TITLE: Development of a molecular assay for *Fusarium oxysporum* f. sp. *lactucae*

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ABSTRACT
Due to similar morphological features, lack of good molecular markers that accurately identify isolates as a pathogen of lettuce and the ability of saprophytic strains to colonize root tissue it can be very difficult to correctly identify isolates of *Fusarium oxysporum* as the pathogen responsible for Fusarium wilt of lettuce. This is particularly problematic when trying to determine if the pathogen is present in a field and estimating the inoculum density so risk of disease can be evaluated prior to planting. While there is only one race of the pathogen currently present in North America (Race 1), there are three additional races encountered in Asia and Europe; having a way to rapidly identify the different pathogen races would be important for preventing their spread to domestic production areas. Lastly, there is limited information available on the genetic variation of the pathogen, which is important information to have to not just track pathogen spread, but also support breeding efforts if there is a correlation between genotype and aggressiveness. This project uses a large database of genomic sequences from a wide range of *F. oxysporum* isolates, including isolates that cause Fusarium wilt of lettuce collected from different regions in the world, and a software program to identify useful sequences that address these problems. The results provide tools for diagnosticians and researchers that will enable accurate identification of the pathogen, facilitate pathogen quantification in soil samples, determine the evolutionary relationship among races of the pathogen and other isolates of *Fusarium oxysporum*, simplify identification of race and clarify genetic variation of the pathogen. The primary objective of this proposal is validation of diagnostic assays for *F. oxysporum* f. sp. *lactucae* Race 1 using two different technologies and optimizing techniques for using one assay for quantification of the pathogen in field soil.

OBJECTIVES:

1. Finish validating the TaqMan real time PCR diagnostic assay for *F. oxysporum* f. sp. *lactucae*. This will provide a rapid and accurate means for pathogen identification.
2. Develop background data needed to use the TaqMan assay to quantify the amount of the pathogen present in soil. This will provide growers and researchers a means to evaluate risk by determining the population levels of the pathogen in their fields and evaluation of crop rotation and other management decisions on these levels.
3. Transfer the TaqMan assay to recombinant polymerase amplification technology to provide a rapid diagnostic assay that can confirm the presence of the pathogen in an infected plant in the field within 20 minutes.

PROCEDURES

The objective of the research proposed in this submission is to fully validate the diagnostic assays (both TaqMan real time PCR and the isothermal RPA assay) and transfer the technology to the end users.

Validation of diagnostic markers

Eight primer pairs selected bioinformatically from regions of race 1 isolate were evaluated for amplification of unique DNA targets in the pathogen but not present in a range of other closely related *formae speciales* (especially the closely related Race 4 isolates) to optimize amplification conditions and test specificity. Specificity was further confirmed by laboratory testing with DNA representing the broad range of taxa in our DNA library representing a good portion of the 64 *formae speciales* we have sequence data for. One locus was selected to focus on, and it was fully validated as specific in multiple laboratory tests with a library of DNAs from other *formae speciales* and race 2, 3 and 4 of f. sp. *lactucae* as well as filed plant samples.

Recombinant polymerase amplification

The TaqMan real time PCR assay specific for only *F. oxysporum* f. sp. *lactucae* race 1 was transferred to the RPA detection chemistry. This is an isothermal technology that can be run directly in the field using a portable unit with a Bluetooth connection to a smart phone with results obtained in as little as 20 minutes without the need for DNA extraction. Specificity and sensitivity of the assay were validated as noted above for the TaqMan qPCR assay, including validation with the same diseased plants.

Quantification of *F. oxysporum* f. sp. *lactucae* in the soil

In order to accurately estimate the level of the pathogen in the soil it is necessary to compare results from soil plating to the strength of the signal generated by the qPCR assay. Given similarities in colony morphology among *F. oxysporum* f. sp. *lactucae* and other taxa in the soil it is not possible to accurately identify the pathogen when growing on agar medium. To enable an accurate way to identify isolates an isolate of the pathogen was selected for transformation with a drug marker to generate a mutant. These isolates would be easily identified when soil is plated on medium amended with the drug as they would be the only ones growing. Unfortunately, after multiple attempts to generate a transformed drug mutant we were not successful. While not ideal, the work around approach we have taken is add a conidial suspension of the pathogen to autoclaved soil amended with corn meal and allow it to incubate

several weeks under moist conditions. Following a gradual dry down of the soil to facilitate conversion of conidia to chlamydospores (the long term resting structure normally found in soil), the soil was ground and plated on a selective media to estimate population densities. This soil was diluted with autoclaved soil to generate a series of soil samples with different inoculum densities. These soils were plated on selective media to confirm pathogen populations in the soil dilution series and had DNA extracted for TaqMan real time PCR analysis.

Genotyping of isolates.

