or zoospores that may be on the root surface. Half of the fresh root (approximately 6-8 rootlets) was analyzed for the presence of *P. graminis* by light microscopy after staining with cotton blue in lacto-phenol. The other half was tested by ELISA at a 1:20 dilution.

Specificity of the antiserum. The antiserum was tested by DAC-ELISA for cross-reactivity against common soil-borne 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 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*, *O. brassicae* and *S. 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 x 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 which 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 x 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, 1/3 of each root sample was stained by boiling fresh sample in lacto-phenol with cotton blue. Remaining portion of the root sample was dried and cut into small fragments, thoroughly mixed, and aliquots of dry weight 1.25 to 5 mg (approximately 1/3 of the root) were triturated in carbonate buffer (1mg 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 (ca. 5 mm 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 Res. Ind., Inc., Burlington, Vermont). Transverse sections of ca. 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) was conjugated with fluorescein 5-isothyocyanate (FITC) (Sigma F-7250) by the following procedure. FITC (15 mg) was dissolved in 1 ml dimethyl sulfoxide (Sigma D-2650) and mixed with 14 ml of 0.1 M sodium carbonate buffer (pH 9.6) (16). One mg of GAR-IgG was dissolved in one 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 1h at 37°C with equal volume of healthy sorghum root extract (dried roots at 0.4% w/v) prepared in conjugate buffer. The immuno-precipitate was removed by centrifugation and the cross-adsorption process repeated three times. IgG for *P. graminis* was extracted from supernatant with neutral ammonium sulphate (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-labelled GAR-IgG was added at a dilution of 1:20 prepared in blocking buffer and incubated for 3 hr 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, with a provision for epifluorescence. Photographs were taken at 80 or 100 X final magnification using a Kodak 400 ASA color reversal film.

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

Culture	code no.	Place of origin	Host	Mean % of cross-reactivity[a]
LOWER FUNGI				
Polymyxa betae	A2641 *[b]	Opprebais, Belgium	Sugar beet	41.1 ± 3.6
	T17 *[b]	Turkey	Sugar beet	42.8 ± 5.1
Polymyxa graminis	**sporosori**			
	B1 *[b]	Loupoigne, Belgium	Barley	99.1 ± 2.0
	C1 *[b]	Ottawa, Canada	Barley	81.1 ± 2.7
	F11 *[b]	Carcassonne, France	Barley	88.0 ± 1.6
	I1 [b]	Patancheru, A.P., India	Sorghum	100. 0 ± 5.8
	I1-1 *[b]	Patancheru, A.P., India	Sorghum	63.3 ± 2.4
	I1-20 *[b]	Patancheru, A.P., India	Sorghum	97.7 ± 5.2
	I1-229 *[b]	Patancheru, A.P., India	Sorghum	73.9 ± 3.3
	I9 [b]	Boraj, Rajasthan, India	Sorghum	8.0 ± 0.9
	P1 [b]	Dudhial, Punjab, Pakistan	Sorghum	7.3 ± 0.6
	J1 [f]	Japan	Wheat	96.6 ± 4.4
	S6 [b]	Bambey, Senegal	Pearl millet	23.7 ± 2.7
	zoospores			
	I1-229 *	Patancheru, A.P., India	Sorghum	42.7 ± 4.3
Spongospora subterranea	A [d]		Potato	98.4 ± 2.9
	B [d]		Potato	110.6 ± 3.0
Olpidium brassicae				
	E1(S24) [c]	Sinthion, Senegal	Sorghum	5.2 ± 1.0
	K51(S21) [c]	Niger	Barley	1.1 ± 0.5
HIGHER FUNGI				
Aspergillus flavus [e]		Patancheru, A.P., India	Soil	4.9 ± 1.4
Aspergillus niger [e]		Patancheru, A.P., India	Soil	3.0 ± 0.9
Fusarium solani [b]		Gembloux, Belgium	Potato	3.6 ± 2.3
Fusarium moniliforme [e]		Patancheru, A.P., India	Sorghum	5.7 ± 1.2
Rhizoctonia solani [b]		Belgium	-	3.6 ± 2.3
Trichoderma viride [e]		Patancheru, A.P., 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. Mean absorbance readings at 405 nm as % of that observed for the homologous isolate, *P. graminis* from India, isolate I_1. Absorbance values were typically 0.087 ± 0.069 (mean ± stdev) for negative control (healthy sorghum roots) and 2.554 ± 0.477 for positive control (isolate I_1, 1 x 10^3 sporosori/ml). Means were calculated from 5 replicates.

