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# Immunofluorescence Assay Detection of the Soybean Rust Pathogen

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**S**oybean rust, caused by the fungal pathogen *Phakopsora pachyrhizi*, was first reported in the continental United States in 2004. Soybean rust is a foliar disease that causes small, chlorotic, irregularly shaped areas on the upper side of leaves while forming raised pustules containing spores (urediniospores) on the underside of the leaves. Soybean rust in the early stages of infection can be difficult to identify because symptoms can be confused with other common soybean diseases including bacterial pustule, Septoria brown spot, downy mildew, and bacterial leaf spot. Soybean yield losses can be significant if the disease is not identified accurately and rapidly.

Numerous strategies are now in place to detect soybean rust including spore trapping via slides placed in sentinel plots with passive and/or rain traps. Many rust spores are similar in shape and appearance to *P. pachyrhizi*, and this is the reason for developing a rapid and sensitive diagnostic protocol for identification. Rapid identification is crucial so that disease management strategies can be implemented as soon as inoculum is identified. An indirect immunofluorescence spore assay (IFSA) for the detection of *P. pachyrhizi* urediniospores is described below. An easy to follow bench top protocol is also included.

## Indirect Immunofluorescence Spore Assay (IFSA)

This assay can be used to detect urediniospores of *P. pachyrhizi* that have been air-trapped onto double-sided tape that is affixed to a glass slide or from bulk urediniospores taken from infected leaves. Urediniospores may be 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, and 4 = bright fluorescence (Appendix, Figure 1). A score of 2 or greater is considered to be a positive reaction. The assay utilizes rabbit polyclonal antibodies (SBR1A, SBR2) produced in response to intact non-germinated (SBR1A) or germinated (SBR2) urediniospores of *P. pachyrhizi*. Both antisera also bind to urediniospores of *P. meibomia*, although SBR2 binds urediniospores of *P. meibomia* more weakly than SBR1A (Appendix, Figures 2–3). The secondary antibody is Alexa Fluor 488 goat anti-rabbit IgG, which is brighter and more photostable than fluorescein derivatives (i.e. FITC). For more information about this assay, see Baysal-Gurel et al. 2008. An immunofluorescence assay to detect urediniospores of *Phakopsora pachyrhizi*. Plant Dis. 92: 1387–1393.

## INSTRUCTIONS

### A. The following equipment and supplies are needed to detect urediniospores of *Phakopsora* species using the indirect IFSA.

- double-sided tape (Scotch® tape, permanent; 3M, Stationery Products Division)
- covered, clean, disposable Petri dishes (Source: VWR Scientific, <http://vwrsp.com>, item number: 25384-302)
- 1 mm thick glass slides (Source: VWR Scientific, <http://vwrsp.com>, item number: 48300-025)
- 1.5-ml microcentrifuge tube (Source: VWR Scientific, <http://vwrsp.com>, item number: 14231-062)
- microtiter plate shaker (Source: VWR Scientific, <http://vwrsp.com>, item number: 57019-600)
- PBS-Tween (Source: Agdia Inc.; <http://www.agdia.com>, item number: ACC 00501)
- primary antibody (SBR1A or SBR2; see below for instructions on how to obtain antibody)
- secondary antibody (Alexa Fluor 488 goat anti-rabbit IgG (H+L); Source: Invitrogen Molecular Probes; <http://probes.invitrogen.com>, item number: A-11034)
- clear nail polish
- rectangular coverslips (24 x 50 mm, No. 1.5 thickness (0.13-0.17 mm); Source: VWR Scientific, <http://vwrsp.com>, item number: 48404-452)
  - epifluorescent microscope
  - Leica DMIRB Microscope\*
  - Optronics Magnafire camera\*
  - Microscope Filter: I3 Blue, Excitation= 450-490, Emission=515
  - Objective magnification; x20
  - exposure: 1.037 second

