

Title: Quantification of soil-borne pathogens of potato using real-time PCR

Submitted to Minnesota Area II Potato Growers and NPPGA

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Research Objectives:

- 1) Develop a multiplex real-time PCR method for the detection and quantification of *V. dahliae*, *C. coccodes*, and *S. subterranea* from field soil.
- 2) Develop soil sampling and soil processing procedures to optimize DNA extraction from soilborne pathogens.

Research Plan:

The PCR primers for *C. coccodes* and *V. dahliae* PCR have been combined into a duplex reaction that will permit the quantification of these two pathogens in a single reaction. Further studies were undertaken to use the powdery scab PCR method of Qu et al. (2006) with the methods already developed in our laboratory.

Mycelia of *V. dahliae* and *C. coccodes* will be added in known quantities to the soil samples. DNA will be extracted from 5 g soil samples with the MegaPrep DNA extraction kit (Mo Bio Inc.) and multiplex real-time PCR will be performed using 10 µL of the soil DNA extract in a 50 µL reaction volume. The amount of each respective pathogen detected in the soil sample will be determined based on standard curves using purified pathogen DNA as template. Serial dilutions of the DNA extracts from pathogen infested soil will be made and examined by real-time PCR to determine threshold levels for reliable detection of each pathogen. DNA extracted from the original soil sample prior to infestation by the four respective pathogens will also be amplified by real-time PCR to examine baseline levels of detection from non-infested soil.

After verifying the sensitivity and specificity of the multiplex real-time PCR assays with purified pathogen DNAs, soil will be collected from potato fields from throughout the region to test for the level *V. dahliae*, *C. coccodes*, and *S. subterranea*. To date 186 fields have been tested for powdery scab and 85 fields have been tested for Verticillium wilt in a beta testing format.

Results:

The real-time PCR assay for the powdery scab pathogen works well over a range of soils. Although we cannot quantify the number of spore balls of *S. subterranea* per gram of soil due to the non-culturability of the pathogen, we have developed a standard curve for the quantity of DNA of the pathogen in soil (Figure 1). We have applied this method across several soil types among the 186 samples we have processed from growers fields and have found the method to be sensitive across all samples processed to date (Figure 2).

The development of a PCR assay for *V. dahliae* that will work with soil detection has been a significant challenge, one we did not face with *C. coccodes* or *S. subterranea*. The detection of microorganisms in soil by PCR provides many challenges, including the presence of inhibiting compounds such as humic acids. These inhibitory compounds have been very problematic for *V. dahliae* detection, more so than for the other pathogens we have been working with in soil extracts. Many researchers have developed protocols for DNA extraction from soil which have been successful for the detection of a target organism using particular primer sets, but these techniques may not work for different organisms or even different primers sets designed to the same organism. The protocol developed in this study was developed by incorporating techniques used in DNA extraction protocols for several different systems and was successful in detecting DNA of *V. dahliae* extracted from soil using a conventional PCR and real-time PCR assay.

While there have been numerous sets of PCR primers developed for the detection of *V. dahliae*, many of these have not been evaluated for efficacy in detecting the organism in soil. Among the two primers sets developed using the TRP gene, TRP1 and TRP7/3, only TRP1 primers were effective at consistently detecting DNA extracted from microsclerotia of *V. dahliae*. While this primer works very well to quantify *V. dahliae* from potato tissue and is a useful tool for potato breeding, we could not make this primer work with DNA extracted from soil. Another primer set designed from the ribosomal intergenic sequences, VDITS1/2, was also successful in detecting microsclerotial DNA, but it too was unsuccessful in detecting microsclerotial DNA extracted from soil. The most successful detection of *V. dahliae* from soil has been with the VertBtF/VertBtR primers. However, the standard curve is too flat and we have difficulty distinguishing between high and low microsclerotial numbers in soil (Figure 3). This ultimately means we have difficulty in separating fields with high amounts of Verticillium from those that have low populations of the pathogen (Figure 4).

Unfortunately, we believe we will have to do further primer development to identify gene sequences that will be useful for detecting *V. dahliae* in soil. We believe this will be possible now that the entire genome of the *V. dahliae* fungus has been sequenced.

