2008

An Immunofluorescence Assay to Detect Urediniospores of *Phakopsora pachyrhizi*

Fulya Baysal-Gurel
Ohio State University

Melanie L. Lewis Ivey
Ohio State University

Anne E. Dorrance
Ohio State University, dorrance.1@osu.edu

Douglas Luster
USDA ARS Foreign Diseases and Weed Science Research Unit

Reid Frederick
USDA ARS Foreign Diseases and Weed Science Research Unit, reid.frederick@usda.gov

See next page for additional authors

An Immunofluorescence Assay to Detect Urediniospores of *Phakopsora pachyrhizi*

Fulya Baysal-Gurel, Melanie L. Lewis Ivey, and **Anne Dorrance,** Department of Plant Pathology, The Ohio State University, Ohio Agricultural Research and Development Center, Wooster; **Douglas Luster** and **Reid Frederick,** USDA ARS Foreign Diseases and Weed Science Research Unit, Ft. Detrick, MD; **Jill Czarnecki,** Naval Medical Research Center, Biological Defense Research Directorate, Silver Spring, MD; **Michael Boehm,** Department of Plant Pathology, The Ohio State University, Columbus; and **Sally A. Miller,** Department of Plant Pathology, The Ohio State University, Ohio Agricultural Research and Development Center, Wooster

ABSTRACT

Baysal-Gurel, F., Ivey, M. L. L., Dorrance, A., Luster, D., Frederick, R., Czarnecki, J., Boehm, M., and Miller, S. A. 2008. An immunofluorescence assay to detect urediniospores of *Phakopsora pachyrhizi*. Plant Dis. 92:1387-1393.

An indirect immunofluorescence spore assay (IFSA) was developed to detect urediniospores of *Phakopsora pachyrhizi*, utilizing rabbit polyclonal antisera produced in response to intact nongerminated (SBR1A) or germinated (SBR2) urediniospores of *P. pachyrhizi.* Both antisera were specific to *Phakopsora* spp. and did not react with other common soybean pathogens or healthy soybean leaf tissue in enzyme-linked immunosorbent assay (ELISA). SBR1A and SBR2 bound to *P. pachyrhizi* and *P. meibomiae* urediniospores were detected with goat anti-rabbit Alexa Fluor 488-tagged antiserum using a Leica DM IRB epifluorescent microscope with an I3 blue filter (excitation 450 to 490 nm, emission 515 nm). The assay was performed on standard glass microscope slides; double-sided tape was superior to a thin coating of petroleum jelly both in retaining spores and in immunofluorescence. The IFSA was used to confirm the identity of *P. pachyrhizi* urediniospores captured on glass slides from passive air samplers from Georgia, Kentucky, and Ohio during 2006.

Additional keywords: field trap, soybean rust

Soybean rust has been reported in Asia, Africa, South and Central America, the United States, and most recently Canada (28,38; A. Tenuta, *personal communication*). Significant yield losses on soybean crops have occurred in Asia, Africa, Australia, and nearly all tropical countries in both the eastern and western hemispheres where soybeans are grown (1–3,21,28, 31,33,34,42). Soybean rust is caused by two *Phakopsora* species, *P. pachyrhizi* Sydow and *P. meibomiae* (Arthur) Arthur, of which *P. pachyrhizi* is the more aggressive (28). *P. meibomiae* is not considered a serious threat to soybean production. In Brazil, *P. pachyrhizi* was estimated to cost growers approximately $1.2 billion in 2003, with $500 million in direct yield losses to the disease and $700 million resulting from use of fungicides (42). It is predicted that *P. pachyrhizi* may negatively impact soybean production in the United States with annual losses between $240 million and $2.0 billion, depending on the severity of the disease (23,28).

P. pachyrhizi can be managed with well-timed fungicide applications (29,30). In the United States, numerous products containing triazole and strobilurin type fungicides have been labeled. Both types of products have good activity when applied close to the time when spores arrive in the field (12,18).

Currently, an emphasis is being placed on tracking the movement of soybean rust through soybean production regions in the United States, and developing predictive models for future fungicide application and timing decisions (41). The IPM PIPE system (16,39), established to monitor and predict diseases such as soybean rust, is dependent upon accurate and timely detection and identification of pathogen propagules to ensure the efficacy of both decision tools and disease management. Methods are needed to efficiently detect and correctly identify and distinguish *P. pachyrhizi* urediniospores from other plant-pathogenic fungal spores for timely and appropriate applications of fungicides.

Techniques for trapping and direct assessment of *P. pachyrhizi* urediniospores would be beneficial to all soybean producers worldwide. Identification of trapped spores of *P. pachyrhizi* currently requires experienced mycologists, specialized equipment, and many hours examining the trapped spores microscopically. Visualization and enumeration of airborne spores through bright field microscopic examination of spores collected in spore traps have been utilized for a number of plant pathogens (15). However, such methods require considerable amounts of time and expertise in spore morphology if accurate counts are to be obtained. The use of this technique is further limited when fungal spores that are morphologically similar are captured together. Technological advances in fungal diagnostics, using either antibody- or nucleic acid-based assays, allow for the accurate differentiation of fungal species (5,14,40).

Air sampling formats can be combined with serological assays to detect and identify airborne fungal spores (17,20). For example, Kennedy et al. (19) developed a direct fungal spore monitoring process in which airborne spores of *Mycosphaerella brassicicola* and *Botrytis cinerea* were trapped in a 96-well enzyme-linked immunosorbent assay (ELISA) plate using a specialized particle sampler and detected by rabbit polyclonal antibodies.

In this study, we report the production of specific polyclonal antibodies for *P. pachyrhizi* and describe the development of an immunofluorescence spore assay (IFSA) for the detection of *P. pachyrhizi* urediniospores in spore trapping systems. This type of assay will greatly assist in the monitoring of *P. pachyrhizi* urediniospores for both fungicide recommendations and confirmation of modeling efforts. Preliminary results of this research have been reported previously (4).

MATERIALS AND METHODS

Microbial cultures. *P. pachyrhizi* and *P. meibomiae* isolates were maintained and propagated at the USDA-ARS Foreign Disease-Weed Science Research Unit (FDWSRU) Biosafety Level 3 (BSL-3) Plant Pathogen Containment Facility at Fort Detrick, MD (26) under an appropriate USDA Animal Plant Health Inspection

Corresponding author: Sally A. Miller
E-mail: miller.769@osu.edu

Accepted for publication 6 June 2008.

doi:10.1094/PDIS-92-10-1387

Service permit. Urediniospores were harvested using a mechanical harvester (9) and both germinated and nongerminated urediniospores were killed in ethylene oxide prior to use in antiserum and assay development according to the conditions of APHIS permit for removing material from the BSL-3 containment facility. All other fungal and bacterial cultures were collected and maintained at The Ohio State University.