The various fungi were provided by: **b** Anne Legrève and Henri. Maraite, Unité de Phytopathologie, UCL, 1348 Louvain-la-Neuve, Belgium (Legrève *et al.*, 1998); **c** Sakina Gharbi and Michel Verhoyen, Unité de Phytopathologie, UCL; **d** Kenneth Bell and Jane Roberts, Department of Fungal and Bacterial Plant Pathology, Scottish Crop Research Institute, Invergowrie, Dundee DD2 5DA, UK; **e** the Cereals and Legumes Pathology Units, ICRISAT; and **f** Yasuo Ohto, Tohoku National Agricultural Experiment Station, Shimokuriyagawa, Morioka, Iwate, 020-0123, Japan.

* Single sporosorus cultures, I_{1-1}, I_{1-20}, and I_{1-229} are derived from the isolate I_1.

Sporosori, resting spores in roots, and zoospores of the obligate parasites *Polymyxa*, *Spongospora* and *Olpidium* were tested at the dilution of 1 x 10^3/ml. Fungi cultured on artificial medium were tested at the 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.

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 x 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 1:2000 (Fig. 2). Therefore this antiserum dilution was used in all future tests.

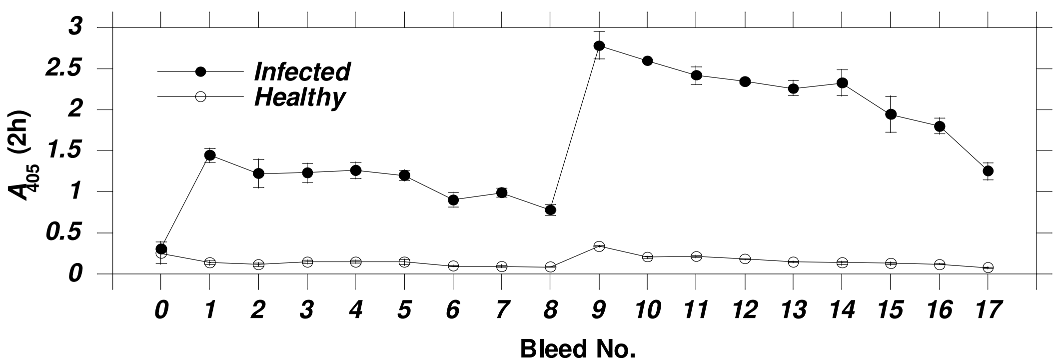


Fig. 1. Progress of *P. graminis* antiserum titer for bleeds collected over time. Bleed 0 is the pre-immune serum. Bleed 1 was collected on day 45. A booster injection was given on day 124 , one month after collecting bleed 8, and bleed 9 was collected 15 days later (day 139). The last bleed No.17 was collected on day 195. Wells were coated with sorghum root extracts containing *P. graminis* (●) (2x10^3 sporosori/ml) and equivalent weight of healthy roots (○). Mean absorbance values (A_{405}) were recorded after 2h of substrate reaction time. The intervals represent the standard deviation (n=3).

The DAC-ELISA system could readily detect 1 sporosorus per well of the ELISA plate. A mean absorbance value (± stdev) of 0.156±0.050 was observed in wells coated with 1 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 one and 10 sporosori per mg gave absorbance values exceeding 0.367±0.006 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 sporosori concentration exceeded ten 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.

