California Leafy Greens Research Program
Research Proposal for 2022-2023

I. IDENTIFICATION

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

B. Funding year: April 1, 2022 to March 31, 2023

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E. Total funds requested: $30,000

F. Locations where research will be performed: USDA-ARS facility in Salinas.

II. IMMEDIATE OBJECTIVES

The immediate objectives are:

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.

Development of a diagnostic marker for *F. oxysporum* f. sp. *lactucae* Race 1was supported by a California Department of Food and Agriculture Specialty Crops Block Grant awarded to the PI that has since expired. Genomic sequencing was completed for a world-wide collection of isolates and a significant amount of bioinformatic work was done to design the assay and confirm its should be specific. Laboratory validation is still in progress.

III. 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 Japan 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 will 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.

IV. RATIONALE

A. Significance, need, and benefit to lettuce industry

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 106 *formae speciales* of host specific *F. oxysporum* have been described, with 30-40 regularly encountered in agricultural production systems.

Due to similarity in morphology, the lack of adequate molecular markers to definitively identify all isolates to *formae specialis* (we have run into problems with pathogens and nonpathogens recovered from a host having the same DNA sequence for the genes commonly used to identify isolates) and commonly recovering saprophytic taxa from the roots of diseased plants; pathogenicity tests are often needed to confirm pathogen identification.

In addition, there is limited ability to identify & quantify particular *formae specialis* in soil plating assays, making it difficult to assess the potential for disease to occur in a field. Plating assays can also 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 there are four races of the pathogen described, with only Race 1 found in California and Arizona. 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 quantification techniques will also help growers and the research community evaluate how cropping practices influence pathogen populations in the soil, thereby contributing to development of a more integrated control program. Development of procedures to differentiate races on a molecular level will also help identify introduction of races currently not present in North America and prevent their distribution. Development of resources for genotyping isolates will assist in investigating the pathogen population structure, tracking movement of new introduced isolates and evaluation if there is a correlation between genotype and aggressiveness of the pathogen.

B. Previous research relevant to the proposal:

The PI has an active research program developing diagnostic assays for a range of strawberry pathogens that will support the efforts of this proposed research. A TaqMan real time PCR diagnostic assay for detection and soil quantification of *Verticillium dahliae* that is accurate down to 1-2 microsclerotia/g soil has been published (Bilodeau et al., 2012) and describes an efficient method for DNA extraction from soil. A TaqMan assay that provides a systematic

approach for detection of *Phytophthora* at a genus and species-specific level was also reported (Bilodeau et al. 2014), the scope of pathogen detection has recently been expanded (Miles et al. 2016, Rojas et al. 2016) and will be evaluated for soil quantification of pathogen inoculum. Projects designing and validating a genotype specific assay for *Macrophomina phaseolina* and an assay for *Fusarium oxysporum* f. sp. *fragariae* for soil quantification have been published (Burkhardt et al. 2018, 2019). The objective is to have the ability to run all these assays concurrently and provide the strawberry industry with a means to evaluate risk of disease from lethal pathogens in a simple multiplexed assay.

In addition to TaqMan real time PCR assays, several rapid isothermal assays have also been developed that allow for detection directly in the field without the need for DNA extraction in as little as 20 minutes. The technology that is used, recombinant polymerase amplification (RPA) can be done with a portable hand-held unit that costs less than $3,000 and is run by an app on a smart phone. The above noted TaqMan assay for *Phytophthora* was modified to work with the RPA technology and validated with a number of field samples (Miles et al. 2015). We currently have a material transfer agreement with the company that produces the RPA technology for them to produce kits with our system for expanded field validation trials. The technology was transferred to Steve Koike's lab when he was located at UC Cooperative Extension in Salinas and is actively being used for detection of *Phytophthora* in strawberry production. In addition, the TaqMan assays for *V. dahliae*, *M. phaseolina* and *F. oxysporum* f. sp. *fragariae* have been transferred to the RPA platform (Burkhardt et al. 2018, 2019). These were transferred to diagnostic labs in the area a workshop held May 2019 so they have the ability to rapidly identify the four major lethal pathogens of strawberry.