Polyclonal antibody production. Four different fractions of *P. pachyrhizi* urediniospores were used to generate five antisera (Table 1). These fractions represented distinct approaches to produce polyclonal antisera against surface and internal antigens of *P. pachyrhizi*. Antisera were generated against surface antigens of intact nongerminated and germinated *P. pachyrhizi* isolate Taiwan 72-1 (TW 72-1) urediniospores, pulverized germinated spores, and spore wall fractions. To generate antibodies against surface antigens of intact, nongerminated spores, urediniospores that were stored frozen in liquid nitrogen were suspended in phosphate-buffered saline (4.3 mM $NaHPO_4$, 1.4 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.3) (PBS) at two different concentrations and killed by treatment with ethylene oxide. To generate antibodies against surface and internal antigens of germinated spores, 1.0 g FW of urediniospores was floated on the surface of 3 liters of sterile distilled H_2O containing 50 µg/ml ampicillin and 50 µg/ml streptomycin in glass pans and allowed to germinate at room temperature overnight. The germinated urediniospore mat was washed in 3 liters of 0.01% Tween 20 vol/vol on a rotary shaker at 100 rpm for 2 h at RT. The germinated spores were collected on Whatman no. 1 filter paper, sterilized overnight with ethylene oxide, and split into two fractions. One-third of the germinated spore fraction was directly suspended in PBS and used for injection. The remaining two-thirds of the germinated spores were resuspended in PBS, flash frozen in liquid nitrogen, and pulverized in a bead beater (Cole Parmer, Vernon Hills, IL) using three 25-s bursts at 2,500 rpm. This pulverized fraction was then split into two fractions. One-half of the pulverized germinated spores were used directly for injection. The other half of the pulverized, germinated spores were resuspended in PBS containing 500 mM NaCl (PBSS), vortexed, centrifuged at 325 × *g*, and the pellet washed with PBSS. This process was repeated three times to remove soluble materials and leave a washed cell wall fraction. The final washed cell wall pellet was resuspended in PBS for injection. The protein content of each fraction was measured using the method of Markwell et al. (24), and protein concentrations were adjusted to optimize the immune response (Table 1). All antiserum production was performed using New Zealand white rabbits by a commercial vendor (Bushover Biologicals, Vassalboro, ME) under contract to the U.S. Navy Biological Defense Research Directorate (BDRD) in Silver Spring, MD conforming to approved animal handling and BDRD Animal Care and Use Committee policies and protocols. Immunization schedules were performed according to the vendor's protocols. Bleeds were conducted until optimal titers were obtained, based upon standardized ELISA screening protocols with target immunogens.

Polyclonal antiserum specificity-indirect ELISA. The five polyclonal antisera (SBR1A, SBR1B, SBR2, SBR3, and SBR4) developed in response to four different *P. pachyrhizi* immunogens were evaluated for specificity using an indirect ELISA protocol with ethylene oxide-killed urediniospores of *P. pachyrhizi* and *P. meibomiae*, and pure culture extracts of four bacterial pathogens (*Pseudomonas syringae* pv. *syringae* van Hall, *P. syringae* pv. *phaseolicola*, *Xanthomonas campestris* pv. *glycines* (Nakano) Dye, *Clavibacter michiganensis* subsp. *insidiosus*) and six fungal pathogens (*Botrytis cinerea* Pers.:Fr., *Colletotrichum acutatum* Simmonds, *C. gloeosporioides* (Penz.) Penz. & Sacc., *Ustilago tritici* (Pers.) Rostr., *Puccinia graminis* f. sp. *tritici* Eriks., *Rhizoctonia solani* Kuhn). Soybean leaf extracts prepared from leaves with bacterial pustules caused by *X. campestris* pv. *glycines*, frogeye leaf spot caused by *Cercospora sojina* K. Harav, bacterial blight caused by *P. syringae* pv. *glycinea* (Coerper) Young, Dye & Wilkie, Septoria leaf spot caused by *Septoria glycines* Hemmi, and powdery mildew caused by *Microsphaera diffusa* Cooke & Peck, and healthy leaves from 183 soybean cultivars were evaluated. In addition, extracts of wheat tissue infected with *U. tritici* (Pers.) Rostr. and *P. graminis* f. sp. *tritici* Eriks were tested.

Germinated and nongerminated urediniospores of *P. pachyrhizi* and *P. meibomiae* were suspended in PBS to a final concentration of 10 µg protein/ml. Pure cultures of each fungal pathogen (except isolates of *Colletotrichum*) were grown in potato dextrose broth (PDB) at room temperature without shaking for 3 to 6 days, depending on the organism. *Colletotrichum* isolates were grown in glucose casein medium as previously described (22). Mycelium from each pathogen was harvested by filtering through sterile Whatman no. 2 filter paper. Cultures were air-dried overnight, ground in liquid nitrogen, and resuspended in PBS to a final concentration of 10 µg protein/ml. Protein concentrations were determined using the Bradford assay (7). Pure cultures of each bacterial pathogen were grown in nutrient broth yeast extract medium without glucose and incubated at 28°C for 36 h with shaking (~250 rpm). Bacteria were harvested by centrifugation (8,100 × *g*, 10 min), and the pellets were resuspended in PBS to final concentrations of 10^6 and 10^2 CFU/ml. Soybean leaf and wheat tissue extracts were prepared by grinding 0.5 g of diseased soybean or wheat tissue in Agdia mesh sample bags containing 3 ml of BEB1 buffer (Agdia, Inc., Elkhart, IN). Extracts were collected, transferred into 1.5-ml sterile microfuge tubes (250-µl aliquots) and stored at –20°C.

Indirect ELISAs were set up by adding 200 µl of each sample to each of two wells of an Immulon 2HB, 96-well flat bottom microtiter plate. Each assay contained healthy tissue extract and BEB1 buffer as negative controls. Microtiter plates were placed into a sealed moist chamber and incubated overnight at 4°C. The microtiter plates were washed six times with PBS containing 0.01% vol/vol Tween 20 (PBS-Tween) and blocked with blocking buffer (PBS containing 5% skim milk) for 1 h at 37°C. Plates were washed again with PBS-Tween six times, 100 µl of diluted primary antibody (1:10 in dilution buffer [PBS-Tween with 5% skim milk]) was added to each well, and the plates were incubated at 37°C for 1 h within a sealed moist chamber. Plates were washed six times with PBS-Tween, 100 µl of diluted alkaline phosphatase conjugate (1:3,000 in dilution buffer) was added to each well, and the plates were incubated at 37°C for 1 h within a sealed moist chamber. Plates were again washed six times with PBS-Tween, and 100 µl of PNP substrate buffer (1 tablet PNP/5 ml of 1× PNP substrate buffer; Agdia) was added to each well. Plates were incubated at 37°C for 15 min and visualized at 405 nm (2-min intervals for 10 min) using an automated microplate reader. A positive sample was scored when the absorbance (405 nm) was at least two times the absorbance of the healthy tissue (negative control).

