CALIFORNIA LEAFY GREENS RESEARCH PROGRAM
Annual Report for Development of a molecular diagnostic assay for *Pythium uncinulatum*

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ABSTRACT

Pythium root rot and wilt of lettuce plants caused by *Pythium uncinulatum* is becoming an increasing problem in Salinas Valley lettuce production. Currently there are no techniques for quantification of the pathogen in the soil and while culturing the pathogen on selective medium can be done to confirm the presence of the pathogen, this takes several days to complete. With this project we propose to use two different molecular diagnostic technologies for developing a diagnostic assay for detection of the pathogen. A TaqMan real time qPCR will be developed first to confirm the uniqueness of the region of the genome targeted by the assay as well as provide a means for quantifying the pathogen in soil samples. Next an isothermal assay using recombinant polymerase amplification (RPA) will be developed using the same region as the TaqMan assay. The advantage of the RPA technology is it does not require DNA extraction and can be completed directly in the field in as little as 20 minutes, thereby providing a rapid, field deployable diagnostic assay.

Significance, need, and benefit to lettuce industry

In 2011 a new disease of lettuce was observed in the Salinas Valley exhibiting symptoms of stunting and yellowing of outer leaves apparent shortly after thinning (Koike and Martin, 2012). As the season progressed the stunting became more severe with a larger percentage of leaves turning yellow. When plants were dug up the feeder and tap roots were rotted and discolored. The pathogen responsible for this disease is *Pythium uncinulatum*, which was first described as a pathogen of lettuce in 1978 in the Netherlands (Blok and van der Plaats-Niterink 1978) and has subsequently been observed in Japan in 2010 (Matsuura and Kanto 2010). The frequency and severity of the disease has been increasing in the Salinas Valley since its first reported observance in 2011 (Koike and Martin 2012). A diagnostic assay for detection using a technology call loop-mediated amplification (LAMP) has been reported (Feng et al. 2019) but it has not been evaluated for quantification of the pathogen in soil samples. It also takes over an hour to run the assay, which limits its utility for direct in-field diagnostics.

Having a rapid means for identification of the pathogen will 1) enable accurate and rapid quantification of the pathogen in field soil and 2) facilitate proper in-field diagnosis of the pathogen, which in turn facilitates the proper selection of oomycete specific chemical controls (as an oomycete, this pathogen is not controlled by fungicides typically used to control fungal pathogens).

Previous research relevant to the proposal:

Much of the background research needed to identify a highly specific region in the *P. uncinulatum* genome to target for development of the diagnostic assay has been completed. PI Martin has Illumina genomic sequence data for a wide range of oomycetes, reflecting over 800 isolates representing 255 species (including *P. uncinulatum* and 133 other *Pythium* species, which is nearly all described species) and 18 genera. The mitochondrial genomes for all of these isolates have been assembled and comparative genomics has identified a region to target for assay development. This approach has been successful for developing diagnostic assays for *Phytophthora* (Miles et al. 2017, Hao et al. 2017, Rojas et al. 2017), downy mildews (*Bremia lactucae*, Kunjeti et al. 2016; *Peronospora effusa*, Klosterman and Martin, unpublished; *Pseudoperonospora cubensis* and *P. humuli*, Martin, unpublished; *Plasmopara destructans*, LeBlanc, Crouch and Martin, unpublished), and *Aphanomyces* (Martin and Chanda, unpublished). While a post doc on the Martin lab, collaborator Miles worked on development of a systematic approach for designing a genus and species-specific diagnostic assay for *Pythium*. With his departure to take a position at Cal State Monterey Bay and then Michigan State University the project was put on the back burner. He is now in a position to reactivate the research and include marker development for *P. uncinulatum* as an objective of the project.

When in the Martin lab collaborator Miles developed the RPA assay for *Phytophthora* (Miles et al. 2015) and has extensive experience working with development of TaqMan qPCR assays (Miles et al 2017). He has also worked closely with the Chilvers lab on development of diagnostic assays using both TaqMan and RPA technologies (Rojas et al. 2017, McCoy et al 2020). He recently led the collaborative effort with the developers of the RPA technology (TwistDx, Oxford, UK) to evaluate preformulated kits they produced for us that included our primers and probe for detection of *Phytophthora*. This was recently published with collaborator McCoy as the lead author (McCoy et al. 2020).