Given the large number of *formae specialis* in *F. oxysporum* (over 104) and the fact that some isolates of the same *formae specialis* evolved independently and are not phylogenetically related it can be difficult to develop molecular diagnostic assays for these pathogens. The approach we used for 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 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. To ensure specificity the diagnostic assays were tested against purified DNA from other *formae specialis* that was provided by a collaborator. The marker system has been highly specific for only f. sp. *fragariae* and was evaluated to determine the relationship between results of the real time PCR assay and inoculum level of the pathogen in the soil (sensitive to below 10 colony forming units per g soil). This same approach was also used for designing an effective genotype specific assay for *M. phaseolina* (a specific genotype is highly aggressive on strawberry and is the predominant pathogen in California so a way to differentiate these isolates from other isolates that are either not pathogenic or aggressive was needed).

C. Long range objectives/time frame:

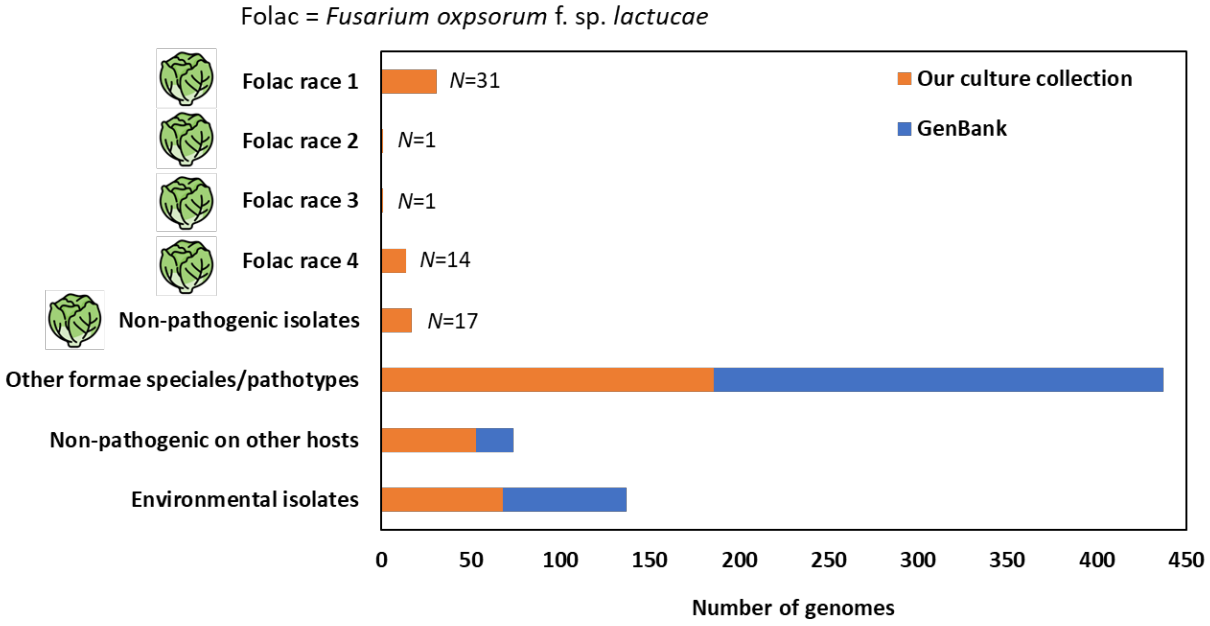
The long-range objective is to develop a more comprehensive molecular resource for *Fusarium oxysporum* f. sp. *lactucae*:

- Validate diagnostic assays capable of identification of Race 1 of the pathogen and transfer technology to end users so they can improve diagnostic capabilities – end of 2022
- Understanding the evolutionary relationships among different races of the pathogen and how these races are related to other *formae specialis* - end of 2022. This is important to ensure appropriate screening for specificity of the diagnostic assay; data also useful for identification of isolates recovered from the field.
- Genotype additional isolates and investigate the population structure of the pathogen.
    - Additional Race 1 isolates from other parts of the world and look to see if there is a correlation between genotype and geographic location. – end of 2022. This will enable researchers to identify if new strains are introduced into the U.S. as well as support breeding efforts if a correlation is found between genotype and aggressiveness of the pathogen.
- As time allows, generate data similar to what was described above for race 2, 3 and 4 of the pathogen. At some point in time these races may be introduced into North America, having the appropriate research tools available will facilitate their identification and management – end of 2023

V. SUMMARY OF PRIOR ACCOMPLISHMENTS WITH *Fusarium oxysporum*

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 750 isolates representing 64 *formae speciales* of *F. oxysporum* and a number of isolates that are not pathogenic on the host of recovery.



