Recovery of *Phakopsora pachyrhizi* Urediniospores From Passive Spore Trap Slides and Extraction of Their DNA for Quantitative PCR

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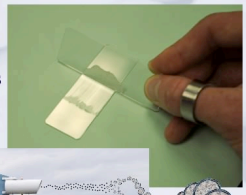
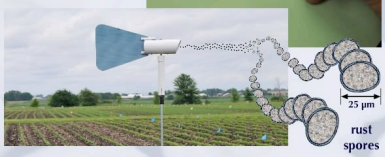
Abstract

Enumeration of rust spores from passive spore traps utilizing white petrolatum-coated slides by traditional microscopic evaluation can represent a serious challenge. Many fungal spores look alike, and clear visualization on the adhesive can be obscured by particulate debris or nonuniformities within the adhesive layer; reports will commonly describe only the number of "rust-like" spores. Molecular methods of *P. pachyrhizi* detection, utilizing both standard PCR and quantitative PCR (qPCR), have been available for several years, but extraction of fungal DNA from petrolatum-embedded spores remained difficult. We now demonstrate the utility of a novel method for recovering the petrolatum layer carrying trapped spores from slides using biodegradable foam strips, with subsequent DNA extraction to yield material suitable for quantification by qPCR. This method permits even single spores of *P. pachyrhizi* to be recovered and detected. False-negative calls were minimized by using a multiplexed exogenous control; no false-positives were observed. This method was successfully employed to assess spore loads in passive traps located at sentinel plots in the USA during the 2008 soybean growing season.

Glass slides coated with Vaseline catch spores in wind-vane traps





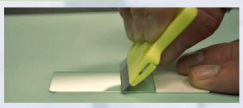
rust spores

Scrape off vaseline and spores:
3 factors ensure complete removal

1. Teflon coating on razor blade

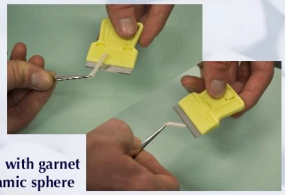
2. Uniform (non-flexing) blade holder

3. Make a single swipe across slide at a 45 degree angle



Transfer to extraction tube and purify DNA

Wipe razor with soluble strip (both sides)



Initial extraction with garnet sand & one ceramic sphere

Rupture spores twice in CLS-Y with 1 hr interval on ice.

Cleanup and concentrate DNA with Qiaquick Spin kit

Experimental

Developing single-spore detection: Individual spores of *Phakopsora pachyrhizi* (either fresh, head-killed, or germinating) were picked from 2% water agar using the tip of a diagonally-cut, vaseline coated, soda straw (either one, two, or eight spores; 10 reps of each) and transferred to strips of soluble, compressed, biodegradable packing foam, and placed into extraction tubes. 1.0 mL of CLS-Y buffer was added along with 1 µg salmon DNA to each tube, and the tubes were then chilled on ice for 10 min. DNA was extracted using two rounds of homogenization in the FastPrep instrument (40 s at a speed setting of 6 m/s) with a 1 hr incubation on ice between rounds, and a final 10 min incubation on ice, and subsequent processing as directed by the manufacturer. Residual inhibitors were removed and further concentration of fungal DNA was achieved by using the Qiaquick PCR Spin kit, with elution in 30 µL. Five µL of eluate was amplified in a Q-PCR instrument using primers and probe specific to *P. pachyrhizi* (Frederick, et al, Phytopathology, 2002), and including a multiplexed internal control for false-negative detection (Haudenshield & Hartman, Phytopathology, 2008). A standard curve of reference *P. pachyrhizi* DNA was used to compare the recovery of spore DNA among the samples and between Q-PCR runs.

Verifying slide-scrape recovery: Individual fresh spores of *P. pachyrhizi* were picked as above, and transferred to vaseline-coated slides. A different analyst then scraped the vaseline and the entrapped spores from the slide using a teflon-coated razor blade held at a 45-degree angle, in a single swipe, using a new blade for each slide. The material collected on the edge of the razor blade was wiped onto a 25 mm x 3 mm strip of compressed, biodegradable packing foam and DNA was extracted and quantified as above.

Assay for Rust by Quantitative PCR

- Include PpaIC-Cy5 internal control to detect false-negatives
- May add BSA at 400 ng/µL to overcome inhibitors
- Include standard curve reference DNA to confirm assay performance

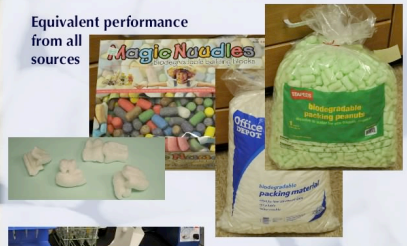
Fluorescent signal: Rust DNA detected and quantified

Results

(1) A number of pilot experiments revealed that all sources of biodegradable packing foam performed equivalently, regardless of color or shape, although those having minimal curl were easier to compress and had a more uniform density than cylindrical types. The cut strips had a mass between 15 and 50 mg, depending upon manufacturer. **(2)** We evaluated a number of single-edge blade holders, and found an inexpensive department-store model (Red Devil, #3224) to be superior in providing uniform pressure along the width of the blade, and to permit easy blade-changing. **(3)** We evaluated a variety of types of razor blades, and found the teflon-coated stainless steel (GEM model 62-0165; Electron Microscopy Sciences) to be superior in sharpness, cleanliness, and in its ability to remove vaseline from glass slides while leaving no visible residue, while also being fairly inexpensive. **(4)** To verify single-spore detection ability, spores were picked and transferred directly to soluble strips for DNA extraction, and Q-PCR amplification. We found that quantification was in proportion to the number of input spores, and that even single spores yielded detectable signal. **(5)** To verify the recovery from coated slides, two or sixteen spores were planted on glass slides (5-6 reps each), recovered by razor-scraping, and normalized to quadruplicate extracts from 10 spores picked and placed directly on soluble strips. After extraction and amplification by Q-PCR, two spores were quantified as 0.87 spores, and sixteen were quantified as 7.3 spores. Thus a recovery rate of *ca.* 45% was observed from the slides, which includes the efficiency of transferring picked spores to the glass slide. **(6)** This method was used to identify and quantify the number of *P. pachyrhizi* spores on coated slides from the 2008 National Soybean Rust Spore Trap Program, when microscopic evaluation indicated the presence of rust-like spores.

Preparation of soluble strips from biodegradable packing foam

Equivalent performance from all sources



Slice into strips (25mm x 3 mm)

Compress to 2 mm thickness

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