# A Multiplexed Immunofluorescence Method Identifies *Phakopsora pachyrhizi* Urediniospores and Determines Their Viability

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## ABSTRACT

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Soybean rust, caused by *Phakopsora pachyrhizi*, occurs concomitantly wherever soybean is grown in the tropical and subtropical regions of the world. After reports of its first occurrence in Brazil in 2001 and the continental United States in 2004, research on the disease and its pathogen has greatly increased. One area of research has focused on capturing urediniospores, primarily by rain collection or wind traps, and detecting them either by microscopic observations or by immunological or molecular techniques. This system of detection has been touted for use as a potential warning system to recommend early applications of fungicides. One shortcoming of the method has been an inability to determine whether the spores are viable. Our study developed a method to detect viable *P. pachyrhizi* urediniospores using an immunofluorescence assay combined with propidium iodide (PI) staining. Antibodies reacted to *P. pachyrhizi* and other *Phakopsora* spp. but did not react with other common soybean pathogens or most other rust fungi tested, based on an indirect immunofluorescence assay using fluorescein isothiocyanate-labeled secondary antibodies. Two vital staining techniques were used to assess viability of *P. pachyrhizi* urediniospores: one combined carboxy fluorescein diacetate (CFDA) and PI, and the other utilized (2-chloro-4-[2,3-dihydro-3-methyl-(benzo-1,3-thiazol-2-yl)-methylidene]-1-phenylquinolinium iodide] (FUN 1). Using the CFDA-PI method, viable spores stained green with CFDA and nonviable spores counterstained red with PI. Using the FUN 1 method, cylindrical intravacuolar structures were induced to form within metabolically active urediniospores, causing them to fluoresce bright red to reddish-orange, whereas dead spores, with no metabolic activity, had an extremely diffused, faint fluorescence. An immunofluorescence technique in combination with PI counterstaining was developed to specifically detect viable *P. pachyrhizi* urediniospores. The method is rapid and reliable, with a potential for application in forecasting soybean rust based on the detection of viable urediniospores.

Soybean rust, caused by the obligate phytopathogenic fungus *Phakopsora pachyrhizi* Syd. & P. Syd., is a major foliar disease of soybean (*Glycine max* (L.) Merr.), reducing soybean yields in many soybean-growing areas throughout the world (19). The pathogen was first reported on *Pachyrhizus erosus* (yam bean, jicama) in Japan in 1902 (20), and documentation of its chronological movement to other global regions includes notable first reports in Brazil in 2001 (53) and the continental United States in 2004 (41), the two countries with the largest soybean production area in the world (15). A second, less-aggressive species, *Phakopsora meibomiae*, also causes soybean rust in Caribbean countries and other countries in the western hemisphere, including Brazil (37), but has not yet been reported in the continental United States.

The most common symptoms of soybean leaf rust are tan to dark-brown or reddish-brown lesions which contain one to many erumpent, globose uredinia, particularly on the undersides of the leaflets (17). Based on disease progress curves, the disease increases dramatically on soybean during the reproductive growth stages and can induce early maturation and defoliation (18). The primary means of managing soybean rust has been with the use of fungicides. Fungicide chemistry, timing, and mode of application have been shown to be critical to reduce disease severity (33,35). Host plant resistance has been another potential management tool, and sources of resistance and breeding for resistance have been reviewed (15,16).

Epidemics of soybean rust appear to be dependent upon a number of factors, including weather conditions, host availability, and overwintering of the fungus on a host that retains foliar vegetation between annual cropping cycles. North American epidemics present interesting questions about *P. pachyrhizi* survival because most of the soybean production is in the temperate zone, where all known hosts die out or die back each year. There is no known alternate host known for *P. pachyrhizi* to overwinter. Since the introduction of the pathogen into the United States in 2004, the fungus perishes annually on frost-sensitive hosts in the temperate region, surviving only in host foliage in warmer, more frost-free southern and coastal areas of the United States and farther south in other subtropical areas. The fungus is known to survive on kudzu (*Pueraria lobata*) (12) but the frost line in the United States varies each year, resulting in unpredictable sources of overwintering inoculum from that host. The fungus relies upon aerial dispersion of urediniospores from overwintering sites in subtropical areas to infect soybean grown in areas to the north. Early detection of *P. pachyrhizi* urediniospores in soybean-production regions is important for predicting the occurrence of soybean rust and for timing fungicide applications to minimize losses (8). To aid in early detection, an online national warning system was designed to monitor sentinel plots across the United States to detect *P. pachyrhizi* urediniospores, predict the disease-spread across the United States, and help growers make disease management decisions, thereby minimizing the losses caused by rust (22,26).

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*The *e*-Xtra logo stands for "electronic extra" and indicates Figures 1, 2, and 5 appear in color online.

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Quantitative polymerase chain reaction (qPCR) assays have been previously reported which specifically detect and quantify DNA from *P. pachyrhizi* and *P. meibomiae* (13). The assays utilize oligonucleotide primers and a fluorogenic 5′-exonuclease linear hydrolysis probe designed to the nucleotide sequence of the 5.8S and internal transcribed spacer 2 region of the nuclear rRNA genes, and can also discriminate between *P. pachyrhizi* and *P. meibomiae*. An immunofluorescence assay utilizing specific polyclonal antibodies (pAbs) has also been previously developed to detect and identify *P. pachyrhizi* urediniospores captured on glass slides obtained from passive air-sampling systems (3). The qPCR and the immunofluorescence assays can detect airborne urediniospores captured by atmospheric sampling of the air or rainwater collection, and can be effective tools for monitoring the movement of the spores during the soybean-growing season. However, these assays do not determine the viability of the urediniospores they detect and, because spore viability is required for disease development, mere detection of urediniospores might elicit hasty management measures, incurring additional costs that ultimately prove unwarranted.

A variety of fluorometric methods to differentiate living from dead microorganisms are available (4), based upon viability indicators, including membrane integrity, enzyme activities, respiration, membrane potential, and intracellular pH. Rotman and Papermaster (40) reported that fluorescein diacetate (FDA), a nonfluorescent fatty acid ester, readily entered into living cells, where it was rapidly hydrolyzed by the esterases, yielding free fluorescein. The polar fluorescein did not exit the cell as fast as the nonpolar FDA entered and, therefore, it accumulated intracellularly and made the cells highly fluorescent. In viable cells with intact membranes and active metabolism, fluroescein produces bright green fluorescence when excited by blue light, in contrast to dead cells, which remain nonfluorescent. Propidium iodide (PI) (2,7-diamino-9-phenyl-10-[diethyl aminopropyl] phen-anthridinium iodide methiodide), is a polar, fluorescent molecule that passes through damaged cell membranes and binds to DNA and RNA by intercalating between the nucleotides with little or no sequence preference. Once bound to nucleic acid, PI fluorescence is enhanced 20- to 30-fold, producing bright red fluorescence. In viable cells, PI is excluded by intact cell membranes and, thus, PI is an effective counterstain to identify nonviable cells. PI is used as a DNA stain in flow cytometry to evaluate the DNA content in cells (30) and, in epifluorescence microscopy, as a nuclear marker (36) and to evaluate cell viability (51). The paired use of FDA and PI has been described (28) to determine vitality of spleen cells in mouse, where the viable cells fluoresced bright green and nonviable cells fluoresced bright red.