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

In addition to the California (eight), Arizona (two), Japan (one) and Europe (three from Italy), race 1 isolates Illumina sequenced previously, we have added data from Florida (six) as well as

nonpathogens recovered from lettuce roots (15 isolates from California and seven from Florida). Data was also previously generated for Race 2 and 3 (one isolate each from Japan). A collaborator in Europe provided 33 cultures of race 1 and 4 to collaborator Geiser representing all areas of the world where these pathogens have been recovered; their genomes were sequenced and added to the database for a total of 72 isolates. The isolate recovered by Kelley Richardson representing a new phenotype has been sequenced.

Identification of unique regions in Race 1 isolates – diagnostic assay development
We have two software pipelines that approach the task differently where a comparative analysis of sequences between the pathogen and all the other isolates we have in our genome database is done to identify unique sequences that can be targeted for development of a diagnostic assays. Using them there are a total of 25 unique loci that passed several rounds of screening to ensure they were not present in other taxa. Eight of these were selected for screening in the lab for developing the TaqMan diagnostic assay.

Soil quantification assays
Prior soil quantification assays used DNA extracted from 0.5 g soil and worked fine for multicellular resting structures of *Verticillium dahliae* and *Macrophomina phaseolina* (Bilodeau et al. 2012, Burkhardt et al. 2018). However, due to the single cell resting structure for *F. oxysporum* greater sensitivity was needed to get the required sensitivity. Mike Matson, a post doc in the Martin lab, has developed a DNA extraction procedure for 15 g of soil that provides sensitivity and accurate detection in the range of 7 colony forming units per g soil, which is suitable for our needs. This experimentation was done with a drug resistant strain of *F. oxysporum* f. sp. *fragariae* to facilitate identification of the pathogen on culture plates and needs to be repeated for *F. oxysporum* f. sp. *lactucae* (see proposed research). Once completed this will provide a means for growers to quantify the amount of the pathogen in their fields, evaluate how rotation practices impact pathogen population densities and make management decisions to minimize losses.

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 closely related taxa; multiple isolates of these will be included in the first round of screening to ensure specificity (this is particularly important if they are recovered as saprophytes form lettuce plants). In a separate collaboration with Dr. Geiser at Penn State University (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. This data is important for making sure we adequately evaluate diagnostic assay specificity.

The 41 genes used for understanding the evolutionary relationships among *F. oxysporum* were examined for their ability to improve identification of unknown isolates. Six genes were identified that more than doubled the ability to discriminate among isolates compared to the current standard approach using the gene translation elongation factor 1alpha. This improves our

ability to identify possible *F. oxysporum* f. sp. *lactucae* isolates from field collections (including identifying if new races are introduced as these loci can differentiate all four races of the pathogen).

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 Race 4 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. The more recently sequenced isolates are currently being added to the dataset. This data supports investigations on the evolution of new genotypes of the pathogen and identify introduction of new genotypes/races into a production area.

VI. 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.

List of *F. oxysporum* isolates tested for specificity

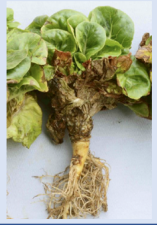
Taxa	Race	# of isolates	Pos/Neg reaction
Folac	1	17	Pos
	4	12	Neg
Non-pathogenic on lettuce		12	Neg
41 other formae speciales		88	Neg
Non-pathogenic on other hosts		13	Neg
Environmental isolates		21	Neg
Fusarium commune (outgroup)		2	Neg
Total		**165**	

Validation of diagnostic markers
Eight primer pairs selected bioinformatically were evaluated for amplification of unique DNA targets in the pathogen but not 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 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. A real time PCR (qPCR) assay was designed using TaqMan probes and demonstrated to detect all race 1 isolates but was highly specific when tested against DNAs from other *formae speciales,* including *F. oxysporum* f. sp. *lactucae* Race 4 isolates. In collaboration with Steve Koike at TriCal Diagnostics the assay was also validated with diseased plants recovered from the field with qPCR results compared to fungal isolations that were made; results supported the accuracy and sensitivity of the assay.

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.

# 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.

> **NOTE:** Both the TaqMan qPCR and RPA assay have been made available to the diagnostic and research community. A manuscript describing the techniques is in preparation with plans to submit to a journal in the near future.

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. We are re-examining procedures and will investigate this again in the future.

Genotyping of isolates.
A highly 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 form different geographical areas.

REFERENCES

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