and the inner part of the spores showing orange-brown color. Scale bar represents 20 µm. **C**, Autofluorescence of *P. graminis* sporosori (orange-brown color) and of the root stele (blue color). Scale bar represents 50 µm. **D**, Section through *Spongospora subterranea* spore balls. Scale bar represents 25 µm.

Initially, a procedure that involved several subcutaneous and intramuscular injections was tested for producing polyclonal antiserum. Despite tests on eight successive bleeds at 1-week intervals, the titer of the serum did not show any sign of increase. A booster given 10 weeks after the seventh injection contributed to a substantial increase in the titer of the antiserum (Fig. 1). Both of the inbred rabbits behaved in a similar manner. In the case of *S. subterranea*, Walsh et al. (46) reported also the necessity of giving a booster injection after a resting period to yield antiserum with a relatively good titer. Therefore, this procedure may be applicable for polyclonal antibody production to other members in the order Plasmodiophorales.

Antiserum did contain antibodies that reacted with healthy root extracts. This nonspecific reaction was eliminated by cross-adsorption with healthy root extracts. Specificity of the antiserum was evaluated by tests with a range of fungi that occur naturally in roots of various hosts utilized in this study. The serum did not react with any of the fungi tested (Table 1) or with a member of the order Chitridales, *O. brassicae*. The serum reacted strongly with another distinct member of the order Plasmodiophorales, *S. subterranea*. Our results confirmed the findings by Wallace et al. (45) that antigens are shared between *P. graminis* and *S. subterranea*. Fortunately, the cross-reactivity of the antiserum with this closely related parasite does not represent an obstacle for the routine detection of *P. graminis* by ELISA in monocotyledonous hosts such as sorghum and millets. Infection by *S. subterranea* is mostly restricted to the families Solanaceae and Chenopodiaceae (18,20–22).

The ELISA procedure adopted was suitable for the estimation of sporosori in root extracts, one of the main objectives of this study. A single sporosorus was adequate to produce OD values that were at least threefold higher than those of comparable healthy root extracts. The procedure facilitated the estimation of *P. graminis* sporosori when it occurred at a concentration exceeding 10 sporosori per mg of dried roots. However, attempts to estimate sporosori in soil samples yielded inconsistent results (data not shown). Our results corroborate those reported for *P. brassicae* (48) and *S. subterranea* (33), when alkaline phosphatase was used for preparing enzyme conjugate. A direct detection and quantification system of *Polymyxa* spp. from soil is currently not available, and extraction of sporosori of members in the order Plasmodiophorales from soil failed to yield satisfactory results, especially when sporosori occurred in low concentrations (2,4,42). *Polymyxa* spp. typically occur at low populations in soils (1 to 50 infective units per g) (2,13) and *Polymyxa* populations appear to be even lower in clump-infested soils (Table 2). Reliability of the ELISA procedure was assessed by making many comparisons with observations of roots for sporosori under a light microscope. Data presented showed perfect correlation between the two methods. Therefore, ELISA is reliable for the detection of sporosori of *P. graminis* in roots. Additionally, the serum reliably detected all the stages of *P. graminis*.

ELISA tests performed on peanut and *C. rotundus* plants grown in a field naturally infested by *P. graminis* concur with results obtained from a previous estimation of *P. graminis* incidence by microscopic observations of roots of these two species. Peanut and *C. rotundus* were selected because of their differential compatibility with *P. graminis*. *P. graminis* was seldom observed in peanut roots, whereas *C. rotundus* was shown to be an excellent host for *P. graminis*, with about 50% of the field-collected plants being infected and generally with high degree of colonization by sporosori (8,26,39).

For studying the epidemiology of *P. graminis*-transmitted viruses, it is essential to develop a reliable method to estimate the inoculum potential in soil. An estimate of the total number of sporosori cannot be used to determine the inoculum potential because of the possibility that some of the sporosori may not be viable. A bioassay system for the estimation of the MPN of infective units already exists for *P. graminis* and *P. betae* (14,31,44). The bioassay depends on the detection of the parasite in roots by light microscopy after exposure to soil inoculum. It can provide a reasonable and sensitive method for the detection and quantification of the vector in soil, but it requires several weeks to be completed and is prohibitive when a large number of samples are to be processed (2). However, the bioassay permits testing for virus presence, thus facilitating the determination of proportion of viruliferous inoculum potential of *P. graminis*. Therefore, the ELISA procedure, with its ability to detect early stages of the *P. graminis* life cycle in conjunction with the bioassay, is likely to reduce the effort and time required for the estimation of viruliferous *P. graminis* in soil. Additionally, ELISA is a simple and cost-effective method readily applicable in most of the developing countries where clump disease occurs. We are currently optimizing this method for quantification of *Polymyxa* spp. in soils from various origins where clump disease occurs, keeping in mind that detection by ELISA will be applicable only to *P. graminis* isolates that react with the antiserum available.

Polymyxa spp. from different parts of the world have been shown to exist as distinct populations that differ in their temperature requirements for optimum development, host range, and in the nucleotide sequence of internal transcribe spacer regions of ribosomal DNA (24). Various isolates were tested by the antiserum produced. Interestingly, isolates from subtropical areas, Rajasthan in India (I_9) and Punjab in Pakistan (P_1), differed from the homologous isolate I_1 (from tropical India) in the intensity of reaction (Table 1). Additionally, two *P. betae* isolates and a *P. graminis* isolate from Senegal also reacted relatively weakly. Therefore, our results confirmed the diversity that was reported to exist in *Polymyxa* spp. It is also interesting to note that I_9 and P_1 originated from soils harboring IPCV-D, a virus serologically distinct from IPCV-H, the virus that occurs in soils where *P. graminis* isolate I_1 originated (8,38). Moreover, the Senegalese *P. graminis* isolate (S_6) was isolated from soils harboring PCV that was shown to be serologically and genomically distinct from IPCV (29,34,37).

Single-sporosorus cultures originating from the same isolate of *P. graminis* (I_1) showed consistent differences in serological reaction (Table 1). Ultrastructural studies have shown that the degree of sporosorus maturation influences spore wall structure (5,6,28). Therefore, the spore wall composition and the type and amount of antigen released during extraction for ELISA may have influenced the results. Additionally, the large variation in the size of sporosori may also exert an influence on the degree of reactivity.

The composition of a resting spore wall of *Polymyxa* spp. is yet to be elucidated. Ciafardini et al. (6) suggested that proteins and perhaps lipids are present in the enzymatically resistant first two layers of the spore wall. Therefore, immunosorbent electron microscopy using antiserum to *P. graminis* and gold labeling is likely to yield valuable results to elucidate the nature of a resting spore wall. When FAT was applied for the detection of various *Polymyxa* isolates, including those that gave weak reaction in ELISA, a clear distinction could be made between those that reacted strongly and those that reacted weakly. The isolates that reacted weakly in ELISA showed specific staining in FAT, confined to a few spots scattered all along the periphery of individual resting spores. It is probable that these antigens are conserved in different *Polymyxa* isolates. Monoclonal antibodies of broad and narrow specificities will help elucidate this.

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