OBJECTIVES

Objective 1: Develop a sensitive and highly specific TaqMan real time qPCR assay for detection of *P. uncinulatum*.

Objective 2: Once the TaqMan assay has been validated and confirms the DNA region targeted is highly specific, the targeted region will be used to develop an isothermal RPA assay for rapid pathogen detection.

Objective 3: The TaqMan assay will be validated for determining the population of the pathogen in field soil.

PROCEDURES

This project will be done in collaboration with colleagues at Michigan State University. Techniques used will be the same as reported in the above cited publications from the participant's labs. The TaqMan real time qPCR assay will be developed first since the parameters needed for designing an effective assay are well known. Validation of this assay will confirm the region of the genome targeted for the assay is highly specific as well as provide a tool for quantifying the pathogen in field soil. The parameters for designing a highly specific RPA assay are less well understood (which is why we use the TaqMan assay to confirm the uniqueness of the targeted DNA sequence), but since the overall design approach of the assay is very similar to TaqMan (using specific primers and a probe) it is straightforward to transfer the TaqMan assay to an RPA assay (different primer combinations will need to be tested, but the general approach has worked well for us in the past). The division of labor for year 1 of this project is:

<u>Martin lab</u>

- Complete bioinformatic work identifying targeted regions of the mitochondrial genome and provide DNA sequence data to MSU collaborators.
- Provide DNA samples of *Pythium* species our MSU collaborators do not have
- Test the TaqMan qPCR assay developed by our MSU collaborators to ensure transferability of the technology, specificity, and sensitivity of detection when the assay is run in another lab

<u>Miles and Chilvers labs</u>

- Design TaqMan qPCR primers and probe specific for *P. uncinulatum*
- Optimize amplification conditions when amplifications are done individually and multiplexed with an internal control to evaluate if PCR inhibitors are present
- Test for specificity and sensitivity using purified DNA and mock plant samples
- Validate with field samples collected from the Salinas Valley
- As time allows, work on using this TaqMan assay for quantification of the pathogen in soil samples.
- As time allows start working on transferring this TaqMan assay to the RPA detection platform

RESULTS and DISCUSSION

Objective 1: Develop a sensitive and highly specific TaqMan real time qPCR assay for detection of *P. uncinulatum.*

Using a comparative genomic approach with assembled mitochondrial genomes representing over 100 *Pythium* species it was observed that *P. uncinulatum* has a potential open reading frame 1,107 bp long that is not found in other species (*orf368*). Searching mitochondrial genomes of other species revealed the sequence of *P. uncinulatum orf368* was unique. Primers and probes were designed for two regions of this sequence and evaluated for specificity of detection with one selected for subsequent optimization and evaluation for specificity reported below.

Sensitivity of detection - a dilution series of purified DNA was evaluated for the relationship between DNA concentration and the amplification cycle when the results became positive (referred to as the Ct). The relationship was linear from 10 to 0.0001 ng (0.0001 ng = 100 fg) but at DNA concentrations of 10 fg amplification was inconsistent so the cut off for accurate and consistent detection of the pathogen was set at a Ct of 32.8 (Figure 1).