The use of 2-chloro-4-(2,3-dihydro-3-methyl-[benzo-1,3-thiazol-2-yl]-methylidene)-1-phenylquinolinium iodide (FUN 1) as a vital stain was first reported with yeast cells using flow cytometry (50). A more detailed quantitative study on its use as a fluorescent probe for vacuole labeling and viability testing of yeasts was reported using several techniques, including fluorometry, flow cytometry, and wide-field and confocal laser-scanning fluorescence microscopy (34). Free FUN 1 is a nonfluorescent molecule in aqueous solution. In metabolically active cells with intact membranes, cylindrical intravacuolar structures (CIVS) are produced after less than 1 h of exposure to FUN 1. These structures move within the vacuolar space and fluoresce orange-red when excited at 470 to 590 mm. In membrane-compromised dead cells, FUN 1 produces green to green-yellow fluorescence; cells with intact membranes and little or no metabolic activity have a diffuse green cytoplasmic fluorescence and lack fluorescent intravacuolar bodies. Calcofluor white M2R, the disodium salt of 4,4′-bis-(4 anilino-bis-diethyl amino-S-triazin-2-y1amino)-2,2′-stilbene disulfonic acid, is an ultraviolet-excitable dye used as a marker of fungal cell walls. It binds to cellulose, chitin, carboxylated polysaccharides, and a variety of other β-linked polymers (23,25,31). FUN 1 has been used to study the viability of cells, either as a single dye (10,11,24) or in combination with calcofluor white M2R counterstaining (21).

The objectives of this study were to (i) develop an immunofluorescence assay for specific detection of *P. pachyrhizi* urediniospores using monoclonal antibodies (mAbs) and pAbs; (ii) develop methods to differentiate viable and nonviable *P. pachyrhizi* urediniospores using 5-carboxyfluorescein diacetate (CFDA), PI, FUN 1, and calcofluor white M2R; and (iii) integrate the immunofluorescence assay with viability staining technique to specifically detect viable *P. pachyrhizi* urediniospores.

## MATERIALS AND METHODS

**Rust isolate.** Urediniospores of isolate FL-07-1 were used for immunization and the development of immunofluorescence and viability assays. This particular U.S. isolate has been used for a number of other studies related to fungal biology (49) and host resistance (38). Urediniospores were increased using the detached-leaf method (47). Leaflets from trifoliolates of 4-week-old soybean plants ('Williams 82') were collected and transferred to petri plates containing 1.5% (wt/vol) water agar medium amended with 6-benzylaminopurine (0.015%, wt/vol) with the abaxial surface of the leaf facing up. These detached leaves were spray inoculated with freshly collected urediniospores using an airbrush (Paasche Airbrush Co., Chicago). The plates were then incubated for 12 h in a tissue chamber (Percival Scientific Inc., Perry, IA) at 20 to 22°C in the dark, after which they were incubated at 20 to 22°C in a 12-h light (380 μmol $m^{-2}$ $s^{-1}$)-and-dark cycle. Lesions with sporulating uredinia formed 2 weeks after inoculation.

**Urediniospore samples.** Urediniospores were collected from inoculated detached soybean leaves 14 to 21 days after inoculation using a vacuum-type spore collector (Barnant, Barrington, IL). To test the various assays, urediniospores were freshly collected (labeled as live spores), collected and heat-killed in a dry bath incubator at 55°C for 10 h (labeled as heat-killed spores), or mixed 1:1 (equal proportions of live and heat-killed spores). The spores were suspended in phosphate-buffered saline (PBS) containing 1% (vol/vol) Tween 20 (PBST) at a concentration of $5 \times 10^4$ spores/ml.

**Production of mAbs and pAbs.** Five adult female Balb/C mice were injected intraperitoneally with 200 μl of intact *P. pachyrhizi* urediniospores ($5 \times 10^5$ spores/ml in 50% [vol/vol] Freund's complete adjuvant [FCA]). After two more immunizations (2 weeks apart), a test bleed was taken and analyzed for immunoreactivity toward *P. pachyrhizi* spores using plate-trapped anitgen enzyme-linked immunosorbent assay (PTA-ELISA) (43). Three weeks later, one mouse was selected and a booster injection (200 μl of $5 \times 10^5$ spores/ml) was administered. Three days later, the mouse was sacrificed, and lymphocytes harvested from the spleen were fused with the X63Ag8.653 myeloma cell line to immortalize them. Stable hybridoma cell lines were selected and isotyping of the mAbs was performed using rabbit anti-mouse immunoglobulin (Ig)G1, IgG2a, IgG2b, IgG3, and IgM antibodies. The antibodies were purified and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis for purity and by PTA-ELISA for antibody activity, and stored at –80°C.

Two female New Zealand white rabbits weighing 1 to 2 kg were immunized by subcutaneous injection with 200 μl of intact *P. pachyrhizi* urediniospores ($5 \times 10^5$ spores/ml in 50% [vol/vol] FCA). Animals were boosted at 21-day intervals with the same immunogen suspended in a mixture of 0.5 ml of phosphate buffer and 0.5 ml of incomplete Freund's adjuvant. Ten days after the third boost, blood was obtained by bleeding the ear vein of the rabbits. Whole blood was collected from the ear vein of the rabbits and by intracardiac puncture 10 days after the fourth injection. Blood samples were allowed to coagulate overnight at 4°C. Then, the serum was separated by centrifugation, and a fraction

was diluted 1 to 5 with 10 mM PBS (137 mM sodium chloride, 2.7 mM potassium chloride, 10 mM sodium hydrogen phosphate, and 2 mM potassium dihydrogen phosphate, pH 7.4) containing 0.01% thimerosal (wt/vol) and kept at 4°C for daily usage. The remainder of each antiserum was purified using saturated ammonium sulfate precipitation, followed by dialysis against 10 mM PBS in 10 liters at 4°C with three buffered changes during 24 h. The purified pAb (Pp-pAb) was stored at –80°C.

**PTA-ELISA.** In all, 100 µl of *P. pachyrhizi* urediniospore suspension ($5 \times 10^5$ spores/ml) in 10 mM PBS was added to each well in 96-well Nunc MaxiSorp plates (Fisher Scientific) and incubated overnight at 37°C. The wells were washed with PBS and blocked with 200 µl of 2.5% (wt/vol) bovine serum albumen in PBS for 2 h at 37°C. Wells were incubated first with 100 µl of primary antibody [pAb or hybridoma supernatant, diluted 1:10 or 1:1, respectively, in PBST] and then with 100 µl of horseradish peroxidase-conjugated goat anti-rabbit antibody diluted 1:1,000 in PBST. Both steps were performed for 1 h at room temperature. After each step, the plates were washed with PBST. Freshly made substrate solution (100 µl) containing 3,3′,5,5′-tetramethylbenzidine was added to each well and the reaction was stopped by adding 100 µl of 2 N sulfuric acid. The absorbance was measured at 450 nm using a uQuant (BioTek Instruments) reader.