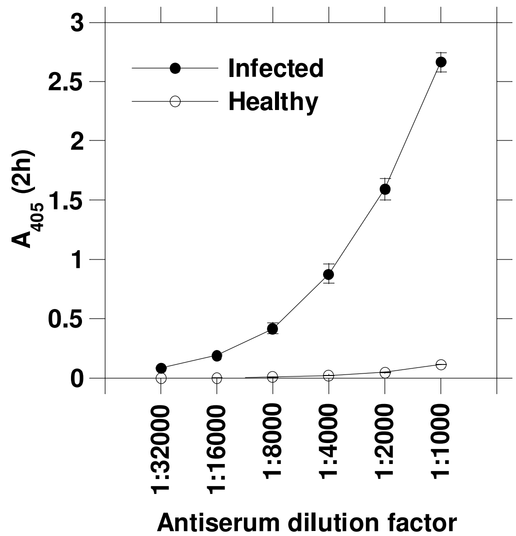


Fig. 2. Relationship between antiserum dilution factor (bleed 15) and A_{405} measured after 2 hours of substrate reaction time in DAC-ELISA. Wells were coated with sorghum root extract containing resting spores (●) (2 x 10^3 sporosori/ml) and equivalent weight of healthy root extract (○).The intervals represent the standard deviation (n=3).

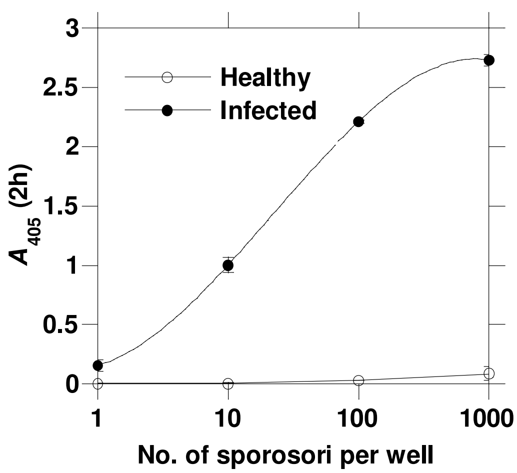


Fig. 3. Relationship of the number of *P. graminis* sporosori (●; fitted curve: $A_{405} = -0.1747\ x^3 + 1.2277\ x^2 - 1.6126\ x + 0.716$) prepared from highly infected sorghum roots, and equivalent weights of healthy sorghum roots (○) with A_{405} measured after 2 hours of substrate reaction time in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

It is apparent from the results presented in Fig. 4 that a ten 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 the estimation of sporosori concentration. Initially it was essential to cross adsorb the serum to eliminate non specific 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.

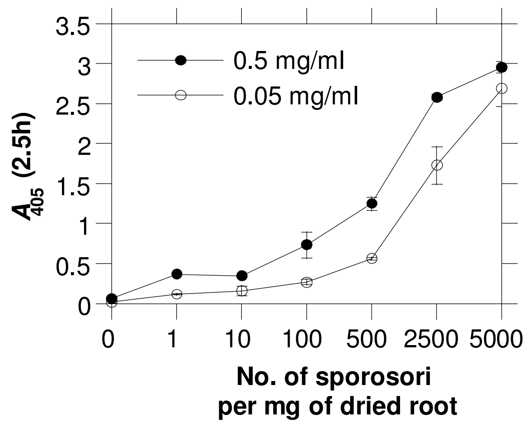


Fig. 4: Detection of *P. graminis* sporosori in spiked root samples. Relationship between the number of sporosori per mg of dried sorghum root and A_{405} measured after 2.5 h of substrate reaction time in DAC-ELISA. Two dilutions were tested: (●) 0.5 mg and (○) 0.05 mg of dried root per ml. The intervals represent the standard deviation (n=3).

Table 2. Comparison between ELISA and observation under a light microscope for the detection of *P. graminis* in roots of plants grown on serial dilutions of soil naturally infested with the *Indian peanut clump virus* (IPCV).

