Detection and Quantification of *Phakopsora pachyrhizi* Spores by Quantitative Real-Time PCR*

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Introduction

Epidemiological models and early detection systems require fast, accurate results on which to base decisions. Spore trapping and counts may provide data for forecasting. Recently developed molecular techniques allow for rapid, reliable detection and quantification of spores. We are currently using quantitative real-time PCR to detect and quantify urediniospores of *Phakopsora pachyrhizi* in water suspensions. The main objective of this study was to improve the sensitivity and precision of qPCR by adding salmon sperm DNA during the extraction procedure, and various coprecipitants during ethanol precipitation.

Materials and Methods

-Fresh *P. pachyrhizi* spores were collected from infected fields at the North Florida Research and Education Center (NFREC), Quincy, FL.

-*P. pachyrhizi* spores were collected from greenhouse grown plants and heat killed at 55°C for 24 hours at the USDA-Foreign Disease-Weed Science Research Unit (FDWSRU), Fort Detrick, MD.

-Spore dilutions of 10^4 spores/ml were prepared in sterile distilled water with 0.04% Tween-20, and confirmed by hemacytometer counts. One hundred µl of dilutions were added to 1.0 ml CLS-Y buffer (FastDNA SPIN kit, Qbiogene, Inc.) and incubated 1 hour prior to DNA extraction.

-0 ug or 1ug sheared salmon sperm DNA (Ambion, Inc.) was added during DNA extraction using the FastPrep system (Qbiogene, Inc.).

-Coprecipitants (1) were used during ethanol precipitation: no coprecipitant, 1 ug sheared salmon sperm DNA, 10 ug sheared salmon sperm DNA, 100 ug tRNA (Sigma), and 20 ug glycogen (Sigma). Pellets were resuspended in 375 µl TE (pH 8.0).

-Four extractions were prepared for each treatment, and each treatment was evaluated by qPCR three times

-1/100 DNA volume (3.75 µl of 375 µl) was tested in qPCR (equivalent to 10 spores), using the protocol developed by Frederick, et al. (2).

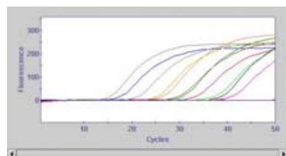

Fig. 1. Example of qPCR graph from a SmartCycler showing level of fluorescense over cycle number.

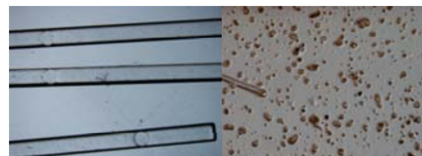

Fig. 2. Rods from a Rotorod spore collecting device (X7.5). Spores captured on silicone grease coated rods (X112).

*Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by the USDA implies no approval of the product to the exclusion of others that may also be suitable.

Results and Discussion

-Large differences in signal strength were evident between the fresh spores and dead spores (Table 1). Heat-killed spores may have degraded DNA. Differences between isolates and spore collection methods also may play a role in DNA quality and quantity.

-Addition of sheared salmon sperm DNA during the extraction procedure improved the sensitivity (lower cycle threshold) and precision (lower standard errors). See example of a graph showing qPCR results for different DNA concentrations (Fig. 1). This may be due to the additional DNA serving as a carrier through the extraction procedure, or it may serve as a substrate for contaminating nucleases.

-Use of additional coprecipitants during ethanol precipitation only slightly increased sensitivity and precision, but the main benefit may be the increased visibility of DNA pellets during precipitation.

-Negative controls (water in extractions and water in qPCR) did not cross the fluorescence threshold if ethanol precipitation was performed. This indicates the high degree of specificity obtained with this system.

Conclusions

We have successfully detected *P. pachyrhizi* at a DNA concentration equivalent to 10 spores at a cycle threshold of 25-27 cycles using fresh spores. Our future experiments will use fresh spores extracted with salmon sperm DNA. We will test samples with known quantities of spores, as well as “unknown” samples to verify the system. We will also test spores embedded on spore traps (see example in Fig. 2) to support the development of an accurate qPCR assay to replace visual observation for the identification and quantification of *P. pachyrhizi* urediniospores.

Table 1. Quantitative PCR cycle threshold (Ct) and standard error (SE) for a DNA dilution equivalent to 10 fresh or heat-killed spores with a combintation of extraction and precipitation additives.

Extraction buffer additive	Coprecipitant added in EtOH precipitation	Fresh spores		Heat-killed spores	
		mean	SE	mean	SE
None	no coprecip	27.82	0.29	36.52	0.25
1ug salmon sperm DNA	no coprecip	26.81	0.15	35.87	0.23
None	1ug salmon sperm DNA	27.59	0.18	38.83	1.01
1ug salmon sperm DNA	1ug salmon sperm DNA	27.57	0.71	37.09	0.80
None	10ug salmon sperm DNA	27.88	0.24	37.18	0.43
1ug salmon sperm DNA	10ug salmon sperm DNA	26.45	0.20	36.05	0.24
None	100ug tRNA	27.35	0.17	36.78	0.30
1ug salmon sperm DNA	100ug tRNA	26.45	0.11	35.99	0.20
None	20ug glycogen	27.34	0.16	37.88	0.45
1ug salmon sperm DNA	20ug glycogen	26.37	0.14	35.61	0.34

Mean and SE from 4 replications

References

1. Sambrook, J. and Russell, D.W. 2001. Molecular Cloning: A Laboratory Manual 3rd Edition, Cold Spring Harbor Laboratory Press, New York. P. A8.13.
2. Frederick, R.D., Snyder, C.L., Peterson, G.L., 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.

Acknowledgements

We gratefully acknowledge the help of the Morel lab (CRIA, Paraguay), the Frederick lab (USDA-FDWSRU, Ft. Detrick, MD), the Isard lab (Penn State, State College, PA), the Marois lab (NFREC, Quincy, FL), and everyone at the Hartman lab (USDA, Urbana, IL); and for the support from USB and USDA-ARS, USDA-APHIS, and USDA-CSREES.