California Leafy Greens Research Program
Project Report for 2017-2018

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

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Abstract
Having the ability to rapidly and accurately diagnose *Fusarium oxysporum* f. sp. *lactucae*, the cause of Fusarium wilt of lettuce, would significantly aid efforts to control this pathogen. Due to similarities in morphology when grown in culture it is very difficult to differentiate strains of the pathogen from other strains in the soil that cannot infect lettuce or saprophytically colonize lettuce tissue. The objective of this project is to develop a molecular diagnostic assay using comparative genomics to identify unique regions of the genome that will be highly specific for the pathogen and provide a rapid means of detection. The approach is to sequence the genomes of a number of pathogenic strains and then compare these DNA sequences with a library of genome sequences that represent a wide range of *Fusarium oxysporum* strains that do not infect lettuce to identify unique regions to use as targets for the molecular assays. The genomes of 10 isolates of race 1 of the pathogen collected from California and Arizona have been sequenced; DNA from other race 1 isolates as well as other races of the pathogen not currently present in the USA (race 2, 3, and 4) obtained from collaborators in Europe and Japan and been sequenced as well. Additional sequencing of Race 1 and 4 isolates from Italy and isolates from Florida is in progress. These sequences are currently being used in a comparative genomics study to select unique regions of the lettuce wilt pathogen to target for development of a diagnostic assay. The Illumina sequence data have also been useful for examining the evolutionary relationships among races of the pathogen and development of a genotyping tool to examine distribution of specific lineages of the pathogen.

Objectives

The range objective is to develop a molecular diagnostic assay for all four races of *Fusarium oxysporum* f. sp. *lactucae* that will be useful for soil quantification of pathogen inoculum in the field as well as rapid in-field diagnostics. The technology will be transferred to the UC Cooperative Extension, commercial diagnostic labs and university researchers so it will be available to the industry.

Procedures

Given the large number of *formae specialis* in *F. oxysporum* and the fact that some isolates of the same *formae specialis* are not phylogenetically related it can be difficult to develop molecular diagnostic assays for these pathogens. The approach we used for the development of specific molecular assays for *F. oxysporum* f. sp. *fragariae* was to sequence the genomes of 5 isolates representing the different evolutionary groupings by Illumina and do a preliminary assembly of the genomes. We also sequenced the genomes of five isolates recovered from strawberry roots that were not pathogenic and used genomic sequence data that had been collected for 13 other *formae specialis* and several saprophytic isolates to identify unique sequences in f. sp. *fragariae.* These loci were then evaluated for specificity for only the pathogen by real-time TaqMan PCR amplification. To ensure specificity, the diagnostic assays were tested against purified DNA from over 45 other closely related *formae specialis* that was provided by collaborators. Thus far the marker system has been highly specific for only f. sp. *fragariae* and is currently being evaluated to determine the relationship between results of the real-time PCR assay and inoculum level of the pathogen in the soil. This approach for soil quantification has been effective for estimating pathogen inoculum densities for *Verticillium dahliae* (Bilodeau et al. 2012) and *Macrophomina phaseolina* (Burkhardt et al. 2018). In addition to the TaqMan real-time PCR assay, an isothermal assay that can be done directly in the field in as little as 10 minutes using a technology called recombinant polymerase amplification (RPA) was developed and provided to the UCCE lab for their use (they also have also been using our RPA assays for *Phytophthora*, *Macrophomina phaseolina* and *Verticillium dahliae*; see Burkhardt et al. 2018, Miles et al. 2015 and Rojas et al. 2017).

Standard techniques will be used for culturing the isolates and extracting DNA. Any additional genomic sequencing has been done at the UC Davis or Michigan State Genome Centers and the data downloaded over the web onto workstations in the PIs office. The necessary computer hardware and software are available on these workstations for processing the data, assembling the genomes and identifying unique regions by comparative genomics for developing specific molecular markers for *formae specialis lactucae*.