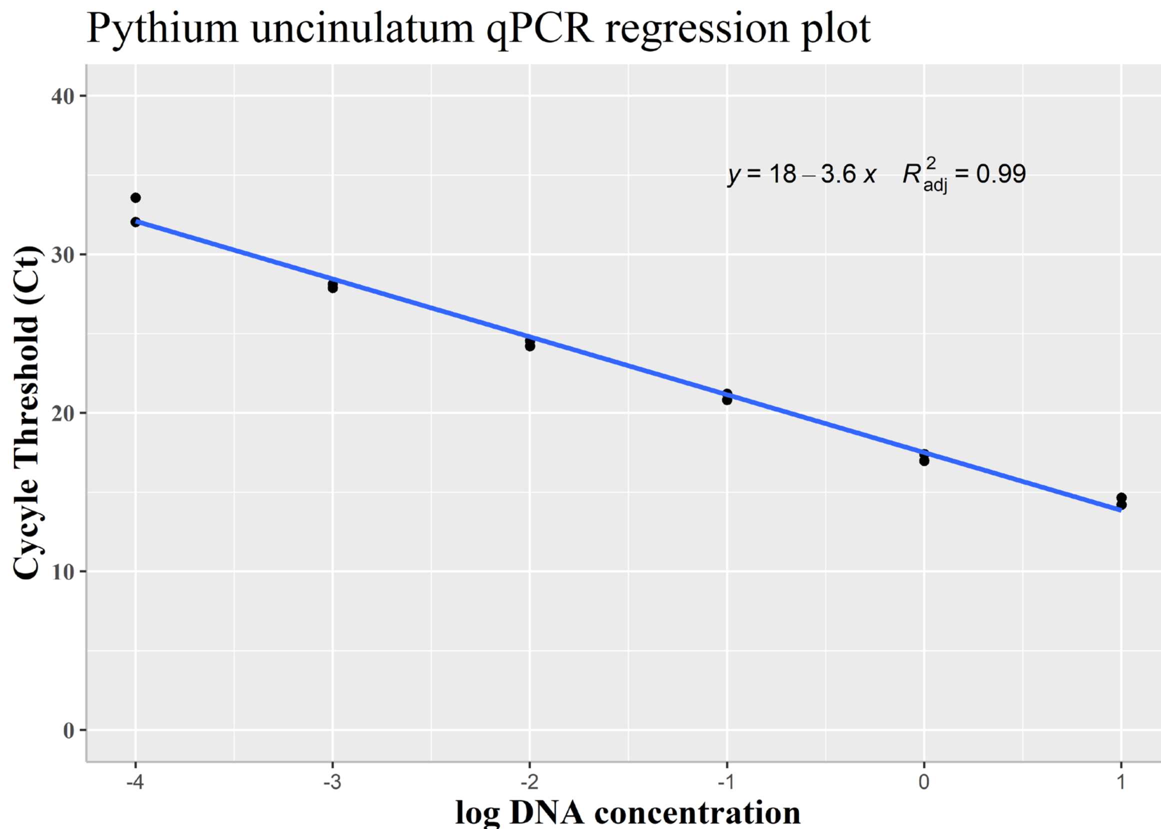

Figure 1. Relationship between *Pythium uncinulatum* DNA concentration (ng/sample) and when the amplification cycle when the results was considered positive (cycle threshold, Ct). DNA concentrations below 0.0001 ng (100 fg) did not have consistent amplification so the Ct for the 100 fg dilution was selected as a cutoff for accurate quantification.

Specificity of detection – Specificity of the diagnostic markers was tested against 36 *Pythium*, two *Phytopythium* and three *Phytophthora* species. At a concentration of 0.1 ng DNA the only sample amplified was for the lettuce pathogen, *Py. uncinulatum* (Table 1). These evaluations were repeated at a concentration of 10 ng DNA with the same results. Currently, the assay has been validated with a single isolate of *Py. uncinulatum,* collection of additional isolates to confirm specificity is currently in progress.

Table 1. Specificity test using 0.1ng of *Py. uncinulatum* DNA. *Pythium* clade refers to the phylogenetic grouping of species in Levesque and de Cock (2004)