IFSA optimization. Several parameters were evaluated to optimize the immunofluorescence staining procedure including: (i) reaction of different primary antisera, (ii) antiserum working dilution with different buffers (PBS and PBS-Tween, Agdia, Inc.), (iii) working dilutions with the

Table 1. Polyclonal antisera developed in response to antigens of *Phakopsora pachyrhizi*

Antiserum	Immunogen preparation	Immunogen amount	Boost amount
SBR1A	Nongerminated urediniospores	5 mg spores FW	2.0 mg spores FW
SBR1B	Nongerminated urediniospores	2.5 mg spores FW	2.0 mg spores FW
SBR2	Germinated urediniospores	0.25 mg protein	0.10 mg protein
SBR3	Pulverized, germinated urediniospores	0.325 mg protein	0.10 mg protein
SBR4	Wall fraction germinated urediniospores	0.05 mg protein	0.125 mg protein

secondary antiserum (Alexa Fluor 488 goat anti-rabbit IgG (H+L), Invitrogen Molecular Probes, Eugene, OR), (iv) primary and secondary antiserum incubation time and temperature, (v) use of a blocking buffer (PBS containing 2% bovine serum albumin [BSA], Sigma Chemical Co., St. Louis, MO) and 5% goat serum (Sigma)], and (vi) type of washing buffer. Spores were observed using an epifluorescence microscope and rated visually using a scale of 1 to 4 where 1 = no fluorescence, 2 = weak fluorescence, 3 = good fluorescence (clearly positive), and 4 = bright fluorescence (Fig. 1).

Assay optimization was conducted using standard glass microscope slides coated with a thin layer of petroleum jelly or affixed with a 51-mm strip of 13- or 19-mm-wide double-sided tape (Scotch Permanent Double-Sided Tape, 3M Corporation, St. Paul, MN). Dry urediniospores were drawn ~1 mm into the tip of a 10-µl glass PCR micropipette and expelled onto the double-sided tape or petroleum jelly. For a urediniospore capture treatment, double-sided tape was pressed onto the petroleum jelly-coated slides to remove the urediniospores and affixed to a glass slide. A 500-µl aliquot of primary antiserum was added to the urediniospores and incubated in a covered petri dish. The glass microscope slide containing primary antiserum-labeled urediniospores was then washed six times (30 to 60 s incubation each time) in washing buffer. A 500-µl aliquot of Alexa Fluor 488-labeled goat anti-rabbit IgG was applied over the surface of the slide and incubated in the dark. The slide was washed six times as described above. One drop (approx. 100 µl) of PBS-Tween was placed over the surface of the slide, which was then was covered with a coverslip. The edges of the coverslip were sealed to the microscope slide with clear nail polish. Samples were stored in the dark at 22°C for up to 2 months.

Polyclonal antiserum specificity-IFSA. Polyclonal antiserum SBR1A was tested for specificity using the IFSA protocol on standard glass microscope slides either coated with petroleum jelly or affixed with double-sided tape. Spores of five fungal pathogens from infected tissue (*Puccinia graminis* f. sp. *tritici*, *Phragmidium* sp., *Ustilago tritici*, *Alternaria* sp., and *Botrytis cinerea*) and ethylene oxide-killed urediniospores of *P. pachyrhizi* isolate TW 72-1 and *P. meibomiae* isolate Puerto Rico 76 (PR 76) were applied to the slides as described above. In addition, urediniospores of 21 *P. pachyrhizi* isolates were tested.

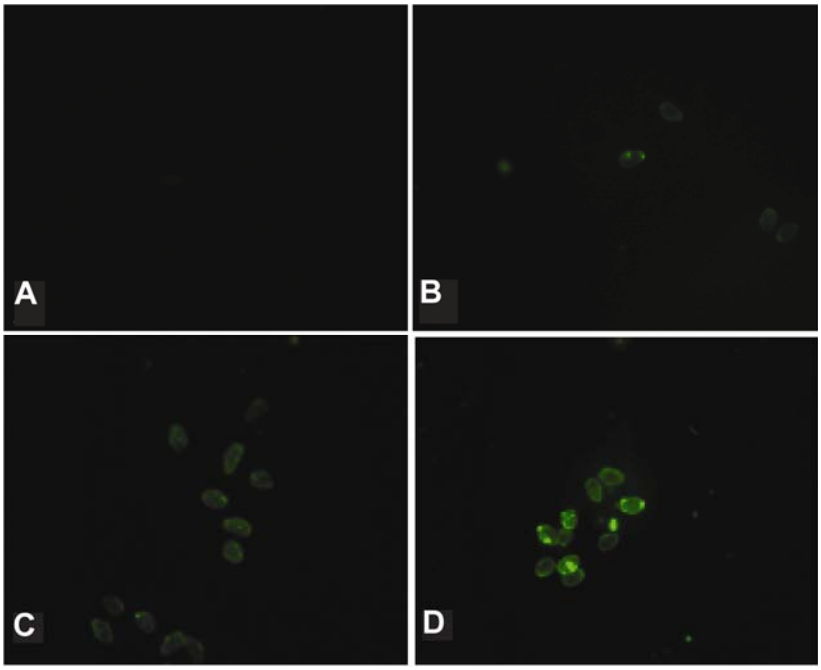

Fig. 1. The visual fluorescence intensity score of urediniospores of *Phakopsora pachyrhizi* determined using an immunofluorescence spore assay (IFSA) (20× objective). **A,** 1 = no fluorescence; **B,** 2 = weak fluorescence; **C,** 3 = good fluorescence (clearly positive); and **D,** 4 = bright fluorescence.

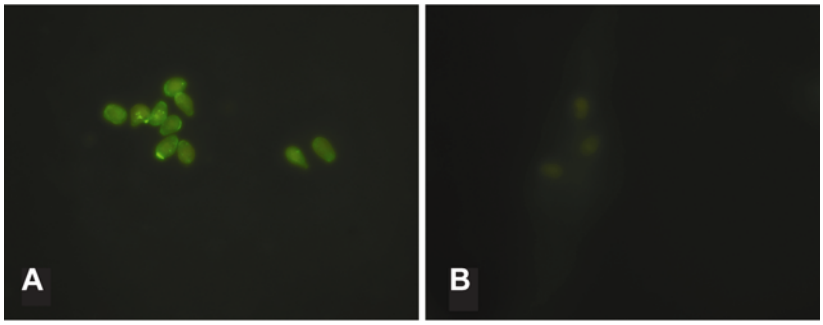

Fig. 2. Visualization of urediniospores of *Phakopsora pachyrhizi* on glass microscope slides in an immunofluorescence spore assay (IFSA) with polyclonal antiserum SBR1A using **A,** double-sided tape, and **B,** petroleum jelly (20× objective).