**Indirect immunofluorescence assay optimization.** Several parameters were evaluated to optimize the immunofluorescence staining procedure, including (i) antibody working dilutions (10, 25, 50, and 100 µg/ml) of Pp-mAb and Pp-pAb, (ii) working dilutions (1:100, 1:150, and 1:200) of secondary antibodies (goat anti-mouse or anti-rabbit fluorescein isothiocyanate [FITC]-labeled secondary antibody solution to detect Pp-mAb or Pp-pAb, respectively), (iii) primary and secondary antibody incubation time (30, 60, and 120 min), (iv) primary and secondary antibody incubation temperature (25 and 37°C), and (v) type of washing buffer (PBS, PBST, and distilled water). Spores were observed using an epifluorescence microscope and rated visually using a scale of 0 to 2, where 0 = no fluorescence, 1 = weak fluorescence, and 2 = good to bright fluorescence.

**Antibody specificity and cross-reactivity studies.** Pp-mAb and Pp-pAb were tested by indirect immunofluorescence for cross-reactivity against eight other pathogenic fungi, including six soybean pathogens (Table 1). In addition, urediniospores of nine other *P. pachyrhizi* isolates (AL07-1, AR08-1, FL07-10, IL06-1, IL08-1, MS06-2, MS07-1, NC07-1, and TX07-1) (48) were also tested (Table 2).

**Direct immunofluorescence.** Pp-mAb and Pp-pAb antibodies were conjugated to FITC using the FluoroTag FITC Conjugation Kit (Sigma-Aldrich, St. Louis) according to the manufacturer's instructions. FITC (50 µg) in 0.1 M carbonate-bicarbonate buffer (pH 9.0) was added to 1 mg of Pp-mAb or Pp-pAb. The mixture was incubated for 3 h in the dark at room temperature with gentle stirring. The labeled protein (FITC-Pp-mAb and FITC-Pp-pAb) was purified from the unconjugated fluorescein using a Sephadex G-25M column, and conjugated antibodies were stored at 4°C in the dark. Live, heat-killed, and a 1:1 mix of urediniospores were

TABLE 1. Specificity of anti-rust antibodies against fungal pathogens in an indirect immunofluorescence assay

| Fungal pathogen | Fungal structure | Host | Reactivity[a] | |
|---|---|---|---|---|
| | | | Pp-mAb | Pp-pAb |
| *Colletotrichum destructivum* | Conidia | Soybean | 0 | 0 |
| *Fusarium solani* | Mycelia | Soybean | 0 | 0 |
| *Gymnosporangium* sp. | Aeciospores | Cedar | 0 | 0 |
| *Macrophomina phaseolina* | Mycelia | Soybean | 0 | 0 |
| *Phakopsora gossypii* | Urediniospores | Cotton | 1 | 1 |
| *P. meibomiae* | Urediniospores | Soybean | 2 | 2 |
| *P. pachyrhizi* | Urediniospores | Soybean | 2 | 2 |
| *P. zizyphii* | Urediniospores | Common jujube | 2 | 2 |
| *Puccinia coronata* | Urediniospores | Quack grass | 0 | 0 |
| *P. graminis* | Urediniospores | Wheat | 0 | 0 |
| *P. polysora* | Urediniospores | Corn | 1 | 1 |
| *P. sorghii* | Urediniospores | Corn | 0 | 0 |
| *P. striiformis* | Urediniospores | Wheat | 0 | 1 |
| *P. triticina* | Urediniospores | Wheat | 0 | 0 |
| *Sclerotinia sclerotiorum* | Mycelia | Soybean | 0 | 0 |
| *Ustilago maydis* | Teliospores | Corn | 0 | 0 |

[a] Mouse monoclonal (Pp-mAb) and rabbit polyclonal (Pp-pAb) antibodies were used and reactivity was detected using secondary antibodies labeled with fluorescein isothiocyanate. Fluorescence of urediniospores was rated visually using a scale of 0 to 2, where 0 = no fluorescence, 1 = weak fluorescence, and 2 = good to bright fluorescence.

TABLE 2. Reactivity of anti-rust antibodies with the urediniospores of *Phakopsora pachyrhizi* isolates in an indirect immunofluorescence assay[a]

| Isolate | Host | County | State | Region | Year | Reactivity[b] | |
|---|---|---|---|---|---|---|---|
| | | | | | | Pp-mAb | Pp-pAb |
| AL07-1 | Soybean | Baldwin | Alabama | South | 2007 | 2 | 2 |
| AR08-1 | Soybean | Crawford | Arkansas | South | 2008 | 2 | 2 |
| FL07-10 | Kudzu | Hernando | Florida | Southeast | 2007 | 2 | 2 |
| IL06-1 | Soybean | Jefferson | Illinois | Central | 2006 | 2 | 2 |
| IL08-1 | Soybean | McLean | Illinois | Central | 2008 | 2 | 2 |
| MS06-2 | Soybean | Warren | Mississippi | South | 2006 | 2 | 2 |
| MS07-1 | Soybean | Humpfreys | Mississippi | South | 2007 | 2 | 2 |
| NC07-1 | Soybean | Lenoir | North Carolina | Southeast | 2007 | 2 | 2 |
| TX07-1 | Soybean | Hidalgo | Texas | South | 2007 | 2 | 2 |
| FL07-1 | Soybean | Gadsden | Florida | Southeast | 2007 | 2 | 2 |

[a] Name, host, location, and origin and year of collection of the isolates were obtained from Twizeyimana et al. (48).

[b] Mouse monoclonal (Pp-mAb) and rabbit polyclonal (Pp-pAb) antibodies were used and reactivity was detected using secondary antibodies labeled with fluorescein isothiocyanate. Fluorescence of urediniospores was rated visually using a scale of 0 to 2, where 0 = no fluorescence, 1 = weak fluorescence, and 2 = good to bright fluorescence.

prepared as previously described. FITC-Pp-pAb or FITC-Pp-mAb diluted with PBS (pH 7.4) was added to 100 µl of spore suspension, achieving a final concentration of 50 µg/ml. The suspensions were incubated at room temperature for 1 h with gentle mixing of the suspensions at regular intervals. They were then washed thrice with 1% PBST by centrifugation at 1,500 × *g* to remove any excess unbound antibody before being observed under a microscope.

**Methods for assessing urediniospore viability.** Urediniospore viability was determined by three methods: (i) the *Funga*Light yeast vitality kit (Molecular Probes Inc., Eugene, OR) containing CFDA, acetoxymethyl ester (AM) and PI; (ii) the LIVE/DEAD yeast viability kit (Molecular Probes Inc.) containing FUN 1 and calcofluor white M2R; and (iii) an in vitro germination test. Three spore samples (live, heat-killed, and a 1:1 mix) were tested for each method.

**CFDA-PI staining.** CFDA and PI were reconstituted in dimethyl sulfoxide and working solutions of 20% CFDA and 40 µM PI were freshly prepared in 10 mM PBS so that the final concentrations of CFDA and PI were 10% and 20 µM, respectively. Three different staining combinations were tested on all three spore types (live, heat-killed, and a 1:1 mix): (i) single-color CFDA staining, 100 µl of CFDA dye to 100 µl of spore suspension; (ii) single-color PI staining, 100 µl of PI dye to 100 µl of spore suspension; and (iii) dual-color staining, 100 µl each of CFDA and PI to 100 µl of spore suspension. Each tube was vortexed gently and all the samples were incubated at room temperature in the dark for 15 min. The slides were prepared and observed under the microscope. Spores were counted as emitting green (live) or red (dead) fluorescence and the images were captured.