Isolate number	Species	*Pythium* clade	Average Ct
C-INSO 2-15	*Py. monospermum*	A	-
AR_262.S.1.6.A	*Py. aphanidermatum*	A	-
NDSO l 10-7	*Py. adherens*	B	-
AR_15.S.2.7.A	*Py. aff. torulosum*	B	-
V-ARSO2 3-46	*Py. angustatum*	B	-
C-ARSO2 5-12	*Py. catenulatum*	B	-
C-MISO2 5-4	*Py. contiguanum*	B	-
CBS_220.94	*Py. folliculosum*	B	-
SDSO 7-31	*Py. kashmirense*	B	-
INSO 1-8A	*Py. lutarium*	B	-
ILSO 5-49B	*Py. oopapillum*	B	-
IASO 10.16.27	*Py. torulosum*	B	-
C-KSSO2 5-11	*Py. acanthicum*	D	-
C-MICO2 5-16	*Py. oligandrum*	D	-
MNSO 2-36	*Py. minus*	E	-
C-MISO2 1-11	*Py. acrogynum*	E	-
V-SDSO2 4-66	*Py. carolinianum*	E	-
ILSO 2-19B	*Py. longisporangium*	E	-
CBS_112350	*Py. ornacarpum*	E	-
CBS_217.94	*Py. radiosum*	E	-
CBS_129728	*Py. selbyi*	E	-
C-NDSO2 3-2	*Py. caroliniarum*	E	-
C-KSSO2 5-20	*Py. longandrum*	E	-
IASO 6-38.41R	*Py. attrantheridium*	F	-
AR_127.S.2.3.A	*Py. irregulare*	F	-
AR_260.S.2.8.A	*Py. kungmingense*	F	-
INSO 4-40	*Py. spinosum*	F	-
NDSO 1-42	*Py. sylvaticum*	F	-
C-NDSO2 6-1	*Py. nagaii*	G	-
CBS_157.69	*Py. undulatum*	H	-
NESO 4-30	*Py. heterothallicum*	I	-
C-ILSO2 3-25	*Py. ultimum*	I	-

KSSO 6-30	*Py. ultimum var. sporangiferum*	I	-
Pyuncin_7	*Py. uncinulatum*	J	22.45
V-INSO2 2-52	*Py. nodosum*	J	-
V-NDSO2 2-55	*Phy. aff. rosacearum*	Phytophthora	
MICO 2-45	*Phy. dreschleri*	Phytophthora	-
KSSO 6-1	*Phy. sansomeana*	Phytophthora	-
C-KSSO2 1-25	*Phytopy. litorale*	Phytopythium	-
V-MISO2 6-46	*Phytopy. megacarpum*	Phytopythium	-
NTF5B-16	*Saprolegnia sp.*	Saprolegnia	-
water only control	water	-	-

Objective 2: Work to fully validate the assay needs to be completed before RPA development and validation are progressed. This includes the collection of more *Pythium uncinulatum* isolates, as well as infected plant samples to conduct final validation on.

Validation of the diagnostic assay in other labs – the procedure has been shared with the Martin lab and the Hasegawa lab for testing with plant samples. This work will continue in the current funding cycle of the grant and if specificity is maintained, development of a field deployable rapid isothermal RPA assay will commence.

Objective 3: The TaqMan assay will be validated for determining the population of the pathogen in field soil.

Pathogen detection in soil - Field soils were collected from naturally infested commercial lettuce fields in the Salinas area with diseased plants. A procedure for extraction DNA from 15 g of soil developed by Mike Matson in the Martin lab was used to provide soil DNA from these samples to the Michigan State collaborators for analysis with the qPCR assay. To evaluate if PCR inhibitors were present in the extracted DNA the samples were run three ways; undiluted, with a 1 to 1 dilution with deionized water and a 1 to 5 dilution. It is important to remember that a lower cycle threshold (Ct) means a higher DNA concentration, so the lower the bars in Figure 2, the higher the inoculum density in this soil.

The pathogen was detected in all soils and with the exception of soils 8 and 9, in all dilutions (Figure 2). Diluting the samples 1 to 1 and 1 to 5 will increase the Ct due to having a lower DNA concentration but if PCR inhibitors are present this dilution may lead to a lowering of the Ct due to a more efficient amplification. In none of these samples was there a reduction in Ct following dilution, suggestion PCR inhibitors, if present, were in low amounts and not having a major impact on results. An exception to this may be in sample 8, where there was a low Ct for one of the undiluted DNAs but inconsistent amplification was observed in all treatments. Given the high Ct for the undiluted DNA in sample 9 it is likely the lack of consistent amplification in the 1 to 5 dilution is due to very low pathogen DNA being present. The horizontal dashed red line represents the threshold level for quantification, below this line an accurate quantification of the pathogen DNA can be made, above it we can only conclude the pathogen was present but at levels too low to quantify.

