Quantification and Single-Spore Detection of Phakopsora pachyrhizi

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Abstract

The microscopic identification and quantification of *Phakopsora pachyrhizi* spores from environmental samples, spore traps, and laboratory specimens can represent a challenge. Such reports, especially from passive spore traps, commonly describe the number of "rust-like" spores; for other forensic samples, visualization is impossible because of the accompanying milieu. Molecular methods of *P. pachyrhizi* detection, utilizing both standard PCR and quantitative PCR (Q-PCR), have been available for several years, and have proven useful in discriminating *P. pachyrhizi* from other rust fungi, and for quantifying DNA and the equivalent number of spores. We report here the validation of a combined method for DNA extraction and analysis by Q-PCR that reliably detects, identifies, and quantifies small numbers, and even single spores, of fresh *P. pachyrhizi* mechanically picked from an agar substrate. Seventy percent of single spores, 95% of spore pairs, and 100% of eight-spore samples were detected using this method. We compare the efficiency of this method for assaying fresh spores with that for heat-killed spores and for actively germinating spores picked from similar agar substrates, as well as for spores picked from white petrolatum-coated glass slides, as used in common spore traps.

Methods

EXTRACTION. Spores of *P. pachyrhizi*, isolate FL07-1, were distributed on the surface of 2.5% (w/v) water-agar plates either by lightly dusting or by spreading in a droplet of 0.05% Tween-20. Individuals or clusters of spores were picked from the surface with the aid of a binocular dissecting microscope by excising a small particle (*ca.* 1–2 mm^3) of agar with the spore(s) on top, using a diagonally-cut plastic soda straw as a scoop. To pick spores from vaseline, the individuals or clusters were scooped using the tip of a scalpel, and the spores scraped from the scalpel blade using a flame-tapered glass microcapillary. The particle of agar (or capillary tip with clump of vaseline) was placed in a FastDNA™ Spin Kit (MP Biomedicals) extraction tube containing garnet sand, a 5 mm ceramic bead, and 1 µg carrier salmon sperm DNA, and the spore DNA was extracted per the manufacturer's instructions using the CLS-Y reagent and two rounds homogenization in the FastPrep FP120A instrument (40 seconds, 6.0 m/s^2), with a 1 hour incubation at room temperature between rounds. In our experience, eluates from the FastDNA kit contain inhibitors to amplification when large volumes are used as template, therefore following elution in water, the spore DNA was further concentrated and purified using QiaQuick™ (Qiagen) spin columns, and eluted in 25 µL of 5 mM Tris, pH 8. A DNA reference standard was prepared from a 0.05% Tween-20 suspension of concentrated, freshly heat-killed, field-collected spores that had been carefully quantified in a hemocytometer, then extracted using the FastDNA™ kit, adjusted to 51.2 Spore-equivalents (SEq) per µL, and then serially diluted 1:8 with 1 µg/mL salmon sperm DNA in 5 mM Tris, pH 8 to a low-end concentration of 0.0125 SEq/µL. [One SEq is the amount of genomic DNA from a single dikaryotic *P. pachyrhizi* spore; given a genome size of 50 Mb, one SEq ≈ 110 fg (110 x 10^{-15} g)]. Aliquots of this reference standard were dispensed and stored at -80°C until use.

Methods

QUANTIFICATION. Real-time PCR was performed using the primers and FAM probe specified by Frederick, et al. [Phytopathology 92(2):217 (2002)], which amplify a 142 nt. region spanning the 5.8S rDNA gene and the adjacent ITS2 sequence, and which discriminates between *P. pachyrhizi* and *P. meibomiae*. We used a Stratagene MX3005P™ thermocycler, with ROX reference dye. 10 µL of eluate was assayed in a final reaction volume of 25 µL, using Platinum™ QPCR Supermix UDG (Invitrogen) with each primer at 300 nM, probe at 30 nM, and $MgCl_2$ at 7 mM. All samples were assayed in duplicate. The cycling regimen was an initial 2 minute 60°C incubation, a subsequent 2 minute 95°C denaturation, followed by 40 cycles of 15 seconds at 95°C → 30 seconds at 60°C. Optical data was collected at the end of each cycle. Ct values were determined by the thermocycler software, using an amplification-based threshold and quantitation of the DNA estimated by comparison to the reference dilution series (a range of 1/16th to 256 SEq per reaction), with an adjustment for volumetric losses during DNA extraction and purification. Excepting the zero controls, the Q-PCR was scored as successful if either or both of the replicates yielded a Ct value by 40 cycles. All similar replicates where amplification was successful were then averaged to determine an overall result (number of spores observed), and internally normalized to yield results for comparison.

Results

When assayed, the DNA reference standard gives the expected amplification curve, as shown in Figure 1. When plotted on a logarithmic scale, the standard curve typically gives a linear response, with a coefficient of correlation in excess of 0.99 (Figure 2). The Ct values for unknown samples are plotted on this standard curve, and the initial quantity is thereby estimated.