*\*fluorescence visualization parameters must be optimized for other equipment*

### B. How to prepare samples from air samplers or bulk urediniospores of *Phakopsora* species for indirect IFSA.

#### 1. Air samplers

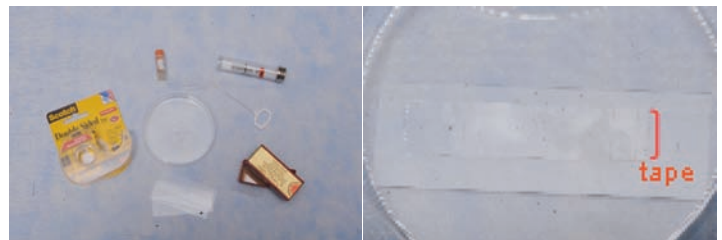
Double-sided tape should be used to capture urediniospores. *Petroleum jelly prevents thorough washing during the IFSA resulting in high background fluorescence.*

#### 2. Leaf lesions

Press a 2-in. strip of double-sided tape directly onto one or more sporulating lesions, then affix the tape, with deposited spores on the upper surface, onto a glass slide.

#### 3. Bulk urediniospores

1. Collect dried urediniospores from the under surface of an infected leaf and transfer to a sterile 1.5 ml microcentrifuge.
2. Apply a 2 in. strip of double-sided tape to a glass slide.
3. Collect urediniospores by inserting a 10 µl micropipette (with plunger completely inserted) containing a clean tip into dried urediniospores and drawing the plunger back ~1mm.
4. Expel dry urediniospores onto the upper surface of the tape attached to the glass slides (Section C) or into a microcentrifuge tube and incubate microcentrifuge tube overnight (16-24 hr) to hydrate spores (Section D).



### C. Indirect IFSA method on glass slides.

*All steps are performed at ambient temperature (~22 °C).*

1. Dilute the primary antibody (SBR1A or SBR2) 1:500 in PBS-Tween.
2. Apply 500 µl of the diluted primary antibody over the upper surface of the tape.



3. Incubate the slide in a covered petri dish, for 2 hr.

4. Wash the slide six times in PBS-Tween as follows:
  - a. Apply ~5 ml PBS-Tween to the upper surface of the tape.
  - b. Incubate 30–60 sec.
  - c. Pour off wash solution.
  - d. Repeat steps 4.a.–4.c. five times.

Note: The inclusion of blocking steps is not needed since the fluorescence achieved without blocking is similar to that achieved with blocking (Baysal-Gurel et al. 2008. *Plant Dis.* 92:1387–1393).

5. Dilute the secondary antibody (Alexa Fluor 488 goat anti-rabbit IgG (H+L)) 1:500-800 in PBS-Tween.



6. Apply 500 µl of the diluted secondary antibody over the upper surface of the tape.

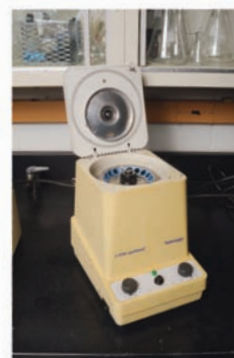


7. Incubate slide in a covered petri dish, in the dark, for 1 hr.
8. Wash the slide six times as described in Step 4.
9. Cover slide with a coverslip.
10. Seal the coverslip permanently to the slide by brushing clear nail polish around the edges of the coverslip.
11. Observe by epifluorescent microscopy.
12. Slides can be stored in the dark at ambient temperature for up to 2 months.

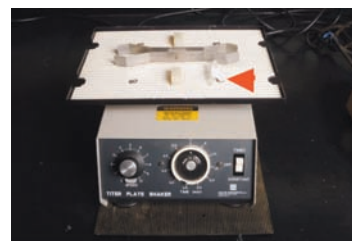
#### D. Indirect IFSA method in microcentrifuge tubes.

All steps are performed at ambient temperature (~22 °C).

1. Pellet urediniospores by centrifugation (5 min. at 16,000 x g).



2. Remove and discard supernatant without disturbing pellet.
3. Wash pelleted spores once as follows:
  - a. Resuspend spores in 1 ml PBS-Tween.
  - b. Pellet urediniospores by centrifugation (3 min. at 16,000 x g).
  - c. Discard wash buffer without disturbing pellet.
4. Dilute the primary antibody (SBR1A or SBR2) 1:500 in PBS-Tween.
5. Add 500 µl of the diluted primary antibody to the washed urediniospores.
6. Incubate samples 2 hr while shaking (~ 230 strokes/min).