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

[a] The soil was sampled on the ICRISAT farm at Patancheru where the *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, +: trace of infection, ++: number of sporosori localized in one region of the root, +++: number of sporosori in various regions of the root.
[c] Ratio between A_{405} observed for the sample (S) and A_{405} observed for healthy sorghum roots (H). Mean absorbance values (± stdev) were typically 0.155±0.106 for negative (healthy sorghum roots) and 1.807±0.274 for positive control (isolate I_1, 1 x 10^3 sporosori/ml).
[d] Quantification of *P. graminis* in soil by the most probable number (MPN) method was compared by the two techniques. The most probable number of infective units (± stdev) of *P. graminis* per liter of dry crushed soil was calculated on the proportion of plants containing sporosori or giving a r≥3 in ELISA test.

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 most probable number 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 few plants showed the presence of *P. graminis* as assessed by low absorbance values in ELISA.

Table 3. Detection of *P. graminis* in field samples by ELISA.

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

[a] Roots of peanut, and *C. rotundus* (nut grass or purple nutsedge) plants were sampled at three occasions during the month of 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. The range is indicated in brackets.

Detection of the various stages of *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 two days after inoculation 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).

Table 4. Detection of the early stages of *P. graminis* life cycle by light microscopy and DAC-ELISA in roots of sorghum plants artificially inoculated each with 4500 sporosori and maintained in automatic immersion system under glasshouse conditions.

Days after inoculation	Microscopy[a]		ELISA[b]				
	ni/N	Life cycle stages	r<3	3≤r≤10	r≥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

Each day half of a fresh sorghum root (approximately 6-8 rootlets) was analyzed by light microscopy after staining and individual rootlets of the remaining root portion were processed by ELISA.

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

[b] Number of rootlets per categories of reaction in ELISA. The samples were classed according to the ratio (r) between A_{405} observed for the sample and A_{405} observed for healthy sorghum root extracts. A_{405} is the mean absorbance values (± stdev) observed for all the rootlets of individual plant and measured after 3h of substrate reaction time.

Mean absorbance values were typically 0.080±0.031 (± stdev) for healthy sorghum root extracts and 2.606±0.834 for infected root extracts (1 x 10^3 sporosori/ml).

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 spore were 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 auto-fluorescence (blue color) which 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.