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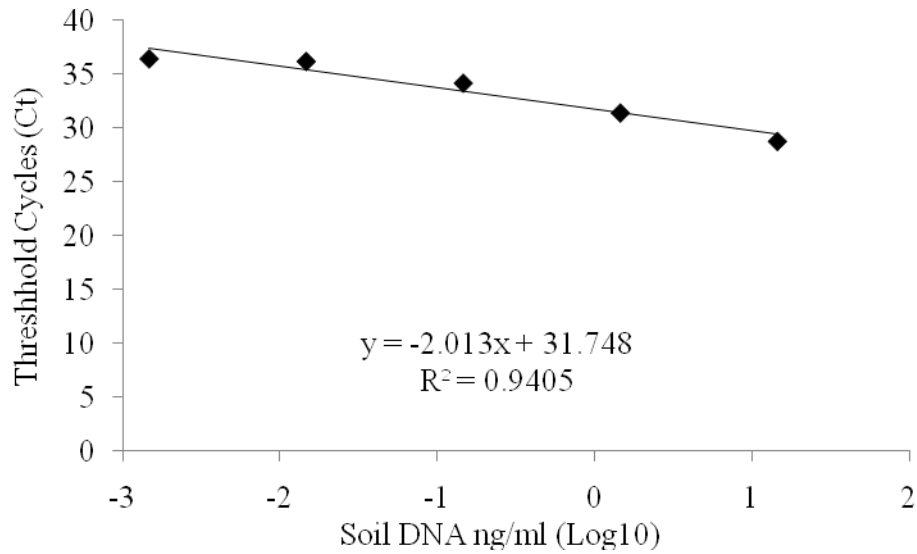


Figure 1. Relationship between ng/DNA from soil and the number of threshold cycles for real-time PCR amplification of *Spongospora subterranea* at 10-fold serial dilutions of DNA using primers SSTqF1/SSR1 and Taqman probe SSTqP1.

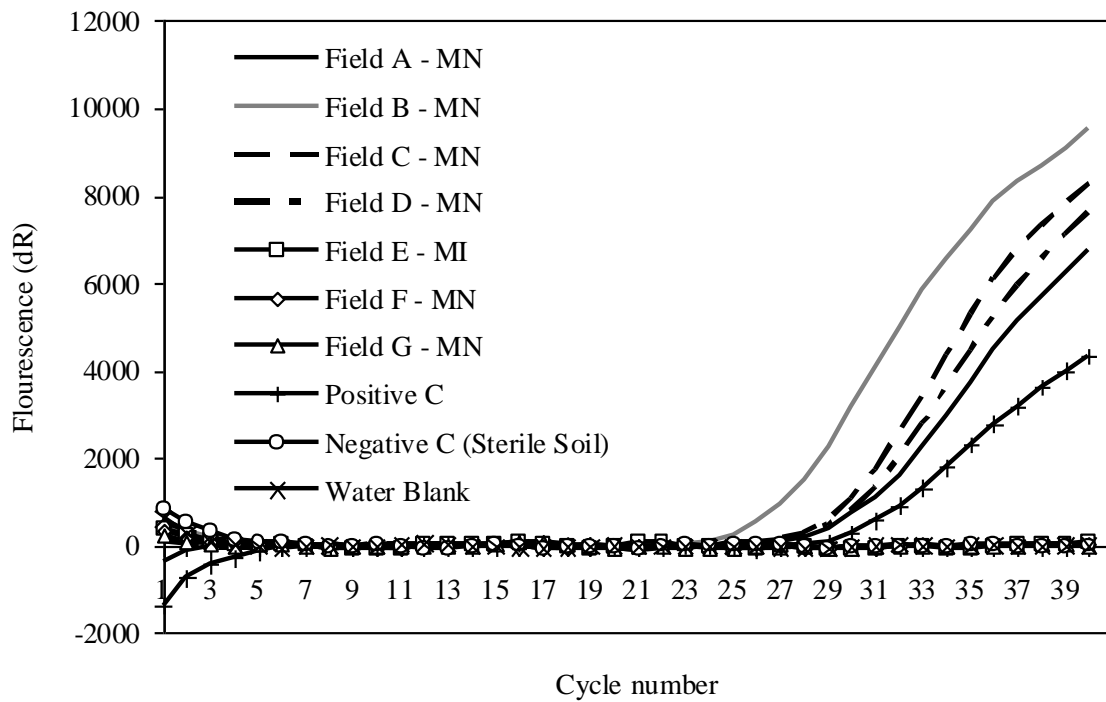


Figure 2. Quantitative PCR amplification of DNA extracted from soil using primers and Taqman probe specific for *Spongospora subterranean*.