7. Pellet urediniospores by centrifugation (5 min. at 16,000 x g).
8. Remove and discard supernatant without disturbing pellet.
9. Wash the pellet containing antibody-labeled urediniospores three times in PBS-Tween as follows:
  - a. Resuspend urediniospores in 1 ml PBS-Tween by pipetting up and down 5-10 times.
  - b. Pellet urediniospores by centrifugation (3 min. at 16,000 x g).

- c. Discard wash buffer without disturbing pellet.
- d. Repeat steps 11.a.–11.c. twice.
10. Dilute the secondary antibody (Alexa Fluor 488 goat anti-rabbit IgG (H+L)) 1:500 in PBS-Tween.
11. Apply 500  $\mu$ l of the diluted secondary antibody to each tube.
12. Incubate samples 1 hr while shaking vigorously in the dark ( $\sim$  230 strokes/min).
13. Pellet urediniospores by centrifugation (5 min. at 16,000 x g).
14. Wash pellet three times as described in Step 11.
15. Resuspend pellet in 25  $\mu$ l PBS-Tween by pipetting or vortexing and store in dark until they are ready to be used.
16. Transfer  $\sim$ 5  $\mu$ l of well-suspended sample to a glass slide and cover with a coverslip.
17. Seal the coverslip permanently to the slide by brushing clear nail polish around the edges of the coverslip.
18. Observe by epifluorescent microscopy.
19. Slides can be stored in the dark at room temperature for up to 2 months.

Photographs in Sections B-D courtesy of Ken Chamberlain, OSU-OARDC.