Table 2. Specificity of polyclonal antisera developed in response to *Phakopsora pachyrhizi* antigens to fungal and bacterial plant pathogen and healthy soybean cultivar extracts in an enzyme-linked immunosorbent assay (ELISA)

Extract	Reactivity in ELISA[a]				
	SBR1A	SBR1B	SBR2	SBR3	SBR4
Phakopsora pachyrhizi isolate TW 72-1	+	+	+	+	+
Phakopsora meibomiae isolate PR 76	+	+	–	–	–
Pseudomonas syringae pv. *syringae* and pv. *phaseolicola*, *Xanthomonas campestris* pv. *glycines*, *Clavibacter michiganensis* subsp. *insidiosus*, *Botrytis cinerea*, *Colletotrichum acutatum*, *C. gloeosporioides, Ustilago tritici*	–	–	–	–	–
Rhizoctonia solani	–	–	–	+	+
Puccinia graminis f. sp. *tritici*	–	–	–	–	+
Diseased soybean tissue: *P. syringae* pv. *glycinea, X. campestris* pv. *glycines*, *Cercospora sojina*, *Septoria glycines*, *Microsphaera diffusa*	–	–	–	–	–
Symptomless soybean tissue: 183 cultivars	–	–	–	–	–

[a] Samples for which the absorbance at 405 nm is greater than or equal to 2× that of the negative control (healthy soybean tissue extract) are considered positive (+). Samples are considered negative (–) when the A_{405} is less than 2× the absorbance of the negative control.

Table 3. Fluorescence intensity of *Phakopsora pachyrhizi* urediniospores in an immunofluorescence spore assay (IFSA) utilizing various assay parameters

Parameter	Variable	Fluorescence intensity[a] 1	2	3	4
Primary antiserum	SBR1A				+
	SBR2			+	
Dilution buffer type	PBS		+		
	PBS-Tween				+
Primary antiserum dilution	1:100				+[b]
	1:250				+[b]
	1:500				+
Secondary antiserum dilution	1:100				+[b]
	1:250				+[b]
	1:500				+
Primary antiserum incubation time	1 h			+	
	2 h				+
	3 h				+
Secondary antiserum incubation time	1 h				+
	2 h				+
	3 h				+[b]
Primary antiserum incubation temperature	22°C				+
	37°C		+		
Secondary antiserum incubation temperature	22°C				+
	37°C		+		
Blocking step: PBS+2% BSA+5% goat serum	Yes				+
	No				+
Washing buffer	PBS		+		
	PBS-Tween				+
	Distilled water	+			

[a] Fluorescence of urediniospores was rated visually using a scale of 1 to 4 where 1 = no fluorescence, 2 = weak fluorescence, 3 = good fluorescence (clearly positive), and 4 = bright fluorescence.
[b] Indicates that the background was cloudy.

Table 4. Reactivity of polyclonal antiserum SBR1A with killed urediniospores of *Phakopsora pachyrhizi* isolates in an immunofluorescence spore assay (IFSA) with polyclonal antiserum SBR1A

Origin	Year	Isolate	Source	Reactivity in IFSA[a]
Louisiana	2004	LA04-1	R. Schneider[b], Ben Hur	4
	2004	LA04-2	R. Schneider, Ben Hur	4
	2004	LA04-3	R. Schneider, St. Gabrielle	4
Alabama	2004	AL04-1	R. Frederick[c], Mobile County	4
	2004	AL04-2	R. Frederick, Baldwin County	4
	2004	AL04-3	R. Frederick, Baldwin County	4
Hawaii	1994	HW94-1	E. Kilgore[d], Oahu	4
	1998	HW98-1	E. Kilgore, Oahu	4
Australia	1979	AU79-1	Unknown	4
Brazil	2001	BZ01-1	J. T. Yorinori[e], Parana	4
China	2003	CH03-1	Z. Ma[f], YunNan Province	4
Colombia	2004	CO04-1	R. Tisnes, Valle del Cauca, Caicedonia	4
India	1973	IN 73-1	D. N. Thapliyal, Pantnagar	4
Paraguay	2001	PG01-2	W. M. Morel[g], Capitan Miranda	4
Philippines	1977	PH 77-1	Bureau of Plant Industries, Los Banos	4
South Africa	2001	SA 01-1	Z. A. Pretorius[h], Natal Province	4
Thailand	2001	TH01-1	S. Poonpolgul[i], Chaingmai	4
	2002	TH02-1	S. Poonpolgul, Chaingmai	4
Taiwan	1972	TW 72-1	Lung-Chi Wu, Taipei	4
	1980	TW 80-2	AVRDC[j], Taiwan	4
Vietnam	2005	VT05-1	N. Binh[k], Hanoi	4
Zimbabwe	2001	ZM 01-1	C. Levy[l], Harare	4

[a] Fluorescence of urediniospores was rated visually using a scale of 1 to 4 where 1 = no fluorescence, 2 = weak fluorescence, 3 = good fluorescence (clearly positive), and 4 = bright fluorescence.
[b] Department of Plant Pathology and Crop Physiology, Louisiana State University, Baton Rouge, LA.
[c] Collections made with the assistance of T. Johnson, R. Wingard, and W. Harrison, Alabama Department of Agriculture and Industries, and E. Sikora, Alabama Cooperative Extension System, Auburn University.
[d] Hawaii Department of Agriculture, Hilo, HI.
[e] Embrapa soja, Londrina, Brazil.
[f] China Agricultural University, Beijing, China.
[g] Centro Regional Investigacion de Agricola, Capitan Miranda, Paraguay.
[h] University of the Free State, Bloemfontein, South Africa.
[i] Thailand Department of Agriculture, Bangkok, Thailand.
[j] Asian Vegetable Research and Development Center, Taipei, Taiwan.
[k] Plant Protection Research Institute, Hanoi, Vietnam.
[l] Commercial Farmers Union of Zimbabwe, Harare, Zimbabwe.

Immunofluorescence microscopy. Images were captured using a Leica DMIRB (Leica Microsystems, Wetzlar, Germany) epifluorescence microscope equipped with an Optronics Magnafire camera (Optronics Engineering, Goleta, CA, or http://optronics.com), digitized with a Magnafire image capture board, and processed with Adobe Photoshop CS (Adobe, Seattle, WA). A USH-102DH-100W ultra-high-pressure mercury lamp (USHIO America, Inc., Cypress, CA) was used as the exciting light source. Images were viewed in the blue excitation range (filter set I_3- excitation filter BP450–490 nm and emission filter BP515 nm) with a 20× objective (N.A. 0.12); the exposure time for image capture was always 1.037 s.

Immunomonitoring of field-trapped spores. Double-sided tape was affixed to standard glass microscope slides, or slides were thinly coated with petroleum jelly, and placed in passive spore traps in Georgia, Kentucky, and Ohio soybean fields during 2006. Each trap was designed to rotate with the wind and was approximately 1.8 m above the soybean canopy. Slides were collected at 7-day intervals and heated in an oven for 48 h at 56°C to kill spores prior to shipment. The IFSA was conducted on each slide and spores were viewed as described above. Nonspecific binding of the secondary antiserum to the petroleum jelly resulted in nonspecific background fluorescence and weak fluorescence of *P. pachyrhizi* urediniospores. Therefore, spores were lifted from petroleum jelly using double-sided tape, which was then fixed onto a new glass microscope slide for IFSA.