There are two approaches we are using to genotype isolates. One approach used a program called MASH to go through raw DNA sequence data and generate a database of the different sequences present (the length is user defined and can range for 20-30 base pairs – referred to as k-mers) and then conducting an analysis that graphically shows the relationship among isolates. A second more accurate way to genotype isolates we have genomic sequence data for is to map the reads from each isolate to a reference isolate and quantify the variation. We can also align the sequences and run a phylogenetic analysis to determine the evolutionary relationship among the isolates. Previously we used a reference sequence extracted from a genome assembly done using short read Illumina sequences representing 37 fragments of DNA with a total length of 7.26 million base pairs (these types of assemblies tend to be highly fragmented). This does a good job of genotyping the isolates but having a larger percentage of the genome, especially representing the more highly evolving regions of the genome, would provide a better resolution of relationships among isolates. Using funds from another source we have sequenced a race 1 and race 4 isolate using two long read sequencing technologies (PacBio HiFidelity and Oxford Nanopore), the data from which has vastly improved the resources for genotyping isolates. The completion of the assemblies will improve our understanding of the relationships among isolates from different geographical areas. This work is in progress.

RESULTS

Validation of specificity of diagnostic assays

The regions selected for designing the diagnostic assay were screened bioinformatically against the genomic sequence database we generated representing over 820 isolates of 60+ formae speciales. In lab testing, both the TaqMan real time PCR assay was confirmed to be specific when tested against a library of DNAs representing a number of other host specific *formae speciales.*

List of *F. oxysporum* isolates tested for specificity

Taxa	Race	# of isolates	Pos/Neg reaction
Folac	1	17	Pos
	4	12	Neg
Non-pathogenic on lettuce		12	Neg
41 other formae speciales		88	Neg
Non-pathogenic on other hosts		13	Neg
Environmental isolates		21	Neg
Fusarium commune (outgroup)		2	Neg
Total		**165**	

Development of the RPA assay
The same region the TaqMan assay was developed from was used to design the RPA assay. It was validated using the same DNA library as the TaqMan assay and found to be highly specific for race 1 of the pathogen.

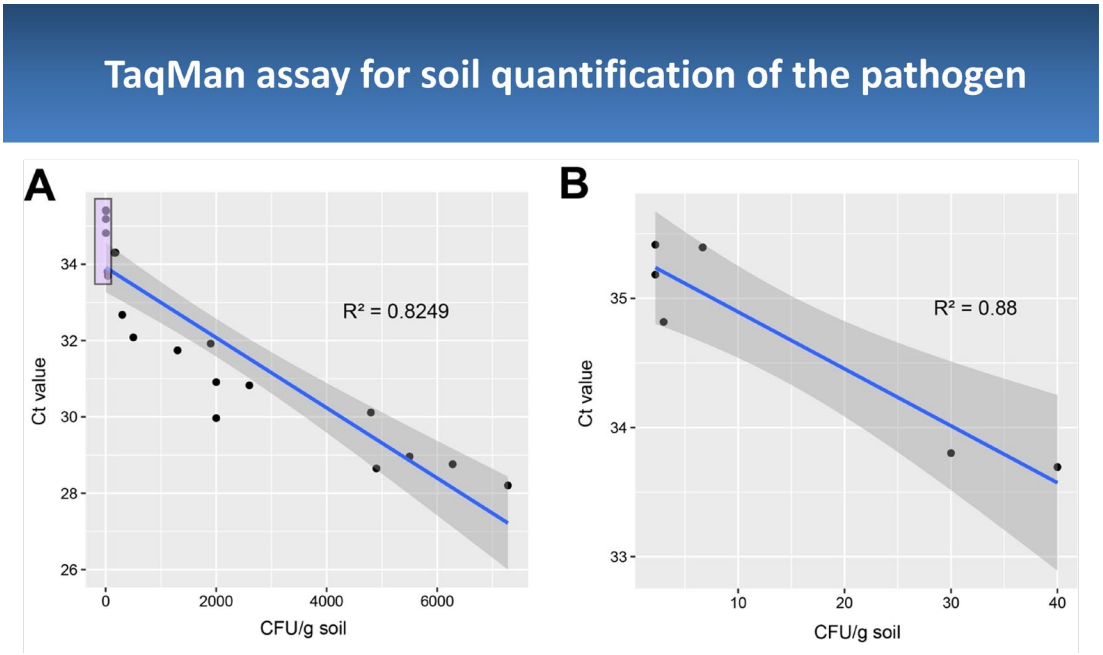
Assay validations with field samples

# of lettuce plants				Result
Symptomatic plants				
32	Positive	Positive	Positive	True positive
3	Negative	Negative	Negative	True negative
1	Positive	Negative	Positive	False negative from TaqMan assay