**FUN 1 and calcofluor white M2R staining.** Working solutions of 40 µM FUN 1 and 40 µM calcofluor white M2R were freshly prepared in 10 mM PBS so that the final concentrations of FUN 1 and calcofluor white M2R were 20 µM each, as per the manufacturers' instructions. Three different staining techniques were tested on all the three spore types (live, heat-killed, and a 1:1 mix): (i) single-color FUN 1 staining, 100 µl of FUN 1 dye to 100 µl of spore suspension; (ii) single-color calcofluor white M2R staining, 100 µl of calcofluor white M2R dye to 100 µl of spore suspension; and (iii) dual-color staining, 100 µl of each FUN 1 and calcofluor white M2R to 100 µl of spore suspension. Each tube was vortexed gently and all the samples were incubated at room temperature protected from light for 30 min. The slides were prepared and observed under the microscope and the images were captured. Spores were counted as emitting green fluorescence with bright orange-red intervacuolar structures (live) or diffused green or red fluorescence devoid of orange-red structures (dead). The bright violet-blue fluorescence corresponds to the cell wall chitin labeled with calcofluor white M2R.

**In vitro germination test.** Live, dead, and a 1:1 mixture of live and dead spores were checked for germination on 1.5% water agar (WA) medium containing 1% BAP in 96-cm petri plates. Three droplets, each containing 10 µl of spore suspension, were placed on the surface of WA medium and incubated in a tissue chamber at 22.5°C in darkness for 24 h. The number of germinated spores was counted using a hemacytometer under a SZX16 microscope (Olympus America Inc., Center Valley, PA) and percent germination was calculated.

**Differentiation of viable and heat-killed *P. pachyrhizi* urediniospores using PI in two-color immunofluorescence.** Viability of urediniospores in a mixture containing a 1:1 ratio of live and heat-killed spores was determined using PI (component B of the *Funga*Light yeast vitality kit). The spores were detected using FITC-labeled antibodies (Pp-mAb and Pp-pAb) in an indirect or direct immunofluorescence assay. A 40-µM working solution of PI was freshly prepared before use, with 100 µl added to an equal volume of the FITC-antibody-labeled urediniospores. The sample was incubated in darkness at room temperature for 15 min. Spores were observed under an epifluorescence microscope and counted as emitting green (live *P. pachyrhizi* spores) or green with red nuclei (dead *P. pachyrhizi* spores) fluorescence, and the images were captured.

**Immunomonitoring of spores trapped on glass slide.** Urediniospores from rust-infected detached leaves were collected at 20, 25, 30, 35, and 40 days after inoculation and dusted on double-sided tape affixed to standard glass microscope slides. Indirect immunofluorescence assays using Pp-mAb and PI staining were conducted on each of three slides per time period as described above. At least 25 urediniospores were counted under an epifluorescence microscope at random for fluorescence signals per microscope slide to determine spore viability. Images were captured as previously described.

**Epifluorescence microscopy.** All samples were examined using an Olympus BX51 microscope (Olympus America Inc.) fitted with appropriate filters for observing labeled urediniospores in both immunofluorescence and viability studies. Spore suspension (15 µl) was put on a microscope slide and a 22-by-22-mm cover glass was placed over it and sealed with nail polish. For each sample, at least 250 spores were counted as fluorescent or nonfluorescent. The experiment was carried out in triplicate and repeated three times. Images were captured using a Retiga 2000R camera (Qimaging, Surrey, Canada) and processed with QCapture Ver. 3.1.1. Spores were first observed under bright-field settings followed by viewing under epifluorescence. Spores labeled with FITC antibodies were observed using a 455-nm excitation filter and a 520-nm barrier filter. Fluorescence from both live and dead spores stained using various combinations of CFDA, PI, and FUN 1 was viewed using a dual bandpass filter set for FITC/PI (mirror unit: U-DM-FI/PI2; Olympus America Inc.). Fluorescence for calcofluor white M2R staining was viewed using a 4′,6-diamidino-2-phenyliadole filter set.

## RESULTS

**Mouse mAbs.** Sera from the immunized mice detected *P. pachyrhizi* urediniospores by ELISA. From the splenocyte fusions, several antibody-producing cell lines were selected on the basis of a positive reaction with *P. pachyrhizi* urediniospore-coated microtiter wells. All cell lines produced IgM-isotype antibodies based on ELISA, the cultures with strong positive reaction were single-cell cloned, and the eight best clones were saved, of which one mAb, Pp-mAb, was selected. The IgM mAbs were purified and the eluted products analyzed by PTA-ELISA for antibody activity. The purified mAb, Pp-mAb, was stored at 4°C.

**Rabbit pAbs.** Terminal bleeds from both rabbits were able to detect *P. pachyrhizi* urediniospores by PTA-ELISA. They were combined, and the purified antibodies were labeled as Pp-pAb.

**Indirect immunofluorescence assay.** Pp-mAb and Pp-pAb reacted positively against freshly collected live urediniospores of *P. pachyrhizi* isolate FL07-1. For both Pp-mAb and Pp-pAb, a minimum concentration of 25 µg/ml was required to observe fluorescence and the intensity increased with an increase in the concentrations of the antibody. All the dilutions of secondary antibody resulted in bright fluorescence of the urediniospores. Increasing the time of incubation of the primary and secondary antibodies from 30 to 60 min increased the fluorescence of urediniospores but increasing the incubation time from 60 to 120 min did not increase the fluorescence intensity. Fluorescence intensity of the urediniospores did not differ with the incubation temperatures. PBST was more effective than PBS and distilled water as a wash buffer, resulting in more intense fluorescence of urediniospores. Based on these results, the final indirect immunofluorescence protocol selected was as follows. A 100-µl urediniospore suspension at a concentration of $5 \times 10^4$ spores/ml was

incubated with a primary antibody at a concentration of 50 µg/ml, with the suspension mixed for 1 h using a rotisserie shaker (Labquake; Thermo Fisher Scientific, Barrington, IL). The suspension was then washed three times with 1% PBST by centrifugation at 1,500 × *g* to remove excess unbound antibody. Secondary antibody was added at a 1:100 dilution. The suspension was triple-washed with 1% PBST by centrifugation at 1,500 × *g*. All the incubation steps were carried out in the dark at room temperature. The samples were stored in darkness at 4°C until further use. Fluorescent signals were observed over the entire surface of *P. pachyrhizi* urediniospores (Fig. 1) using both Pp-pAb and Pp-mAb, with greater intensity often associated with echinulations (Fig. 1E). There were no visual differences between the fluorescent signals produced by Pp-pAb and Pp-mAb. The antibodies successfully detected urediniospores that were freshly collected, heat killed, and a 1:1 mix by imparting bright fluorescence.