RESULTS

Polyclonal antiserum specificity-indirect ELISA. Antisera SBR1A and SBR1B, produced against nongerminated urediniospores, reacted positively with both *P. pachyrhizi* and *P. meibomiae* urediniospores in ELISA, while antiserum SBR2, produced against intact germinated urediniospores, reacted only with *P. pachyrhizi* urediniospores. Antisera SBR3 and SBR4, produced against a crude extract of pulverized germinated urediniospores and cell walls of pulverized germinated urediniospores, respectively, reacted with urediniospores of both *Phakopsora* species, but also cross-reacted with *R. solani* and *P. graminis* f. sp. *tritici*. None of the antisera reacted with *B. cinerea*, *C. acutatum*, *C. gloeosporioides, U. tritici*, the four bacterial pathogens, or leaf extracts of any of the 183 healthy soybean cultivars tested (Table 2).

Immunofluorescence spore assay optimization. Both primary antisera (SBR1A and SBR2) reacted with urediniospores of *P. pachyrhizi*; however, urediniospores treated with SBR1A were brighter, with an immunofluorescence score of 4, than those treated with SBR2, with an immuno-

fluorescence score of 3 (Table 3). PBS-Tween was more effective than PBS as a dilution buffer and wash buffer, resulting in more intense fluorescence of urediniospores (intensity score of 4) than for PBS (intensity score of 2). All of the dilutions of the primary and secondary antiserum resulted in bright fluorescence with urediniospores of *P. pachyrhizi*. Increasing the time of the primary antiserum incubation from 1 to 2 h increased the fluorescence of urediniospores of *P. pachyrhizi*, but increasing the incubation time from 2 to 3 h did not increase the fluorescence intensity. Fluorescence intensity of *P. pachyrhizi* urediniospores did not differ with secondary antibody incubation time. An incubation temperature of 37°C for the primary and secondary antiserum resulted in a weak fluorescence (intensity score of 2) compared to fluorescence at 22°C (intensity score of 4). Inclusion of a blocking step (PBS + 2% BSA + 5% goat serum) for 1 h prior to the primary antiserum incubation step at 22°C did not improve fluorescence intensity compared to the nonblocked control.

The final IFSA protocol chosen was as follows: a 500-µl aliquot of SBR1A primary antiserum diluted 1:500 in PBS-Tween was placed over a sample affixed to a thin layer of petroleum jelly or double-sided tape on a standard glass microscope slide and incubated for 2 h. The slides were then washed six times with PBS-Tween, and 500 µl of a 1:500 dilution of Alexa Fluor 488 in PBS-Tween was added to the slides and incubated for 1 h. Slides were washed six times as described above. All incubation steps were carried out in the dark at 22°C.

Significantly fewer urediniospores were lost during IFSA when samples were affixed to microscope slides directly using double-sided tape or petroleum jelly than when they were captured on petroleum jelly and transferred to tape. On average, 25.0% of urediniospores were lost when transferred to tape from petroleum jelly-coated slides, while only 3.7 and 5.2% of urediniospores affixed directly to tape or petroleum jelly, respectively, were lost. Nonspecific binding of the secondary antiserum to the petroleum jelly resulted in background fluorescence and weak specific fluorescence of *P. pachyrhizi* urediniospores (Fig. 2).

Polyclonal antiserum specificity-IFSA. Fluorescent signals were observed over the entire surface of *P. pachyrhizi* urediniospores, with small patches of greater intensity often observed (Fig. 1D). Urediniospores of all of the 22 *P. pachyrhizi* isolates tested produced high fluorescence intensity scores (scores for all isolates = 4) with SBR1A antiserum in the IFSA (Table 4). Urediniospores of the *P. meibomiae* isolate PR 76 reacted positively with SBR1A antiserum, but the signal was weaker (intensity score of 3) than observed for urediniospores of *P. pachyrhizi*. None of the spores of nontarget species, including *Ustilago* and *Puccinia* spp., reacted positively (Table 5; Fig. 3).

Immunomonitoring of field-trapped spores. Thirty-six samples (microscope slides) were received from air samplers stationed in Georgia, Kentucky, and Ohio. Under bright field microscopy, a wide range of airborne spores, pollen, bacteria, insect, and particulate material was observed on each of the double-sided tape-affixed and petroleum jelly-coated glass microscope slides from the field spore traps (Fig. 4). *P. pachyrhizi* urediniospores could not be easily distinguished from other fungal spores trapped on the slides using bright field microscopy. In some cases, urediniospores were distorted by desiccation (Fig. 4A and E). After each slide was immunolabeled, *P. pachyrhizi* urediniospores fluoresced brightly (Fig. 4B, D, and F) while other fungal spores, pollen grains, bacteria, insects, and particulate material did not fluoresce. *P. pachyrhizi* urediniospores were often observed in clumps on the microscope slides. Of the 36 slides received, two were positive for *P. pachyrhizi* urediniospores from Georgia (GA12 and GA15), one from Kentucky (KY3), and six from Ohio (OH6, OH7, OH8, OH11, OH12, and OH16) (Table 6).

Table 5. Specificity of polyclonal antiserum SBR1A against fungal spores in an immunofluorescence spore assay (IFSA)

Fungal pathogen	Host	Reactivity in IFSA[a]
Phakopsora pachyrhizi isolate TW 72-1	Soybean (*Glycine max*)	4
Phakopsora meibomiae isolate PR 76	Soybean (*Glycine max*)	3
Puccinia graminis f. sp. *tritici*	Wheat (*Triticum aestivum*)	1
Phragmidium sp.	Rose (*Rosa* spp.)	1
Ustilago tritici	Wheat (*Triticum aestivum*)	1
Alternaria sp.	Tomato (*Solanum lycopersicum*)	1
Botrytis cinerea	Tomato (*Solanum lycopersicum*)	1

[a] Fluorescence of urediniospores was rated visually using a scale of 1 to 4 where 1 = no fluorescence, 2 = weak fluorescence, 3 = good fluorescence (clearly positive), and 4 = bright fluorescence.