Results

Sequence database for bioinformatic screening

The sequence database we have developed for screening for unique sequences of *F. oxysporum* f. sp. *lactucae* now contains data for over 240 isolates representing 47 *formae specialis* of *F. oxysporum* and isolates that are not pathogenic on the host of recovery (more than 5 TB in size). This will significantly improve our ability to bioinformatically identify unique sequences to target for the development of molecular assays.

A CDFA Specialty Crops Block Grant was awarded to PI Martin in 2018 titled "Molecular detection and quantification of *Fusarium oxysporum* vascular wilt pathogens." A post-doctoral position has been filled, and a collaboration with a researcher at Penn State University who is the curator of the Fusarium Culture Collection has been established; Tom Gordon is also part of this project. This grant will support expansion of the database to include sequence data from a larger number of taxa, which in turn will help in development and validation of diagnostic markers for the different races of *F. oxysporum* f. sp. *lactucae* at no cost to the California Leafy Green Research Program.

Sequencing of *F. oxysporum* f. sp. *lactucae* isolates

A total of 10 California and Arizona isolates have been Illumina sequenced. Collaborations were established with researchers in Italy and Japan to obtain DNA from geographically diverse Race 1 isolates (four isolates from Europe and Japan) as well as Race 2, 3 (Japan, one isolate each) and Race 4 (Europe, two isolates) isolates. These isolates have been sequenced by Illumina and added to the dataset for analysis. A shipment of 15 DNAs of Race 1 and 4 isolates from Italy has been received, as has 14 isolates from Florida (and 10 isolates recovered from lettuce but not pathogenic on this crop; these are important to ensure specificity of the assay that is developed). These DNAs are being prepared for Illumina sequencing.

Collaborations have been established with Stephanie Slinski (Yuma Center for Excellence in Desert Agriculture) for her to collect isolates of the pathogen from different production areas of Arizona and provide them to Tom Gordon for pathogenicity testing on lettuce. We are looking for both *F. oxysporum* f. sp. *lactucae* and isolates of *F. oxysporum* that are recovered from lettuce but not pathogenic.

Evolutionary relationships among isolates

It is helpful to understand the evolutionary relationships among the four races as well as in comparisons with other *formae speciales* as this helps to identify other taxa to include in the first round of screening to ensure specificity. In a separate collaboration with Dr. Geiser at Penn State University (an evolutionary biologist with an emphasis on *Fusarium*) he has provided information on a suite of 41 genes that he uses for phylogenetic analysis of *F. oxysporum*. Using these genes, Race 1 and 4 were found to be closely related with Races 2 and 3 differentiated on separate clades. Data for additional *formae speciales* are in the process of being added to the dataset to provide a more comprehensive analysis.

Genotyping of isolates.

The ability to genotype isolates would be useful for evaluating variation among isolates of a particular race, identify if there are regional populations of the pathogen (useful for examining correlation with virulence and following pathogen movement) and allow for analysis of population structure within a field. Having Illumina sequence data for all isolates under study provides an opportunity to identify conserved regions in the genome of all isolates that can be used to genotype individuals. Using comparative genomics a total of 37 fragments of chromosomes totaling 7.26 million base pairs were found to be useful for this purpose. Comparison of 13 Race 1 isolates (California, Arizona, Europe and Japan) revealed a high degree of conservation with only a total of 5-10 differences noted (all single nucleotide

polymorphisms). Examining two Race 4 isolates (Europe) revealed no differences between isolates but 247 differences when compared to Race 1 isolates. In contrast, Race 2 and 3 isolates differed from Race 1 at 108,177 and 89,401 positions, respectively.

Screening for sequences unique to specific races of the pathogen

The genomic data for *F. oxysporum* f. sp. *lactucae* Race 1 has been used in our software pipeline to select regions in this race's genome that are not present in other *formae speciales.* There were a total of 540 potential unique target loci over 500 bp that were identified. Since it would be helpful to have a diagnostic assay that would detect all races of the pathogen, the data from Race 4, the race most closely related to Race 1, was screened against these 540 targets but only 11 loci were present in both races; subsequent testing revealed only 4 of these were present in all isolates. There were no loci present in all four races of the pathogen. We are awaiting the additional Illumina sequence data for isolates recovered in Italy and Florida (including nonpathogenic isolates recovered from lettuce) so these sequences can be included in the screening before lab work is started validating Race 1 specific detection assays.