In the first experiment, individual fresh *P. pachyrhizi* spores were picked from water-agar, either idividually, or in groups of two or eight. Ten of each count were picked (along with six empty control tubes) and quantified; the entire experiment was repeated once by another analyst. As shown in Table 1, zero, one, two, and eight spores were quantified as 0, 0.9, 2.5, or 8.1 spores, with 100, 70, 95, and 100% reaction success, respectively. Although there was variation, each count was clustered well enough to discriminate between one and two spores.

In the second experiment, fresh *P. pachyrhizi* spores were allowed to begin to germinate for 1-2 hr on water-agar, and only those spores where a germ tube was visible were picked, either individually, or in groups of two or eight. Ten of each count were picked (along with six control tubes containing only a comparable piece of agar). As shown in Table 1, zero, one, two, and eight spores were quantified as 0, 1.3, 2.3, and 14 spores, with 100, 90, 100, and 100% reaction success, respectively. Again, the counts were clustered sufficiently to discriminate one spore from two.

In the third experiment, fresh *P. pachyrhizi* spores were sprinkled on a glass microscope slide coated with vaseline, and individual spores were plucked from the vaseline and quantified. As shown in Table 1, zero, one, and three spores were quantified as 0.1, 0.45, and 0.45 spores respectively, with all reactions successful. We find that while detection of *P. pachyrhizi* was unambiguous, we were not able to discriminate between one and three spores.

Figure 1.

P. pachyrhizi Reference Standard Dilution Series (1:8)

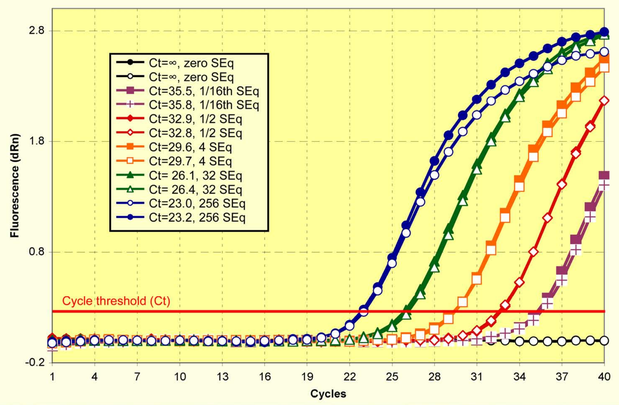

Figure 2.

Estimation of Initial Quantity via Reference DNA Dilution Series

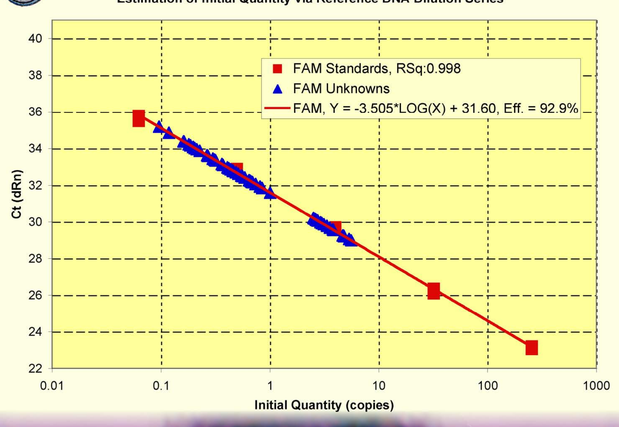

Table 1.

Compiled results of spore pick quantifications.

Spore Type	No. by Count	% Succ.	Est. by Assay	Std. Dev.	Nrmlzd. No. Obs.	No. of Reps.
Fresh (Non-germ.)	0	100	0.00	.001	0.0	12
	1	70	0.93	.46	0.74	14
	2	95	2.50	1.4	2.0	19
	8	100	8.09	3.2	6.47	20
Fresh (Germ.)	0	100	.002	.004	0.0	6
	1	90	1.35	.79	1.2	9
	2	100	2.25	.92	2.0	10
	8	100	13.7	4.6	12.2	10
Fresh (Vaseline)	0	100	0.05	.07	0.11	5
	1	100	0.45	.51	1.0	5
	3	100	0.45	.31	1.0	5

Conclusions

We have described a method to prepare a DNA reference standard solution useful in absolute quantification of Asian Soybean Rust spores, a method for picking spores from vaseline and water-agar substrates and for extracting & concentrating their DNA, and a method for assaying the isolated DNA to estimate the number of spores present in the initial sample. Our assay system will successfully quantify even single spores when picked from water-agar, and with greater efficiency if the spores are germinated. When spores are picked from vaseline-coated slides, such as those in passive spore traps, our system has the sensitivity to detect single spores, but is not able to differentiate between low numbers of spores. This may be because of a protective effect of the vaseline, a sequestering of the spore cytoplasm, or other unidentified factors. Additional experimentation will be performed toward resolving the question.

We find a 70-95% rate of successful amplification for single and double spores, and are currently preparing a multiplexed, internal control reporter so that reaction integrity can be affirmed, and to reveal any potential loss of template DNA during extraction or handling.

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