Table 6. Soybean rust immunofluorescence spore assay (IFSA) results for air-trapped spores on slides from Georgia, Kentucky, and Ohio, 2006

Slide identification	Trapping date	Slide type	Soybean rust spores in IFSA[a]
GA 1	08.16.2006-08.23.2006	Double-sided tape	1
GA 2	08.02.2006-08.09.2006	Double-sided tape	1
GA 3	08.15.2006-08.22.2006	Double-sided tape	1
GA 4	08.01.2006-08.08.2006	Double-sided tape	1
GA 5	07.31.2006-08.07.2006	Double-sided tape	1
GA 6	08.14.2006-08.21.2006	Double-sided tape	1
GA 7	07.31.2006-08.07.2006	Double-sided tape	1
GA 8	08.14.2006-08.21.2006	Double-sided tape	1
GA 9	08.01.2006-08.08.2006	Double-sided tape	1
GA 10	08.10.2006-08.17.2006	Double-sided tape	1
GA 11	08.10.2006-08.17.2006	Double-sided tape	1
GA 12	08.02.2006-08.09.2006	Double-sided tape	4
GA 13	08.16.2006-08.23.2006	Double-sided tape	1
GA 14	08.15.2006-08.22.2006	Double-sided tape	1
GA 15	08.01.2006-08.08.2006	Double-sided tape	4
GA 16	08.10.2006-08.17.2006	Double-sided tape	1
KY 1	07.28.2006-08.04.2006	Double-sided tape	1
KY 2	08.04.2006-08.11.2006	Double-sided tape	1
KY 3	07.11.2006-07.21.2006	Double-sided tape	4
KY 4	08.21.2006-08.25.2006	Double-sided tape	1
OH 1	09.01.2006-09.08.2006	Petroleum jelly	1
OH 2	08.24.2006-08.30.2006	Petroleum jelly	1
OH 3	10.10.2006-10.17.2006	Petroleum jelly	1
OH 4	09.09.2006-09.16.2006	Petroleum jelly	1
OH 5	09.16.2006-09.23.2006	Petroleum jelly	1
OH 6	07.25.2006-07.31.2006	Petroleum jelly	3
OH 7	No date provided	Petroleum jelly	4
OH 8	No date provided	Petroleum jelly	4
OH 9	09.29.2006-10.05.2006	Petroleum jelly	1
OH 10	08.21.2006-08.23.2006	Double-sided tape	1
OH 11	No date provided	Double-sided tape	4
OH 12	09.05.2006-09.11.2006	Double-sided tape	4
OH 13	09.11.2006-09.18.2006	Double-sided tape	1
OH 14	09.05.2006-09.11.2006	Double-sided tape	1
OH 15	09.25.2006-10.03.2006	Double-sided tape	1
OH 16	10.03.2006-10.10.2006	Double-sided tape	4

[a] Fluorescence of urediniospores was rated visually using a scale of 1 to 4 where 1 = no fluorescence, 2 = weak fluorescence, 3 = good fluorescence (clearly positive), and 4 = bright fluorescence.

DISCUSSION

The ability to detect and quantify targeted airborne fungal spores rapidly and reliably will be useful in research and critical to the accuracy of disease-forecasting systems. Serological methods are potentially useful for detecting and quantifying airborne bioparticles (8,10,13,25). However, although immunological methods have been used to detect airborne allergens (32), there have been only a few applications for detection and identification of airborne spores of plant pathogens. Immunofluorescence has been used to identify spores of *Botrytis cinerea* deposited on the trapping surface of a Burkard spore sampler (11). Spores were collected on modified tape in a Burkard sampler exposed in an infected onion crop and labeled directly on the tape using a *Botrytis*-specific monoclonal antibody (6). Kennedy et al. (20) also used immunofluorescence to detect *Mycosphaerella brassicicola* ascospores on Burkard spore trap tapes coated with bovine serum albumin as a support medium and blocking agent. In an alternative approach, Schmechel et al. (35) developed a prototype rotating-arm sampler, designed for use with ELISA, to detect and enumerate airborne spores. The trap was tested in wind tunnel experiments with *Alternaria brassicae* conidia utilizing a monoclonal antibody against germinating conidia of this pathogen (36,37). More recently, a new particle-trapping device, the microtiter immunospore trap, was developed by the Burkard Manufacturing Co., Rickmansworth, UK (19). The device uses a suction system to collect airborne particles directly into the wells of a microtiter plate. Kennedy et al. (19) demonstrated the utility of this device for detecting airborne spores of *M. brassicicola* and *B. cinerea* by ELISA. We found in this study that *P. pachyrhizi* urediniospores captured in a passive air sampler could be quickly and easily identified. The advantage of immunofluorescence over ELISA is that immunolabeled spores can be directly visualized and counted. Further, a passive air sampler such as the one used in this study is simple, inexpensive, and fully adapted to urediniospore capture on slides placed inside.

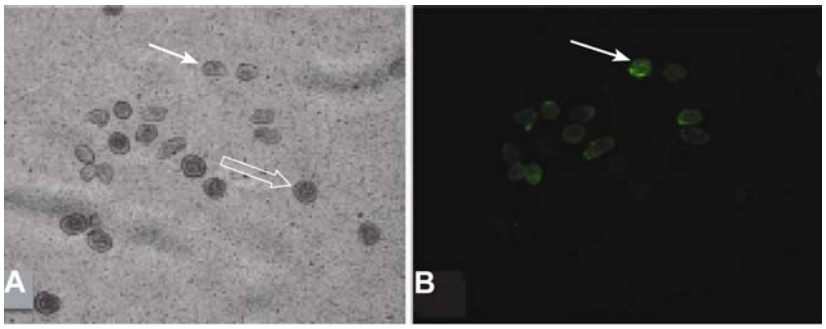

Fig. 3. Visualization of *Phakopsora pachyrhizi* (example shown by solid arrows) and *Puccinia graminis* f. sp. *tritici* (example shown by open arrow) with double-sided tape using **A,** bright field microscopy, and **B,** immunofluorescence spore assay (IFSA) with polyclonal antiserum SBR1A (20× objective).