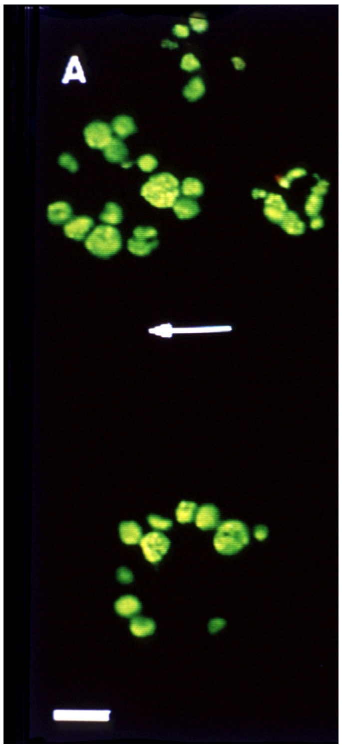
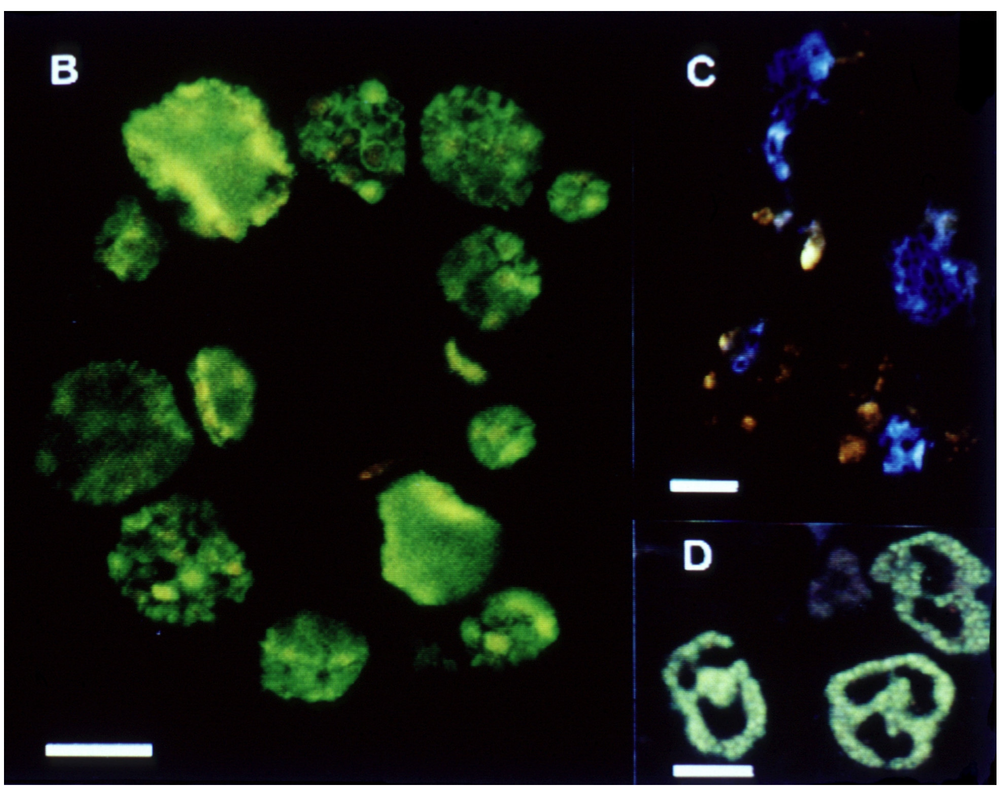


Fig. 5. Detection of sporosori by fluorescent antibody technique using FITC (light micrographs). Homologous reaction with *P. graminis,* isolate I_1 from Patancheru, India; **A**, Transversal sections through four infected and one non-infected (arrow) sorghum roots. Scale bar represents 50 µm; **B**, 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**, Auto-fluorescence of *P. graminis* sporosori (orange-brown color) and of the root stele (blue color). Scale bar represents 50 µm. **D**, Section through *S. subterranea* spore balls. Scale bar represents 25 µm.



Determination of cross-reactivity of the antiserum with a range of *Polymyxa* spp. isolates, *S. subterranea*, and *O. brassicae*. By both ELISA and FAT *O. brassicae* gave negative reactions. Interestingly 2 isolates of *S. subterranea* reacted strongly in both the 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 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 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 involve use of such hazardous chemicals as phenol. More recently, to facilitate rapid *Polymyxa* spp. detection, molecular techniques have been developed (35, 36, 47). These techniques even though very specific, are expensive, require special skills and equipments 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 presented data on estimation of *P. graminis* sporosori by immuno-chemical 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 consequent release of antigens. Indeed, previous rabbit immunizations with non-sonicated spores gave sera of very low titer and of poor specificity (results not presented). During sonication process some of the sporosori have 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 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). Initially a procedure which involved several subcutaneous and intramuscular injections was tested for producing polyclonal antiserum. Despite tests on 8 successive bleeds at one week intervals, the titer of the serum did not show any sign of increase. A booster given 10 weeks after the 7^{th} injection contributed to 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 Plasmodiophorales.

Antiserum did contain antibodies that reacted with healthy root extracts. This non specific reaction was eliminated by cross adsorption with healthy root extracts. Specificity of the antiserum was evaluated by tests with a range of fungi which occur naturally in roots of various hosts utilized in this study. The serum did not react with any of the fungi tested (Table 1) and with a member of the Chitridales, *O. brassicae*. The serum reacted strongly with another distinct member of the 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 routine detection of *P graminis* by ELISA in monocotyledonous hosts such as sorghum and millets. Infection by *S subterranea* is mostly restricted to Solanaceae and Chenopodiaceae (18, 20, 21, 22).