### Appendix of IF Images

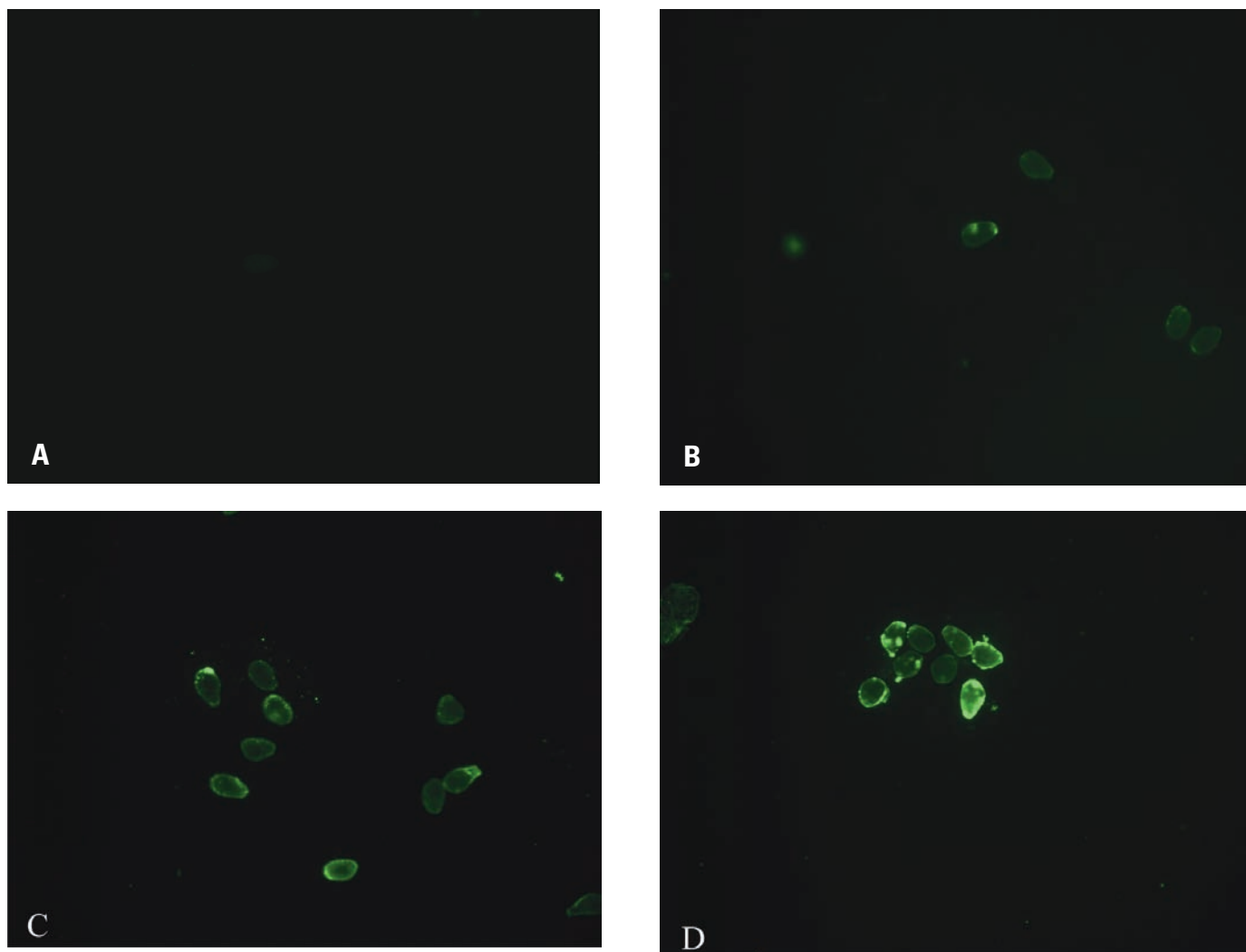


Figure 1. The fluorescence intensity score of urediniospores of *P. pachyrhizi* by immunofluorescence microscopy A) 1= no fluorescence, B) 2= weak fluorescence, C) 3= good fluorescence, and D) 4= bright fluorescence.

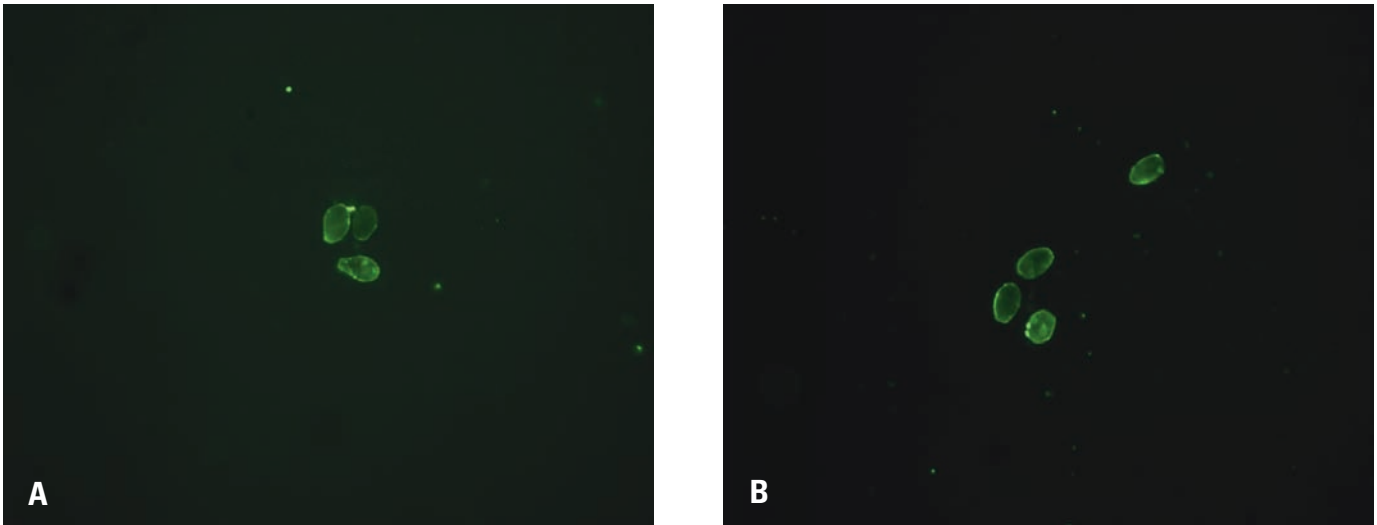


Figure 2. Immunofluorescence detection of urediniospores of *P. pachyrhizi* using the primary antibodies SBR1A (A) and SBR2 (B).