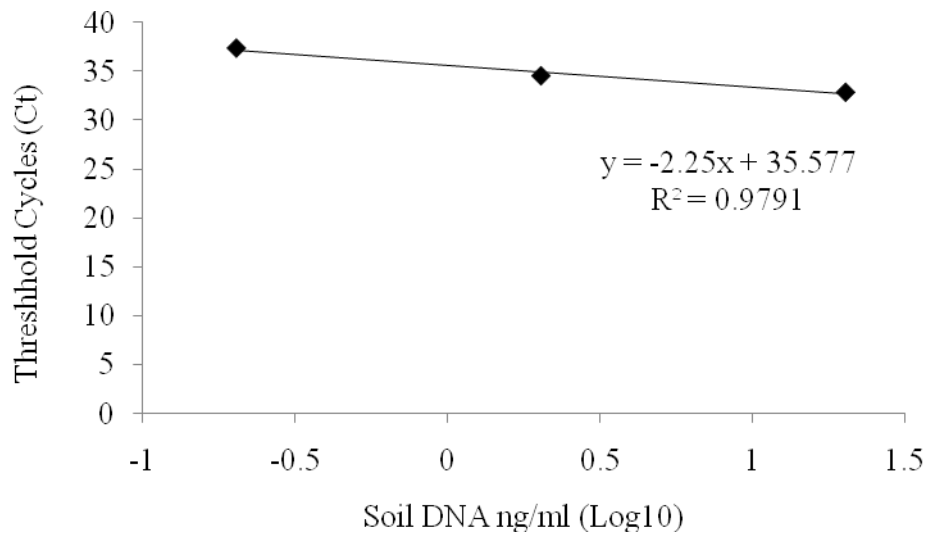


Figure 3. Relationship between ng/DNA from soil and the number of threshold cycles for real-time PCR amplification of *Verticillium dahliae* at 10-fold serial dilutions of DNA using primers VertBtF/VertBtR and Taqman probe VertBtP.

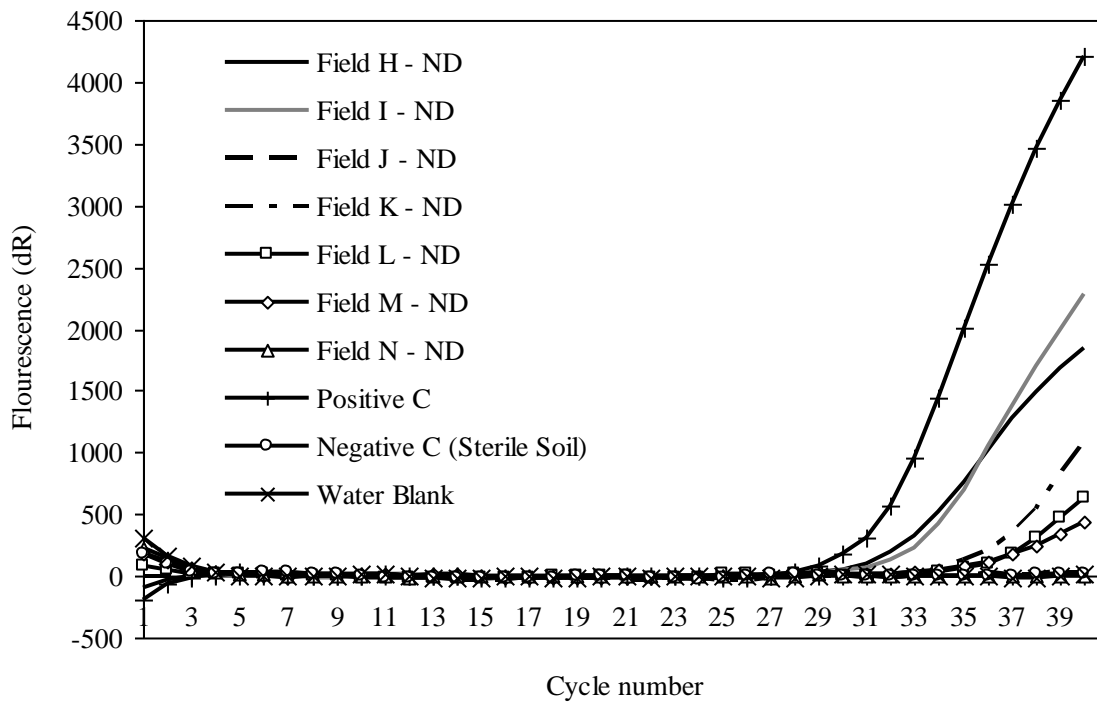


Figure 4. Quantitative PCR amplification of DNA extracted from soil using primers VertBtF/VertBtR and Taqman probe VertBtP specific for *Verticillium dahliae*.