The ELISA procedure adopted was suitable for estimation of sporosori in root extracts, one of the main objectives of this study. A single sporosorus was adequate to produce OD values that are at least 3 fold higher than those of comparable healthy root extracts. The procedure facilitated 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 (not reported). 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* from soil is currently not available and extraction of sporosori of Plamodiophorales from soil failed to yield satisfactory results especially when sporosori occur in low concentration (2, 4, 42). *Polymyxa* typically occurs at low populations in soils (1-50 infective units/g) (2, 13) and *Polymyxa* populations appear to be even lower in clump infested soils (Table 2). Reliability of 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 detected reliably 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 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. S*eldom *P. graminis* was 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 epidemiology of *P. graminis* transmitted viruses, it is essential to develop a reliable method to estimate inoculum potential in soil. An estimate of 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 estimation of the most probable number 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 detection and quantification of the vector in soil but it requires several weeks to be completed and is prohibitive when 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 *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* 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 sub-tropical 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 which occurs in soils where *P. graminis* isolate I_1 originated (8, 38). Moreover the Senegalese *P. graminis* (S_6) was isolated from soils harboring PCV which 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 resting spore wall of *Polymyxa* 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 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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Epidemiology

Chapter 2

Geographical distribution of the peanut clump disease in Asia

Abstract

Surveys were conducted in Asia to determine the geographical distribution of the peanut clump disease. The countries visited included India, Pakistan and Thailand. The Durgapura isolate (IPCV-D) is a member of the most widespread serotype of IPCV. Viruses serologically related to IPCV-D occur in the Indian States of Gujarat (Talod), Rajasthan (Boraj, Dausa, Durgapura, Kallavas and Ranoli), Andhra Pradesh (Bapatla, Chinnaganjam, Ganapavaram, Pallipalem, Ramapuram and Tsundupalle), Tamil Nadu (Pondicherry) and in the Punjab Province of Pakistan in Attock, Chakwal and Rawalpindi districts. A second serotype, which occurs in Punjab in India (Jalandhar, Ludhiana and Sangrur), is represented by the Ludhiana isolate (IPCV-L). It is also present in Pakistan in Attock district. In Hyderabad, Andhra Pradesh, a third serotype occurs (IPCV-H). The disease in India is very severe in Rajasthan where groundnut is rotated with cereals and particularly with wheat or barley. In Punjab the disease caused severe damage to groundnut until the early eighties when the wheat-groundnut rotation was replaced by a wheat-rice system. The disease was not encountered in Thailand where groundnut is mostly grown during the post rainy season and rotated with rainy season crops of rice.

Introduction and Methodology

Knowledge on the occurrence of clump disease in Asia was limited because virus detection in the developing countries where clump disease presumably occurs is often hampered by a lack of equipment and skills for proper identification of viruses by the national agricultural research systems (NARS) (Reddy, 1990, Reddy and Gowda, 1996, Reddy *et al.*, 1997). In the absence of sufficient data on the distribution and spread of peanut clump disease, the necessity of conducting research on this virus disease and its economic importance were sometimes questioned. Therefore, surveys were conducted in India, Pakistan and Thailand in Asia to collect the necessary information to evaluate the disease importance. The surveys in India and Pakistan were mostly requested by NARS, whereas the survey conducted in Thailand was opportunistic and conducted at the occasion of the 4th Meeting of the International Working Group on Groundnut Viruses (Khon Kaen University, Thailand, 12-14 March 1995).