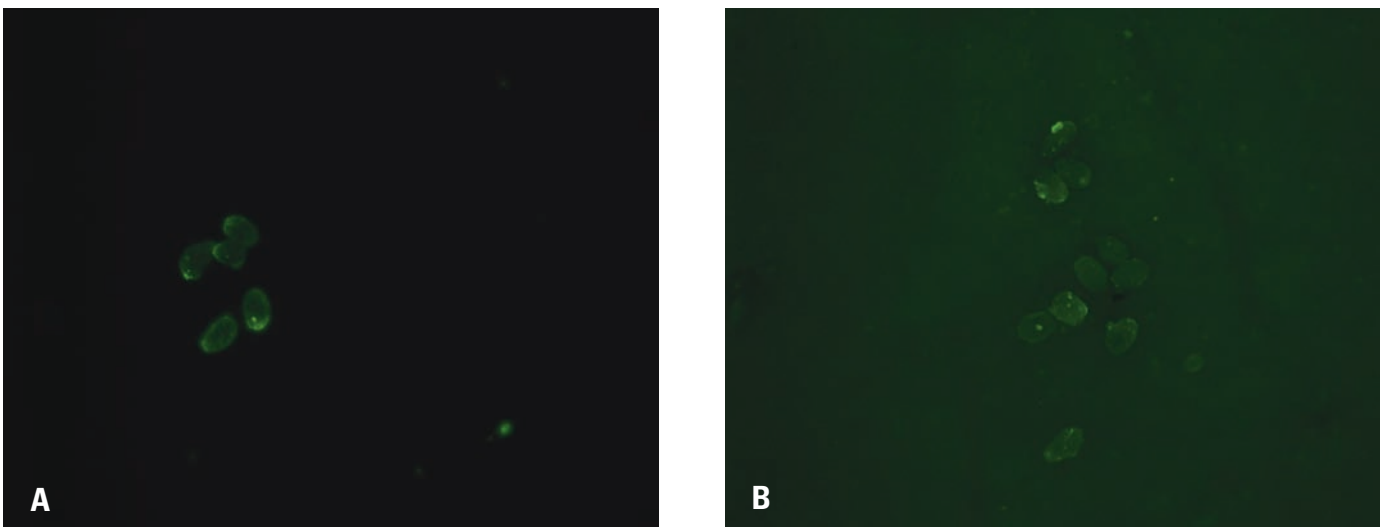


Figure 3. Immunofluorescence detection of urediniospores of *P. meibomia* using the primary antibodies SBR1A (A) and SBR2 (B).

### HOW TO OBTAIN POLYCLONAL ANTIBODIES

Polyclonal antibodies (~1 mg) may be obtained upon request by contacting:

**Dr. Anne Dorrance**  
**Department of Plant Pathology, The Ohio State University –OARDC**  
**1680 Madison Avenue**  
**Wooster, OH 44691**  
**330-202-3560**  
**dorrance.1@osu.edu**

## Bench Protocol: Indirect IFSA

### Glass slides and Microcentrifuge Protocol



New users are advised to familiarize themselves with the detailed protocols before using this bench protocol.

#### Notes

- All centrifuge steps are at 16, 000 x g in a conventional tabletop microcentrifuge.
- All incubations are performed at ambient room temperature (~22 °C).
- Primary and secondary antibodies are diluted 1:500 in PBS-Tween.

#### Procedure

1. Add 500 µl of the primary antibody to the upper surface of the tape or to the washed urediniospores in the microfuge tube.
2. Incubate 2 h in a covered petri dish or with vigorous shaking.
3. Wash the slide six times in PBS-Tween or the pelleted urediniospores three times in PBS-Tween.
4. Add 500 µl of the secondary antibody to the upper surface of the tape or to the washed urediniospores in the microfuge tube.
5. Incubate 1 h in a covered petri dish or with vigorous shaking, in the dark.
6. Wash the slide six times in PBS-Tween or the pelleted urediniospores three times in PBS-Tween.
7. Cover the glass slide with a coverslip or transfer 5 µl from microfuge tube to glass slide and cover with the coverslip.
8. Seal the coverslip permanently to the slide with clear nail polish.
9. Observe by epifluorescent microscopy:
  - Leica DMIRB Microscope
  - Optronics Magnafire camera
  - Microscope Filter: I3 Blue, Excitation= 450-490, Emission=515
  - Objective magnification; x20
  - exposure: 1.037 second

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