Evaluation of published diagnostic assays for the pathogen

Ortega et al. (2018) recently published a manuscript describing an isothermal diagnostic assay for detection of *F. oxysporum* f. sp. *lactucae*. Unfortunately, the specifics of how the assay works was not provided (for example, no information on amplification primers) but the region used for developing the assay was identified. Using this region to run through our pipeline to evaluate specificity suggest the assay may not be specific; several additional *formae speciales* they did not include in their specificity screen had identical sequences (f. sp. *melonis, fragariae*, and several other *formae speciales*…).

Discussion

Fusarium oxysporum is an important lethal vascular wilt pathogen of a wide range of specialty crops in CA with limited options aside from host resistance for their control. Morphological variation among isolates is not observed, but host range differences are, with host specific isolates classified as "*formae specialis*" (or f. sp.) according to the host they infect. For example, isolates that infect only strawberry are *F. oxysporum* f. sp. *fragariae,* those that infect only lettuce are *F. oxysporum* f. sp. *lactucae*. Approximately 100 *formae speciales* of host specific *F. oxysporum* have been described, with 30-40 regularly encountered in agricultural production systems.

There is limited ability to identify & quantify particular *formae speciales* in soil plating assays, making it difficult to assess the potential for disease to occur in a field. In addition, plating assays can take days to complete, and do not always provide an accurate soil quantification due to the presence of isolates that are nonpathogenic on the host under study. The development of a molecular diagnostic assay would address potential problems with plating assays.

Fusarium wilt of lettuce had been reported only from Japan until 1993 when it was observed from several fields in the area of Huron, CA. The pathogen has since spread to

Arizona in 2001 and is becoming an increasing production problem in the California and Arizona production areas. Currently, only race 1 isolates have been identified in the US production areas, but isolates representing race 2, 3 and 4 have been reported in Japan and Europe; Gilardi et al. 2017). Having the ability to rapidly and accurately determine if the pathogen is not just found in a field, but also the amount of it that is present will help growers evaluate the risk of disease based on quantitative knowledge of the pathogen. The availability of rapid detection techniques (the isothermal RPA assay) will also help growers and the research community rapidly diagnose diseased plants and evaluate how cropping practices influence pathogen populations in the soil, thereby contributing to the development of a more integrated control program.

The genomes of ten isolates of *formae speciales lactucae* race 1 recovered from California and Arizona have been sequenced with Illumina technology. Additional race 1 isolates from Italy (3) and Japan (1) as well as isolates representing race 2 (Japan, 1 isolate), race 3 (Japan, 1 isolate) and race 4 (Italy, 2 isolates) have been sequenced as well with sequencing in progress for additional isolates from Italy and Florida. Comparative genomics is in progress, once unique regions present in all races of the pathogen have been identified they will be tested for specificity using TaqMan real-time PCR against a broad array of *formae speciales* we currently have DNA for. Once the TaqMan assay has been fully validated the marker will be transferred to the isothermal RPA platform so a rapid, in-field detection assay will be available to diagnosticians.

Of the isolates sequences thus far the following conclusions can be drawn: 1) Race 1 and 4 isolates are closely related and more distant from Race 2 and 3 isolates and 2) the Race 1 isolates from California, Arizona, Italy and Japan are the same clone (only 5-10 single nucleotide polymorphisms out of 7.26 million bp).

While only race 1 is currently present in the USA our intention is to develop a marker that will identify all four races so the tools will be available for detecting the other races in the event they are introduced into our production areas. Preliminary phylogenetic analysis using the genomic data indicates that race 1 and 4 are closely related with race 2 and 3 a little less so. We are in the process of expanding this analysis to include a broader array of *formae speciales*. In the event a single assay is not able to detect Race 1 and 4, assays for individual races will be developed.

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