Surveys were conducted in fields along or near roads. The fields were scored for clump symptoms by taking observations across the two diagonals of the field and at the borders. Groundnut and weed plants as well as soil samples were collected from disease sites. In most cases, whole plants were uprooted, labelled, sealed in polythene bags and kept on ice in an ice-box. Back to the laboratory, all plants were analysed for virus presence by ELISA using the three sera available for IPCV detection and a serum produced against a PCV isolate (Nolt *et al.*, 1988, Reddy *et al.*, 1998). Groundnut plants were further analysed by bioassays such as sap inoculation onto leaves of french bean plants in which IPCV causes veinal necrosis, or by grafting branches onto healthy groundnut rootstock followed by observation of symptoms in newly formed branches. Soils sampled at the disease sites were analysed to determine their texture (Table 2). The soil from Pakistan was sent to Belgium for *P. graminis* isolation (Legrève et al., 1999). A summary of the survey sites in Pakistan and Thailand is presented in Table 1 and illustrated in Fig. 1 and 2.

Survey for peanut clump disease in North Thailand in postrainy season crops of groundnut

Groundnut is grown in the postrainy season in central and north Thailand, followed by rice, the latter being the major rainy season crop in the country. In March 1995, we surveyed the regions of Chiang Mai, Langpang, Kalasin and Wang Nam Yen. Groundnut is grown only if irrigation is available, near rivers, lakes, canals or at the foot hills. The soil in paddy fields is heavy and if allowed to dry may prevent entry of pegs. For this reason the crop is irrigated by flooding the field as in case of rice, at least 3 times during the growing season. In all the fields visited, the crop was found to be quite healthy. The peanut clump disease has never been observed. *Peanut bud necrosis virus* (PBNV) affected the crop up to 5 % in some fields. *Peanut Stripe Virus* (PStV) was rarely present. Aphids were sometimes heavily present. The main problem that we noticed was excessive weeds in most of the fields. Weeding is rarely done and in crops at harvesting stage we counted only 2 to 3 pods per plant. From our observations, the peanut clump disease is not present in postrainy season crops of groundnut. *P. graminis* could not be detected in graminaceous weeds collected during the survey in Thailand.

Table 1. Areas surveyed in Thailand (March 1995) and Pakistan (July 1995) for peanut clump disease.

Country	City or Village	Number of fields	Crop stage	Observation	IPCV serotype
Thailand	Lampang area:				
	Bandan	3	60 days	PBNV (5%), PStV (<5%)	-
	Ban Huey Rai	2	harvest	weeds	-
	Sanklang	3 small fields	60 days	weeds	-
	Hangchat	1	60 days	PBNV, aphids	-
	Chiang Mai area:	3	60 days	PBNV, mites	-
	Kalasin area:	5	>40 days	PBNV, PStV, mineral deficiency	-
	East of Bangkok:	2	60 days	PBNV	-
Pakistan	Chakwal area:				
	Dudhial	3	90 days	IPCV < 10%, PBNV	IPCV-D
	Bhaun	3	90 days	stunting <1%	-
	ARF of Bhaun	2	90 days	stunting <1%	-
	BARI	3	90 days	stunting <1%	IPCV-D IPCV-L
	Mari	1	90 days	-	-
	Chach	3	90 days	stunting <1%	-
	Chach to Islamabad	>20	90 days	stunting <1%	-
	Fateh Jang area:	19	90 days	PBNV 5-10%	-
	Attock area: 60 000 ha of groundnut	>20 large fields	60-90 days	Very healthy looking crops	
	NARC-Islamabad	1	2-3 weeks	IPCV >20%, chlorosis due to Fe deficiency	IPCV-D

PBNV: *Peanut bud necrosis virus*, PStV: *Peanut stripe virus*.
BARI: Barani Agricultural Research Institute, Chakwal, Pakistan.
NARC: National Agricultural Research Center, Islamabad, Pakistan.
ARF Bhaun: Adaptive Research Farm of Bhaun, Pakistan.
IPCV-Talod and IPCV-Durgapura are serologically related (serotype IPCV-D).