Quantification of the positive soil DNA samples showed that *Py. uncinulatum* was present at approximately $1x10^{-3}$ to $1x10^{-5}$ ng; how this relates to inoculum density in the soil has yet to be determined. This provides evidence that this assay can be used to determine the presence, absence, and abundance of *Py. uncinulatum* within soil DNA extractions. Further work will be needed to fine tune this diagnostic qPCR assay for quantification of *Py. uncinulatum* within soil samples. This work would include addition of an internal control to make the assay a multiplexed detection of the pathogen DNA coupled with control DNA to evaluate if PCR inhibitors were present and affecting the accuracy of results (Bilodeau et al. 2012). The correlation between the Ct and pathogen inoculum density is also needed to evaluate risk to the grower (Bilodeau et al, 2012, Burkhardt et al. 2018).

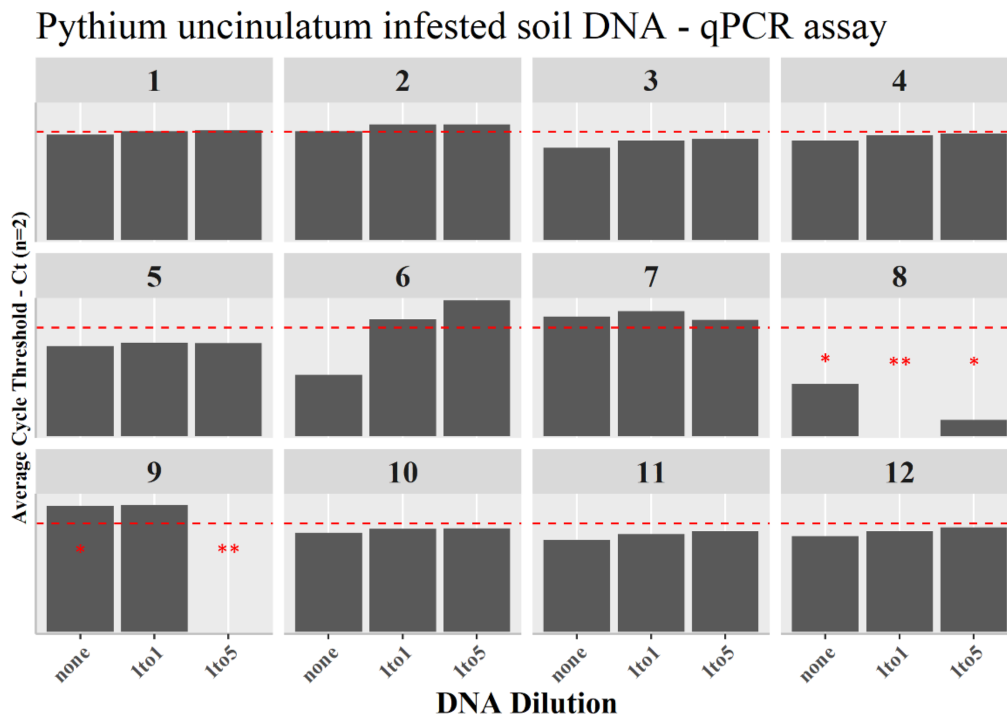

Figure 2. Results of the qPCR assay for *Py. uncinulatum* for 12 soil samples collected from commercial lettuce fields in the Salinas Valley exhibiting disease symptoms caused by this pathogen. DNA samples were run in three configurations, undiluted DNA, diluted 1 to 1 with water, and diluted 1 to 5 with water. The horizontal red dashed line represents the threshold for accurate quantification (Ct 32.8). **Important to note that the lower the cycle threshold (Ct) the earlier the detection occurred due to greater amounts of the pathogen's DNA being present (there was a higher inoculum density in these samples).** Graphs with an * are samples with inconsistent amplification.

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