**Antibody specificity and cross-reactivity studies.** Based upon the fluorescence intensity, the antibodies did not react with any of the non-rust soybean pathogens *Colletotrichum destructivum*, *Fusarium solani*, *Macrophomina phaseolina*, *Phytophthora sojae*, and *Sclerotinia sclerotiorum*; or non-soybean rust pathogens *Gymnosporangium* sp., *Puccinia coronata*, *Puccinia graminis*, *Puccinia sorghii*, *Puccinia striiformis* (except weakly with Pp-pAb), *Puccinia triticina*, and *Usilago maydis* (Table 1). The antibodies did react weakly with *Phakopsora gossypii* and *Puccinia polysora*, and strongly with *Phakopsora meibomiae*, *P. pachyrhizi*, and *P. zizyphii*. Urediniospores of all of the 10 *P. pachyrhizi* isolates tested produced high fluorescence intensity with both Pp-mAb and Pp-pAb (Table 2).

**Direct immunofluorescence.** FITC-Pp-pAb and FITC-Pp-mAb reacted positively with *P. pachyrhizi* urediniospores, with bright fluorescence observed over the entire surface and, occasionally, very strong green fluorescence of the entire spore (Fig. 1D). The antibodies detected live and dead spores and a 1:1 spore mix (data not shown) without any visual differences in the fluorescent signals. Similar fluorescence of the spores using both

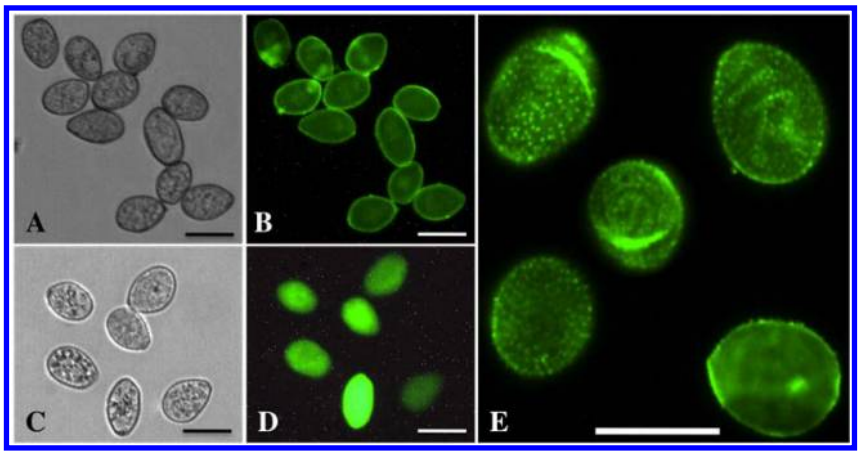


**Fig. 1.** Detection of urediniospores of *Phakopsora pachyrhizi* isolate FL07-1 by immunofluorescence. A 1:1 spore mixture consisting of freshly collected live and dead spores (heat-killed at 55°C for 10 h) were detected. **A and B,** Indirect immunofluorescence using anti-rust polyclonal antibodies. **C and D,** Direct immunofluorescence using monoclonal antibody (mAb) labeled with fluorescein isothiocyanate (FITC) (FITC-Pp-mAb). **E,** Echinulations of urediniospores fluorescing green in an indirect immunofluorescence assay using Pp-mAb antibodies. **A and C,** Visualized using an Olympus BX51 microscope under bright field. **B and D,** Visualized using an Olympus BX51 microscope under bright field, then under a dual-bandpass filter set for FITC-propidium iodide. Scale bar represents 20 µm.

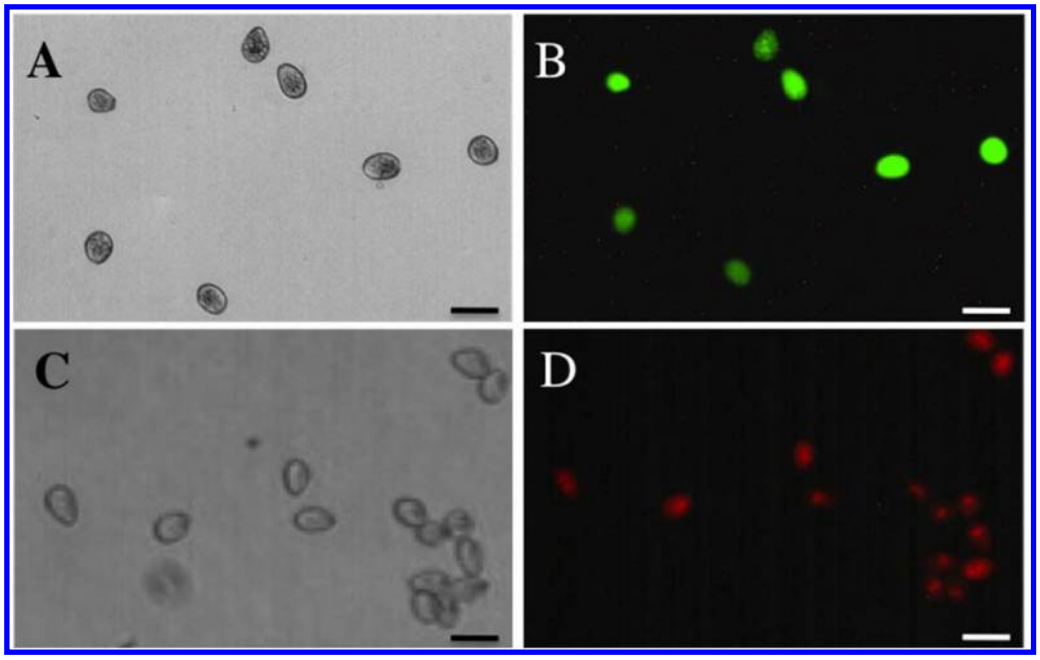


**Fig. 2.** Viability of urediniospores of *Phakopsora pachyrhizi* isolate FL07-1 using 5-carboxyfluorescein diacetate (CFDA) and propidium iodide (PI) staining. **A and B,** Freshly collected live spores fluoresced green indicating their viability. **C and D,** Dead spores that were heat-killed at 55°C for 10 h fluoresced red, indicating their nonviability. **A and C,** Visualized using an Olympus BX51 microscope under bright field. **B and D,** Visualized using an Olympus BX51 microscope under bright field, then under dual-bandpass filter set for fluorescein isothiocyanate and PI. Scale bar represents 20 µm.

indirect and direct immunofluorescence techniques indicated that the reactivity of the antibodies was not affected by FITC labeling because both techniques had good to bright fluorescence.

**Methods for assessing urediniospores viability: CFDA-PI staining.** The use of CFDA and PI provided a clear, differential staining of *P. pachyrhizi* urediniospores (Fig. 2). Dual staining of freshly collected live spores with CFDA and PI resulted in viable spores fluorescing green (Fig. 2B), indicating membrane integrity. The heat-killed dead spores or, more often, the nuclei of nonviable urediniospores fluoresced red because of compromised membranes, indicating their nonviability (Fig. 2D). There were no significant differences ($P > 0.05$) in the viabilities within the freshly collected live spores, within the heat-killed dead spores, and within the 1:1 mix containing both when assessed by CFDA, PI, and CFDA-PI methods.