1 = culture based; 2 = TaqMan qPCR; 3 = RPA

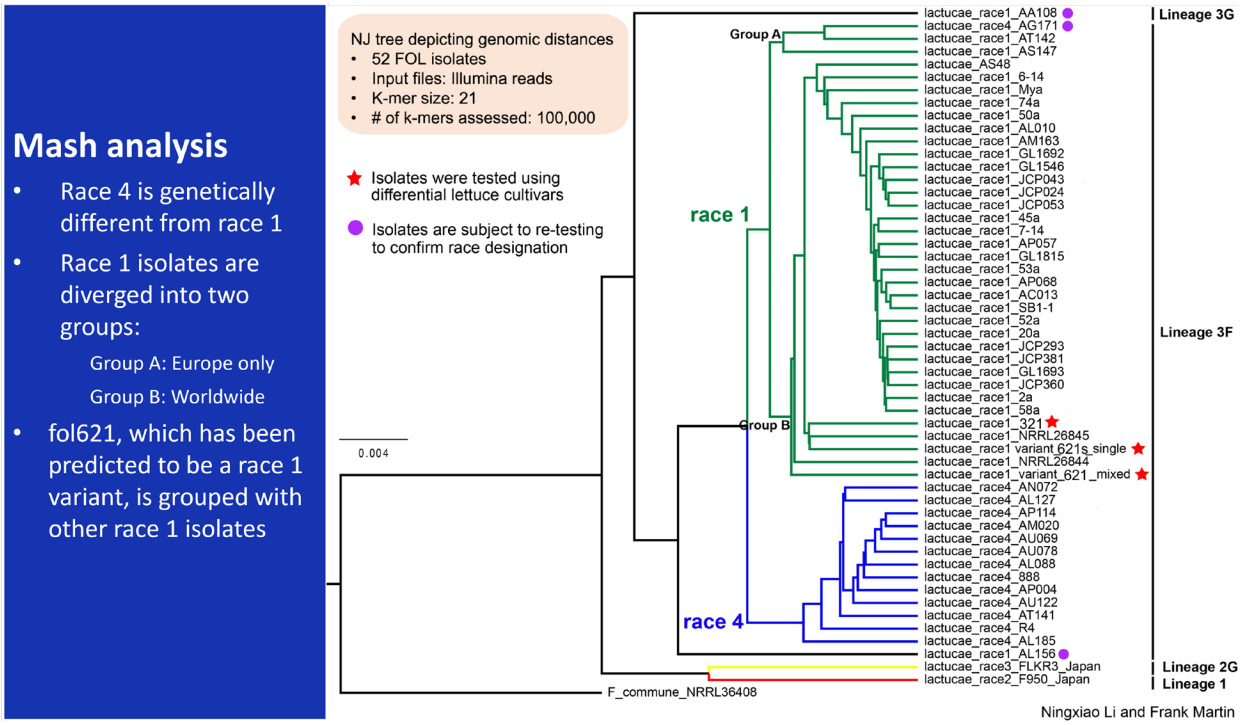
The negative result from the TaqMan assay for one sample was likely due to an error in where the tissue samples was taken for DNA extraction, as DNA extracted from the culture recovered from this plant and RPA extract were both positive when tested with the TaqMan assay.

Soil quantification assay
The results obtained using the autoclaved soil amended with the pathogen were similar to what was observed with *F. oxysporum* f. sp. *fragariae* using a drug-resistant isolate of the pathogen (Matson et al. 2024).



Genotyping of race 1 isolates

MASH analysis clearly separated out race 1 and race 4 isolates. There were a few isolates where the race designation given to us by cooperators did not correlate with where the isolate grouped. More detailed analysis with comparison of genome assemblies and the genotyping by mapping reads to a reference isolate support the placement of isolates and misidentification of race designations. Pathogenicity tests are being run to clarify. The recent race 1 variant 621 was not differentiated from other race 1 isolates in this type of analysis but is under further investigation with the genomic sequence data.



Conclusion

Two different types of diagnostic assays were developed and fully validated:

- TaqMan real time PCR assay – is a lab-based assay that is considered the gold standard technology for diagnostic assays due to its high level of specificity and sensitivity. It requires DNA extraction but can be completed within a day if the sample is provided early enough and the lab can accommodate a rapid turn-around.
    - When used with plant samples the assay has a plant targeted internal control template to confirm if the DNA extraction is pure enough for amplification (thereby preventing false negatives when pathogen detection is not observed because of contaminants in the DNA; Bilodeau et al. 2014, Miles et al. 2017, Burkhardt et al. 2017, 2018).
    - When used with extracted DNA from soil to quantify the amount of the pathogen present there is an internal control DNA that is added to the assay to evaluate the purity of the DNA to confirm the accuracy of results obtained (Bilodeau et al. 2011).
- RPA isothermal assay – this technology can determine if the pathogen is present in as little as 20 minutes from the time a sample is provided with out the need for DNA

extraction. The assay can be completed in the field using a portable device with a Blue-tooth connection to a smart phone. It is equally specific as the TaqMan assay but is not as sensitive; like the TaqMan assay does have a plant internal control to confirm the sample cane be amplified (thereby preventing false negatives; Miles et al. 2017). It is best suitable for pathogen detection in plants; at this point it is not advisable to use for soil quantification due to the level of assay sensitivity,

Isolate genotyping - MASH analysis was effective in visualizing the relationship among isolates and clarification of race designations. It did not separate isolate 621, the recently described race 1 variant. A more comprehensive genotypic analysis using the entire genome is in progress.

NOTE: Both the TaqMan qPCR and RPA assay have been made available to the diagnostic and research community. A manuscript describing the diagnostic assays is in press in the journal *Phytopathology*: https://apsjournals.apsnet.org/doi/10.1094/PHYTO-04-24-0142-R.

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