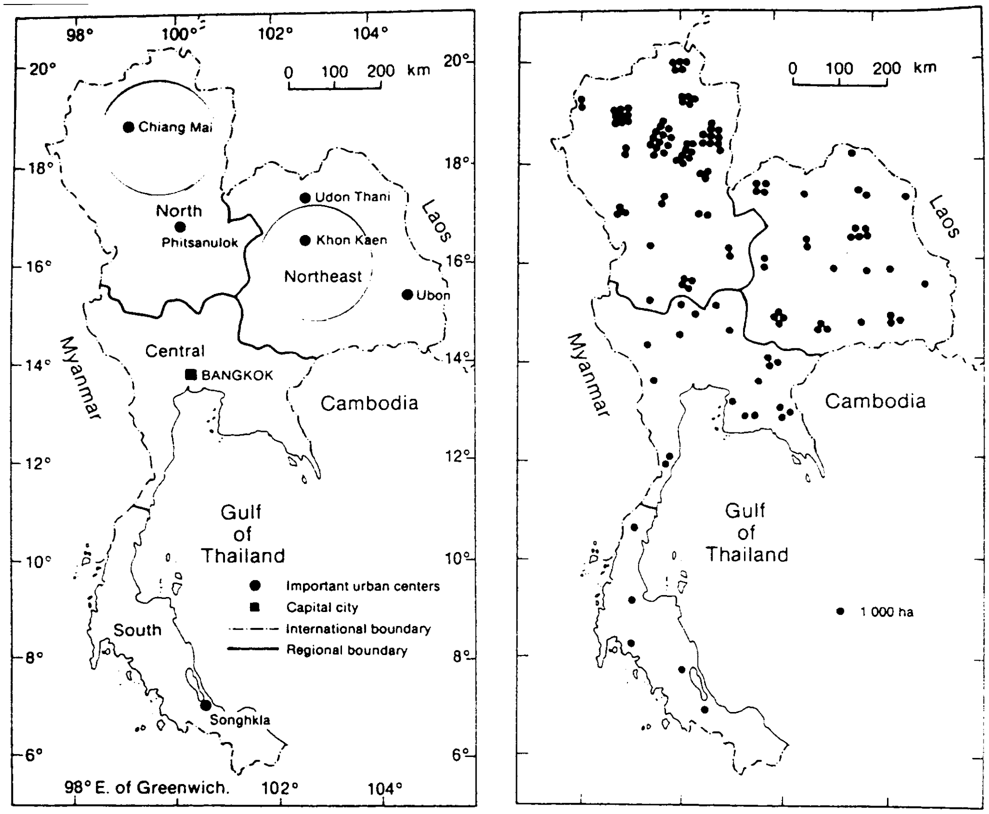

Figure 1. Administrative divisions of Thailand (left), groundnut distribution (•) and areas surveyed in March 1995 (right).

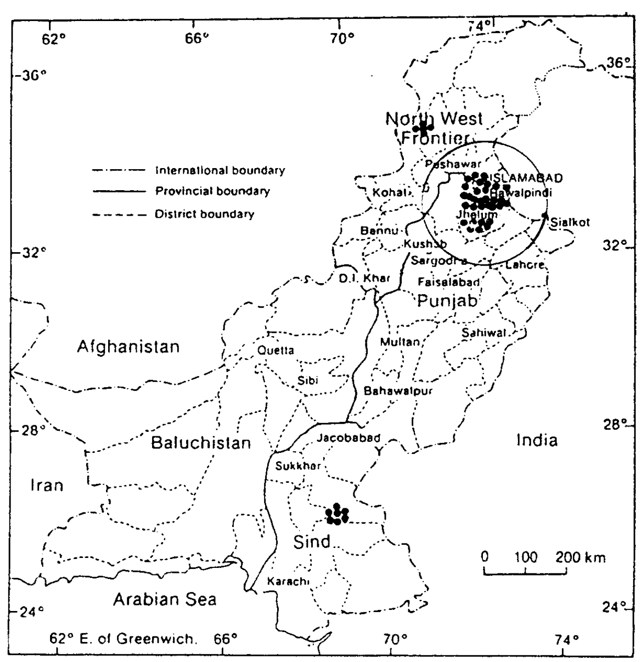

Figure 2. Administrative divisions of Pakistan, groundnut distribution (•) and areas surveyed in July 1995.