**FUN 1 and calcofluor white M2R staining.** The viability of freshly collected live and heat-killed spores was assessed by FUN 1 and calcofluor white M2R dual staining (Fig. 3). The intravaculoar structures in live spores, both ungerminated and germinated, fluoresced bright red to reddish-orange because of metabolical activity with intact membranes (Fig. 3B). Dead spores, with no metabolic activity, had a diffuse, faint fluorescence (Fig. 3E). Calcofluor white M2R did not stain nongerminated live and heat-killed dead spores, clearly indicating the absence of cellulose and chitin in the cell walls of urediniospores (Fig. 3F). However, the germ tubes of germinating urediniospores stained blue (Fig. 3C).

**In vitro germination test.** The urediniospores began germinating within 60 min after incubation. A spore was considered germinated when the length of the germ tube was twice the length of the urediniospore. By 6 h after incubation, spore germination was approximately 60% and, by 24 h, the germ tubes fused resulting in a complex network.

No significant differences ($P > 0.05$) were observed in spore viability levels among the three methods: CFDA-PI staining (live = 91.1% ± 1.5, heat-killed = 0%, 1:1 spore mix = 42.9% ± 1.9), FUN 1 staining (live = 89.7% ± 1.8, heat-killed = 0%, 1:1 spore mix = 41.9% ± 1.9), and in vitro germination test (live = 93.7% ± 1.3, heat-killed = 0%, 1:1 spore mix = 46.6% ± 1.6). Both CFDA-PI dual staining and FUN 1 staining methods can differentiate viable and nonviable spores in a mixture consisting

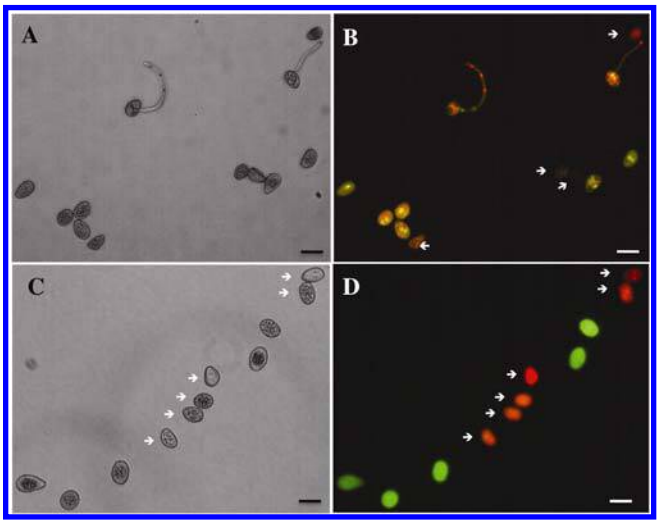


**Fig. 4.** Viability of urediniospores of *Phakopsora pachyrhizi* isolate FL07-1 using 2-chloro-4-(2,3-dihydro-3-methyl-[benzo-1,3-thiazol-2-yl]-methylidene)-1-phenylquinolinium iodide (FUN 1) staining and dual staining by 5-carboxyfluorescein diacetate (CFDA) and propidium iodide (PI). A 1:1 spore mixture consisting of freshly collected live and dead spores (heat killed at 55°C for 10 h) were used. **A and B,** Spore mixture was stained with FUN 1, and live spores fluoresced green, with bright orange-red intravacuolar staining. Dead spores emitted a diffused fluorescence devoid of intervaculoar staining (indicated with arrows). **C and D,** Spore mixture was stained with CFDA and PI, and live spores emitted green fluorescence. Dead spores fluoresced red (indicated with arrows). **A and C,** Visualized using an Olympus BX51 microscope under bright field. **B and D,** Visualized using an Olympus BX51 microscope under bright field, then under dual-bandpass filter set for fluorescein isothiocyanate and PI. Scale bar represents 20 µm.

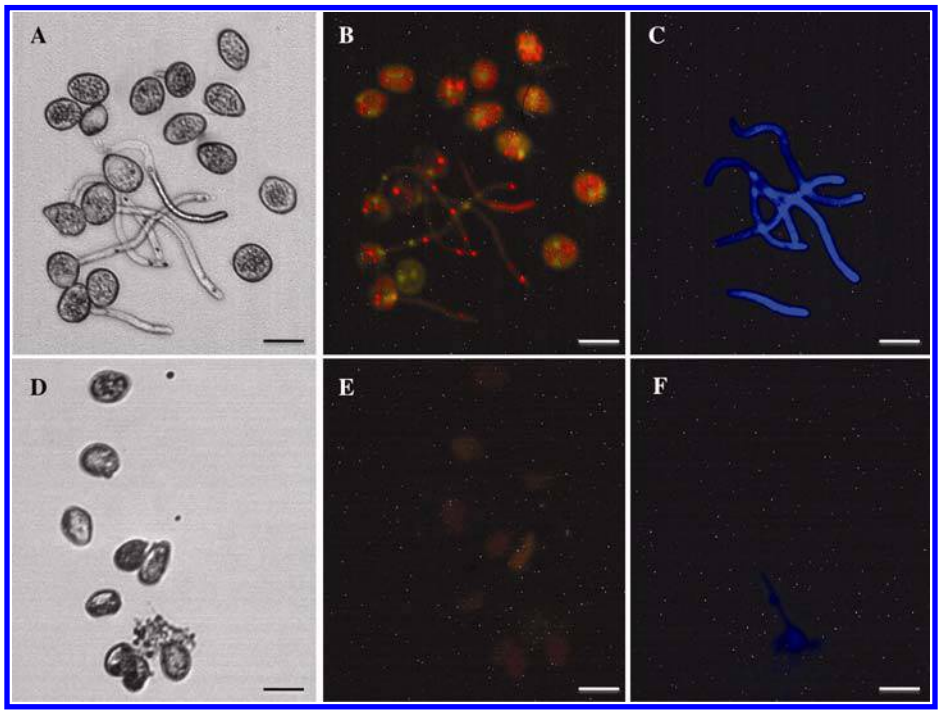


**Fig. 3.** Viability of urediniospores of *Phakopsora pachyrhizi* isolate FL07-1 using 2-chloro-4-(2,3-dihydro-3-methyl-[benzo-1,3-thiazol-2-yl]-methylidene)-1-phenylquinolinium iodide (FUN 1) and calcofluor white M2R. **A to C,** Urediniospores under bright field, dual-bandpass filter set for fluorescein isothiocyanate (FITC) and propidium iodide (PI), and filter set for 4′,6-diamidino-2-phenyliadole (DAPI), respectively. Intravaculaor structures of live spores and germlings in **B** fluoresced reddish orange. **C,** Germ tubes fluoresced bluish-violet because of chitin. **D to F,** Dead spores that were heat-killed at 55°C for 10 h under bright field, dual-bandpass filter set for FITC/PI, and filter set for DAPI, respectively. **E and F,** Dead spores did not fluoresce with FUN 1 and calcofluor white M2R, respectively. Spores were visualized using an Olympus BX 51 microscope and images were captured. Scale bar represents 20 µm.

of both live and dead spores (Fig. 4), similar to in vitro germination. Whereas the in vitro germination required at least 2 to 6 h, the CFDA-PI dual staining and FUN 1 staining assessed the viabilities of the urediniospores within 30 min.