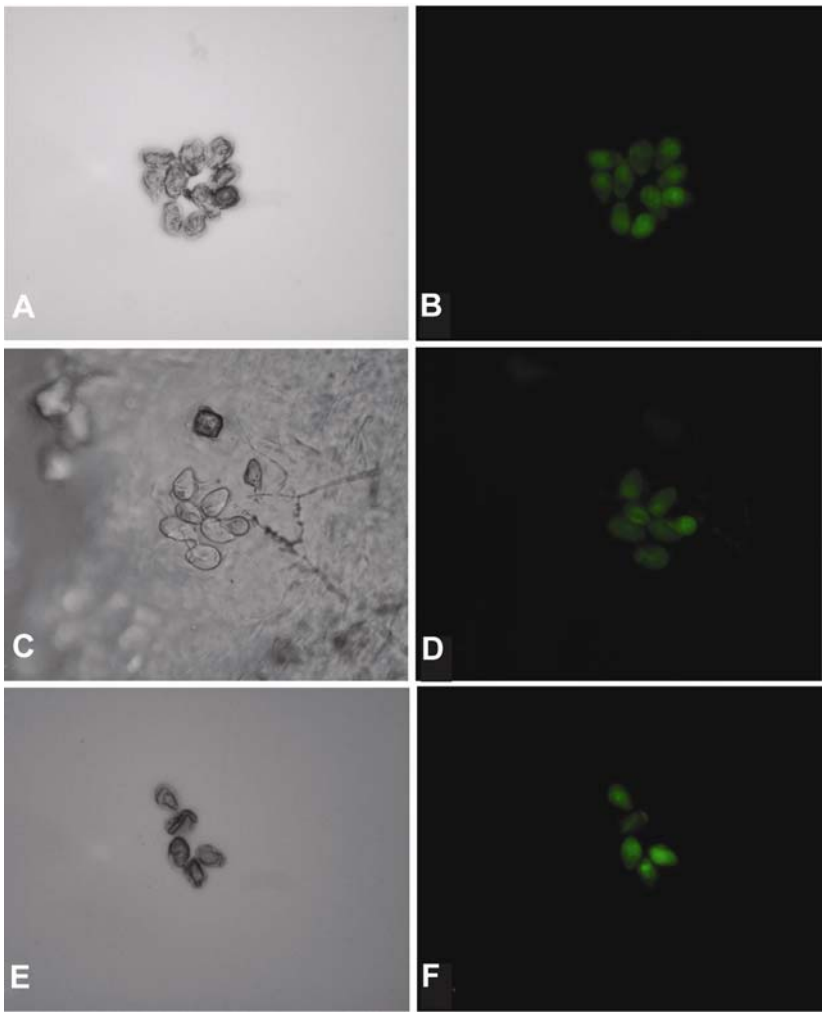

Fig. 4. Immunofluorescence spore assay (IFSA) of field-trapped soybean rust urediniospores on glass microscope slides with double-sided tape. Images in the left column were taken using bright field microscopy; images in the right column were taken of the same slides using epifluorescence microscopy. Field samples were from Ohio **(A and B)**, Kentucky **(C and D)**, and Georgia **(E and F)** (20× objective).

The polyclonal antisera developed to different fungal immunogen preparations displayed a range of specificity. The most specific antisera were developed against pulverized, nongerminated urediniospores of *P. pachyrhizi*. This approach may be particularly appropriate for an immunofluorescence assay because it appears to enrich for antibodies to urediniospore surface epitopes. Meyer and Dewey (27) demonstrated that genus-specific monoclonal antibodies capable of binding surface epitopes of *Botrytis cinerea* conidia were most reactive in ELISA and immunofluorescence assays.

We found that the efficiency of visualization of immunofluorescent urediniospores on microscope slides affixed with double-sided tape was substantially superior to that of slides coated with petroleum jelly. In experiments in which the primary antiserum was omitted, the secondary antiserum conjugated with Alexa Fluor 488 appeared to bind nonspecifically with urediniospores on petroleum jelly-coated slides (data not shown). It is likely that the petroleum jelly interfered with effective washing of the secondary antibody. While retention of urediniospores on tape and petroleum jelly throughout IFSA was similar (approximately 95 to 97% retention), transferring urediniospores from petroleum jelly to tape resulted in significant losses of spores (approximately

75% retention). The air samplers currently being used for *P. pachyrhizi* urediniospores sampling were originally designed to use petroleum jelly-coated slides, but our results clearly demonstrate that double-sided tape is the superior urediniospore capture material where IFSA will be used. Preliminary results from field tests with double-sided tape or petroleum jelly-coated glass microscope slides collected from field tests in 2006 demonstrate that the IFSA combined with spore trapping can be an effective tool for monitoring *P. pachyrhizi* urediniospores movement during the soybean growing season.

In future studies, we will investigate the use of monoclonal antibodies specific to the urediniospores of *P. pachyrhizi*. The monoclonal antibodies will be assessed in the test format described above, and the potential for a continuous detection assay will also be investigated. It is clear that immunological techniques have the potential to play a significant role in the detection and monitoring of airborne plant pathogens such as the soybean rust pathogen, *P. pachyrhizi*.

ACKNOWLEDGMENTS

We thank Tea Meulia for assistance with fluorescent microscopy techniques, and Ohio Agricultural Research and Development Center Molecular and Cellular Imaging Center for providing microscopy and image analysis facilities. This project was sponsored through soybean check-off dollars from Iowa Soybean Association and United Soybean Board. Salaries and research support provided by state and federal funds appropriated to the Ohio Agricultural Research and Development Center, The Ohio State University. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture or The Ohio State University.