Overall, CFDA, PI, CFDA-PI, and FUN 1 assessed the viabilities of urediniospores that were comparable with the results of in vitro germination tests. The similarity in the viability estimates between in vitro germination and PI staining validates the possible use of PI along with the immunofluorescence assay to detect viable *P. pachyrhizi* urediniospores.

**Differentiation of viable and nonviable *P. pachyrhizi* urediniospores using PI in two-color immunofluorescence.** Single-dye staining by PI was used in combination with direct or indirect immunofluorescence to identify viable *P. pachyrhizi* urediniospores. Viable spores fluoresced green because of reactivity with Pp-antibodies and dead spores fluoresced green with reddish nuclei, indicating their nonviability and making it possible to simultaneously distinguish live and dead urediniospores (Fig. 5).

**Immunomonitoring of captured spores on glass slide.** Viable urediniospores affixed to double-sided tape fluoresced bright green and nonviable spores fluoresced green with reddish nuclei (Fig. 6). There were no visual differences in the fluorescence signals between the urediniospores affixed to the double-sided tape and the urediniospores that were assayed for viability in microcentrifuge tubes. On average, 0.9 and 2.5% of urediniospores affixed to the tape were lost during direct and indirect immunofluorescence assays, respectively. All the urediniospores trapped on the glass slides reacted with Pp-mAb, emitting bright green fluorescent signals using indirect immunofluorescence assay, regardless of the age of the spores (Table 3). Overall, age of the cultures affected the viability of the urediniospores: viability was reduced from 91 to 40% from 20 to 40 days after inoculation, respectively (Table 3).

## DISCUSSION

In this study, mAbs and pAbs were produced against intact urediniospores of *P. pachyrhizi*. Two immunofluorescence assays, direct and indirect, were developed using FITC-labeled antibodies to detect the spores. Vital staining techniques using CFDA, PI, FUN 1, and calcofluor white M2R were tested for the first time to

TABLE 3. Immunofluorescence assay and propidium iodide (PI) staining of urediniospores of *Phakopsora pachyrhizi* isolate FL07-1 trapped on glass slides based on samples taken over time post inoculation

| DPI[a] | Reactivity[b] | Viability (%)[c] |
|---|---|---|
| 20 | 2 | 91 ± 3.5 |
| 25 | 2 | 96 ± 4.0 |
| 30 | 2 | 81 ± 0.6 |
| 35 | 2 | 48 ± 2.3 |
| 40 | 2 | 40 ± 4.6 |

[a] Days postinoculation (DPI) on detached soybean leaves.
[b] Mouse anti-rust monoclonal antibodies were used and reactivity was detected using secondary antibodies labeled with fluorescein isothiocyanate. Fluorescence of urediniospores was rated visually using a scale of 0 to 2, where 0 = no fluorescence, 1 = weak fluorescence, and 2 = good to bright fluorescence (clearly positive).
[c] Viability of urediniospores based on PI stain.

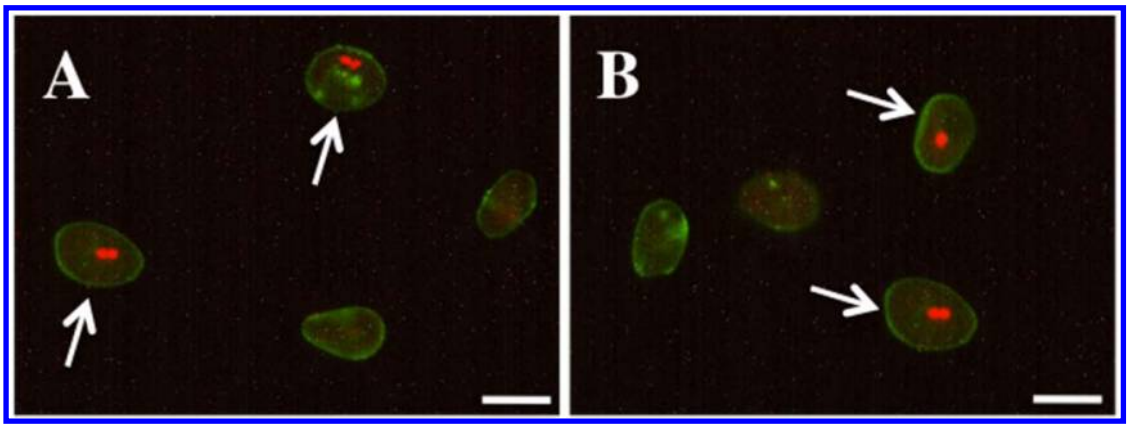


**Fig. 5.** Two-color fluorescence assay to detect the urediniospores of *Phakopsora pachyrhizi* isolate FL07-1 using indirect immunofluorescence and propidium iodide (PI) staining. A spore mix consisting of a 1:1 ratio of freshly collected live and dead spores that were heat killed at 55°C for 10 h was first incubated with and **A,** anti-rust polyclonal and **B,** anti-rust monoclonal antibodies and detected using a secondary antibody conjugated with fluorescein isothiocyanate (FITC). Spores were then incubated with PI for 15 min in darkness and viewed using an Olympus BX51 microscope fitted with a dual-bandpass filter set for FITC/PI. Viable spores fluoresced green and nonviable spores fluoresced green with their nuclei stained red, indicating compromised membranes that allowed the permeability of the PI dye. Scale bar represents 20 µm.

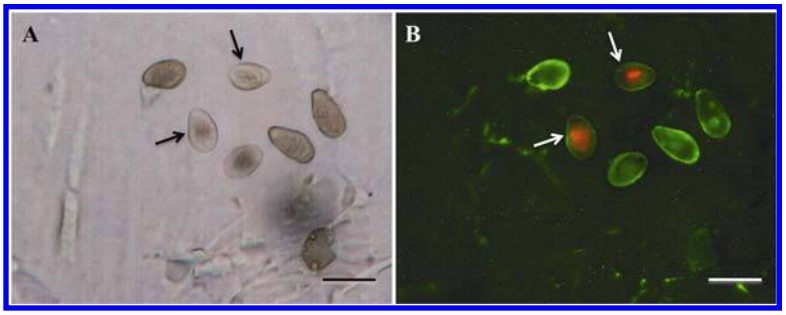


**Fig. 6.** Two-color fluorescence assay to detect the urediniospores of *Phakopsora pachyrhizi* isolate FL07-1 captured on double-sided tape affixed to a glass microscope slide. Urediniospores from 35-day-old infected soybean leaves were incubated with anti-rust monoclonal antibodies and detected using a secondary antibody conjugated with fluorescein isothiocyanate (FITC). Spores were then incubated with propidium iodide (PI) for 15 min in darkness. **A and B,** Spores detected using indirect immunofluorescence and PI staining. Viable spores fluoresced green and nonviable spores (denoted by arrows) fluoresced green with their nuclei stained red, indicating compromised membranes that allowed the permeability of the PI dye. **A,** Visualized using an Olympus BX51 microscope under bright field. **B,** Visualized using an Olympus BX51 microscope under bright field, then under a dual-bandpass filter set for FITC/PI. Scale bar represents 20 µm.

differentiate live and dead urediniospores, and a two-color immunofluorescence assay combined with PI staining was developed to specifically detect viable *P. pachyrhizi* urediniospores with a sensitivity of single-spore detection.

Bioaerosols containing bacteria, fungi, viruses, and/or pollen may adversely affect human health, agriculture, water, and atmospheric chemistry (1). Early detection and identification of plant-pathogenic fungi enables farmers to make more informed decisions on when and how chemicals can be applied to prevent epidemics. Several spore trapping and sampling devices, such as the Andersen sampler model, Burkard volumetric spore trap, Hirst-type spore trap, Teflon filters, S.A.S. sampler, and single-stage collectors, were developed to collect airborne spores of fungi, including *Alternaria*, *Botrytis*, *Cladosporium*, *Didymella*, and *Ganoderma* spp. (42). Integrating these sampling devices with antibody-based or nucleic-acid-based diagnostic methods would facilitate the deployment of detection systems for identification and quantification of specific pathogens.

Immunoassays are established technologies for the detection of several fungal plant pathogens, including *Rhizoctonia solani* (5), *S. sclerotiorum* (27), and *Venturia inaequalis* (39). A particle-trapping device known as the microtiter immunospore trap (Burkard Manufacturing Co., Rickmansworth, UK) collects airborne particles directly into the wells of a microtiter plate and enables rapid quantification of spores of *Botrytis cinerea* and *Mycosphaerella brassicola* (29) by ELISA without any processing of the sample. Immunosensors based on surface plasmon resonance, cantilever, and quartz crystal microbalance technologies are available to detect *Puccinia striiformis* and *Phytophthora infestans* (43,44). These antibody-based biosensors with improved sensitivity hold great promise but, currently, there are some limitations inherent in these technologies in aspects of sensor design and sensing elements that must be resolved before they can be deployed. A detailed review of the recent technological advances in antibody-based sensors is available elsewhere (45). Immunofluorescence has been used to identify spores of *Botrytis* spp. (9) and *M. brassicola* (29) deposited on the trapping surface of a Burkard spore sampler. More recently, immunofluorescence was used to detect the urediniospores of *P. pachyrhizi* captured in passive air samplers (3). The advantage of immunofluorescence over other immunological methods is that immunolabeled spores can be directly visualized and quantified simultaneously. It is clear that immunological techniques have the potential to play a significant role in the detection and quantification of fungal spores. However, they cannot differentiate between live and dead spores unless integrated with vital stains that can assess viability based on indicators like membrane integrity, enzyme activities, and respiration.

The CFDA-PI method informs on both enzyme activity and membrane integrity of the spores. CFDA requires cellular esterase activity in addition to intact membrane and PI, a nucleic acid intercalating dye, is known to pass only through the membranes of dead cells. The FDA-PI dual-staining method was used in a number of studies to determine the viability of myxosporean and actinosporean spores (52) and a wide variety of fungal spores, including *Alternaria brassicae, C. gloeosporioides* f. sp. *malvae*, *Leptosphaeria maculans*, and *S. sclerotiorum* (6), to name a few. We investigated the use of CFDA-PI staining and were successfully able to distinguish between viable and nonviable urediniospores in a mixture containing live and dead spores, and these results were in agreement with the in vitro germination tests. There were no significant differences among the CFDA-PI, CFDA, and PI staining methods in discriminating live and heat-killed spores, thus confirming that the dyes can be used either singly or in combination. It was important to validate the use of PI as a viability indicator before integrating it with the immunofluorescence technique. The main advantages of the CFDA-PI assay are its speed, high sensitivity, and simplicity.

Fluorescence labeling with FUN 1 stain provides spectral and morphological information not available with other single-dye fluorescence-based methods for viability determination in yeasts and fungi (34). Biochemical processing of FUN 1 by live urediniospores yielded orange-red CIVS, confirming that the CIVS are a function of metabolic activity in the cell. FUN 1 allowed a clear discrimination between live and dead urediniospores. The applications of FUN 1 can be extended to study the effect of environmental conditions such as light and moisture on the viability of urediniospores. We could not use FUN 1 with FITC-labeled antibodies for specific detection of viable *P. pachyrhizi* urediniospores because of an overlap in their emission spectra leading to cross-talk between their fluorescence signals. Calcofluor white M2R stained the germ tubes and did not stain the spore walls of both live and dead urediniospores, thus limiting its application as a viability indicator. No further studies were conducted to integrate the calcofluor white M2R staining with immunofluorescence.

More recently, methods and models have been developed that can detect soybean rust or predict the occurrence of the disease. For example, an integrated atmospheric model that couples a long-range atmospheric spore transport and deposition module with soybean leaf canopy wetness was developed in Minnesota for predicting the risk of occurrence of soybean rust (46). An image-processing method for detecting soybean rust from multispectral images of soybean leaves was based on the ratio of infected area and rust color index as symptom indicators for quantifying rust severity (7). Immunosensors developed based on surface plasmon resonance and electrochemical impedance spectroscopy can detect *P. pachyrhizi* urediniospores or mycelia in the initial stages of infection with a potential for early diagnosis (32). Barnes et al. (2) developed a qPCR assay sensitive enough to detect a single *P. pachyrhizi* urediniospore to evaluate precipitation collected at national atmospheric deposition program or national trends network stations located across the major growing regions of the continental United States to assess patterns of spore deposition during the soybean-growing season. However, these methods cannot differentiate between live and dead spores. The fluorescence techniques described here provide a new method for specific detection of viable *P. pachyrhizi* urediniospores. To our knowledge, this is the first report on detecting *P. pachyrhizi* urediniospores by a direct immunofluorescence assay using antibodies labeled with FITC. Staining the immunolabeled spores with PI was successful in distinguishing live from dead urediniospores. Simultaneous labeling of spores with antibodies and PI does not interfere with the specificity of the antigen-antibody binding, because FITC-labeled antibodies specifically recognize the antigenic determinants on the cell surface while PI binds to the nuclei inside the spores. Preliminary results from the urediniospores captured on double-sided tape demonstrated that an immunofluorescence assay combined with PI staining can be an effective tool for detecting viable *P. pachyrhizi* urediniospores. This has the potential to be used to monitor the movement of spores spread not only by conventional means but also by nonconventional means such as clothing (14). We also noted that the methods described here do not preclude follow-up quantification and speciation of spore extracts by qPCR as a double check in situations where a redundant analysis might be warranted, such as forensic applications. Soybean rust is a devastating disease in several soybean-growing regions of Africa, Asia, Australia, and South America, and it has the potential to cause significant yield losses in those countries and is a potential threat to U.S. production. The fluorescent assays we developed are rapid and reliable with a potential for application in forecasting soybean rust. In future studies, we will integrate this method with passive spore sampling to develop an effective tool with a potential to detect and monitor the movement of viable *P. pachyrhizi* urediniospores during the soybean growing season. Early detection, coupled with

timely application of fungicides, would slow the spread of the pathogen and minimize yield losses.

## ACKNOWLEDGMENTS

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