LITERATURE CITED

1. Akinsanmi, O. A., Ladipo, J. L., and Oyekan, P. O. 2001. First report of soybean rust (*Phakopsora pachyrhizi*) in Nigeria. Plant Dis. 85:97.
2. Asian Vegetable Research and Development Center. 1987. Bibliography of soybean rust, 1985–1986. AVRDC Publication No. 87-277. Shanhua, Tainan, Taiwan, ROC.
3. Asian Vegetable Research and Development Center. 1992. Annotated bibliography of soybean rust (*Phakopsora pachyrhizi* Sydow). AVRDC Publication No. 92:372. Shanhua, Tainan, Taiwan, ROC.
4. Baysal, F., Dorrance, A., Ivey, M. L. L., Luster, D., Frederick, R., Czarnecki, J., Boehm, M., and Miller, S. A. 2007. An immunofluorescence assay to detect soybean rust urediniospores. (Abstr.) Phytopathology 97:S9.
5. Beck, J., Ligon, J. J., Ettienne, L., and Binder, A. 1996. Detection of crop fungal pathogens by polymerase chain reaction technology. Pages 111-118 in: Diagnostics in Crop Production. BCPC Symposium No. 65. G. Marshall, ed. British Crop Protection Council, Farnham, UK.
6. Bossi, R., and Dewey, F. M. 1992. Development of a monoclonal antibody-based immunodetection assay for *Botrytis cinerea*. Plant Pathol. 41:472-482.
7. Bradford, M. M. 1976. A rapid and sensitive method for the estimation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248-254.
8. Burge, H. A., and Solomon, W. R. 1987. Sampling and analysis of biological aerosols. Atmos. Environ. 21:451-456.
9. Cherry, E., and Peet, C. 1966. An efficient device for the rapid collection of fungal spores from infected plants. Phytopathology 56:1102-1103.
10. DeCosemo, G. A. L., Stewart, I. W., Griffiths, W. D., and Deans, J. S. 1992. The assessment of airborne microorganisms. J. Aerosol Sci. 23:S683-686.
11. Dewey, F. M. 1996. Production and use of monoclonal antibodies for the detection of fungi. Pages 85-91 in: Diagnostics in Crop Production. BCPC Symposium Proceedings No. 65. G. Marshall, ed. British Crop Protection Council, Farnham, UK.
12. Dorrance, A. E., Draper, M. A., and Hershman, D. E., eds. 2005. Using Foliar Fungicides to Manage Soybean Rust. NC-504 Land Grant Universities Cooperating. Bull. SR-2005.
13. Flannigan, B. 1997. Air sampling for fungi in indoor environments. J. Aerosol Sci. 28:381-392.
14. Frederick, R. D., Snyder, C. L., Peterson, G. L., and Bonde, M. R. 2002. Polymerase chain reaction assays for the detection and discrimination of the soybean rust pathogens *Phakopsora pachyrhizi* and *P. meibomiae*. Phytopathology 92:217-227.
15. Hirst, J. M. 1953. Changes in Atmospheric Spore Content: Diurnal Periodicity and the Effects of Weather. Trans. Br. Mycol. Soc. 36:375-392.
16. Isard, S. A., Russo, J. M., and DeWolf, E. D. 2006. The establishment of a national Pest Information Platform for Extension and Education. Plant Health Progress Online: doi:10.1094/PHP-2006-0915-01-RV.
17. Jamaux, I., and Spire, D. 1994. Development of a polyclonal antibody-based immunoassay for the early detection of *Sclerotinia sclerotiorum* in rapeseed petals. Plant Pathol. 43:847-862.
18. Jost, P., Harris, G., Harrison, K., Kemerait, R., McPherson, R., Prostko, E., Roberts, P., Raymer, P., Shumaker, G., and Sumner, P. 2005. Georgia Soybean Production Guide. University of Georgia Cooperative Extension Bull. CSS-05-02.
19. Kennedy, R., Wakeham, A. J., Byrne, K. G., Meyer, U. M., and Dewey, F. M. 2000. A new method to monitor airborne inoculum of the fungal plant pathogens *Mycosphaerella brassicicola* and *Botrytis cinerea*. Appl. Environ. Microbiol. 66:2996-3000.
20. Kennedy, R., Wakeham, A. J., and Cullington, J. E. 1999. Production and immunodetection of ascospores of *Mycosphaerella brassicicola*: Ringspot of vegetable crucifers. Plant Pathol. 48:297-307.
21. Levy, C. 2003. Measures to control soybean rust in Southern Africa and an initial investigation of the meteorological factors that favor its development. (Abstr.) Phytopathology 93:S103.
22. Lewis Ivey, M. L., Nava Diaz, C., and Miller, S. A. 2004. Identification and management of *Colletotrichum acutatum* on immature bell peppers. Plant Dis. 88:1198-1204.
23. Livingston, M., Johnson, R., Daberkow, S., Roberts, M., Ash, M., and Breneman, V. 2004. Economic and policy implications of windborne entry of Asian soybean rust into the United States. USDA Economic Research Service OCS-04D-42. http://www.ers.usda.gov.
24. Markwell, M. A., Haas, S. M., Tolbert, N. E., and Bieber, L. L. 1981. Protein determination in membrane and lipoprotein samples: Manual and automated procedures. Methods Enzymol. 72:296-303.
25. McCartney, H. A., Fitt, B. D. L., and Schmechel, D. 1997. Sampling bioaerosols in plant pathology. J. Aerosol Sci. 28:349-364.
26. Melching, J. S., Bromfield, K. R., and Kingslover, C. H. 1983. The plant pathogen containment facility at Frederick, Maryland. Plant Dis. 67:717-722.
27. Meyer, U., and Dewey, F. M. 2000. Efficacy of different immunogens for raising monoclonal antibodies to *Botrytis cinerea*. Mycol Res 104:979-987.
28. Miles, M. R., Hartman, G. L., and Frederick, R. D. 2003. Soybean rust: Is the U.S. crop at risk? American Phytopathological Society. Online feature. http://www.apsnet.org/online/feature/rust.
29. Miles, M. R., Hartman, G. L., Levy, C., and Morel, W. 2003. Current status of soybean rust control by fungicides. Pestic. Outlook 14:197-200.
30. Miles, M. R., Levy, C., Morel, W., Mueller, T., Steinlage, T., van Rij, N., Frederick, R. D., and Hartman, G. L. 2007. International fungicide efficacy trials for the management of soybean rust. Plant Dis. 91:1450-1458.
31. Morel, W. P. 2001. Roya de la soja. Ministerio de Agricultura y Ganaderia, Subsecretaria de Agricultura, Direccion de Investigacion Agricola, Centro Regional de Investigacion Agricola-CRIA, Capitan Miranda, Itapua, Paraguay. Comunicado Tecnico-Report Official, Serie Fitopathologia, 1, Junio de 2001.
32. Mullins, J., and Emberlin, J. 1997. Sampling pollens. J. Aerosol Sci. 28:365-370.
33. Pretorius, Z. A., Kloppers, F. J., and Frederick, R. D. 2001. First report of soybean rust in South Africa. Plant Dis. 85:1288.
34. Rossi, R. L. 2003. First report of *Phakopsora pachyrhizi*, the causal organism of soybean rust in the province of Misiones, Argentina. Plant Dis. 87:102.
35. Schmechel, D., McCartney, H. A., and Halsey, K. 1994. The development of immunological techniques for the detection and evaluation of fungal disease inoculum in oilseed rape crops. Pages 247-253 in: Modern Detection Methods for Plant Pathogenic Fungi: Identification, Detection and Quantification. A. Schots, F. M. Dewey, and R. Oliver, eds. CAB International, Wallingford, UK.
36. Schmechel, D., McCartney, H. A., and Magan, N. 1996. A novel approach for immunomonitoring air-borne fungal pathogens. Pages 93-98 in: Diagnostics in Crop Production. BCPC Symposium Proceedings No. 65. G. Marshall, ed. British Crop Protection Council, Farnham, UK.
37. Schmechel, D., McCartney, H. A., and Magan, N. 1997. The production and characterization of monoclonal antibodies against *Alternaria brassicae* (Berk.) Sacc., the cause of dark leaf and pod spot in oilseed rape. Food Agric. Immunol. 9:219-232.
38. Schneider, R. W., Hollier, C. A., Whitam, H. K., Palm, M. E., McKemy, J. M., Hernández, J. R., Levy, L., and DeVries-Paterson, R. 2005. First report of soybean rust caused by *Phakopsora pachyrhizi* in the continental United States. Plant Dis. 89:774.
39. U.S. Department of Agriculture. 2007. Pest information platform for extension and education. Available April 1, 2007, from http://www.sbrusa.net/cgi-bin/sbr/public.cgi.
40. Werres, S., and Steffens, C. 1994. Immunological techniques used with fungal plant pathogens: Aspect of antigens, antibodies and assays for diagnosis. Ann. Appl. Biol. 125:615-643.
41. Yang, X. B., and Pan, Z. 2007. Soybean rust update and outlook, June 2007. Integrated Crop Management, the IC-498(14).
42. Yorinori, J. T., Paiva, W. M., Frederick, R. D., Costamilan, L. M., Bertagnolli, P. F., Hartman, G. E., Godoy, C. V., and Nunes, J., Jr. 2005. Epidemics of soybean rust (*Phakopsora pachyrhizi*) in Brazil and Paraguay from 2001 to 2003. Plant Dis. 89:675-677.