USDA ARS
Crop Improvement and Protection Research Unit

Rapid Molecular Diagnostics for Strawberry Soilborne Pathogens

CSUMB

Phytophthora cactorum
Photo: F. J. Louws

Verticillium dahliae (wilt)

Photos: Steve Koike

Macrophomina phaseolina (charcoal rot)

Fusarium oxysporum (Wilt)

UC CE

Timothy Miles, Alyssa Burkhardt,
Frank Martin, Marina Ramon, Steve Koike,
Stacy Mauzey

Diagnosis:

Macrophomina
Fusarium
Verticillium
Phytophthora

?

?

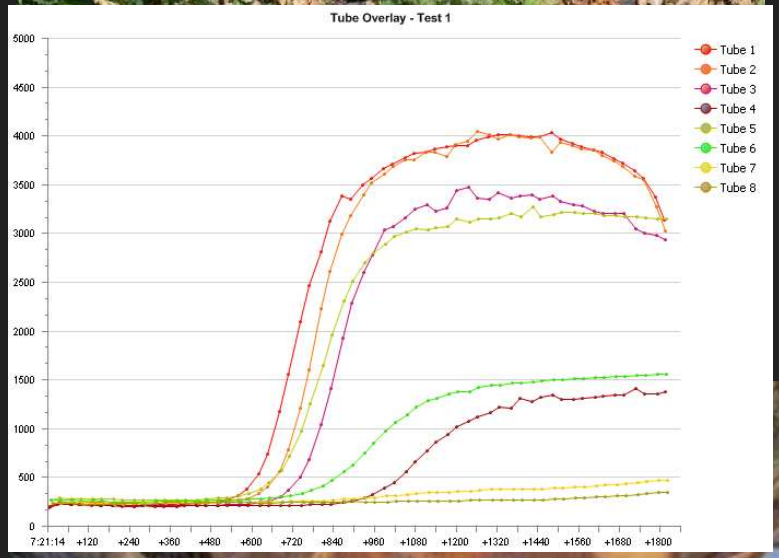
?

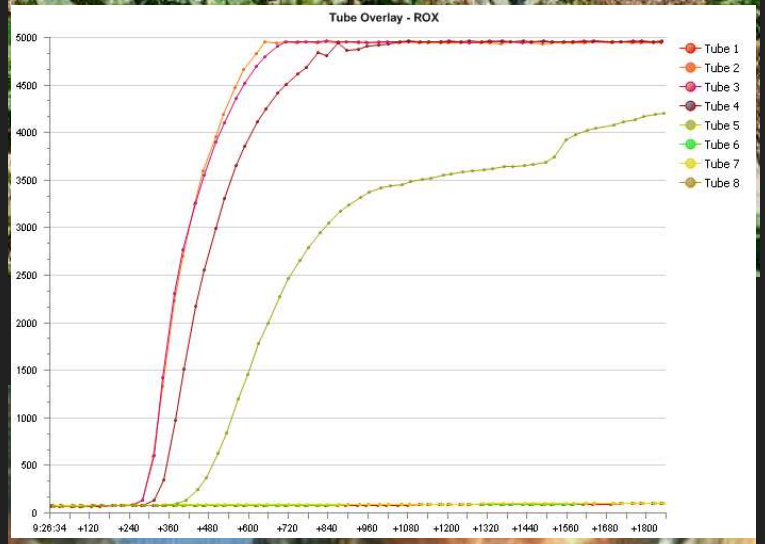
Why develop rapid molecular diagnostics?

- Multiple plant pathogens can cause the same symptoms
- Plating infected plant samples on media is effective, but time consuming
- GOAL: Develop **rapid** and **specific** molecular assays to detect major soilborne diseases of strawberry
  - DNA-based Methods:
    - TaqMan real-time quantitative PCR assay
    - Rapid Isothermal Recombinase Polymerase Amplification (RPA) Assay

Macrophomina or *Fusarium*?

Molecular Tests Could Tell You!



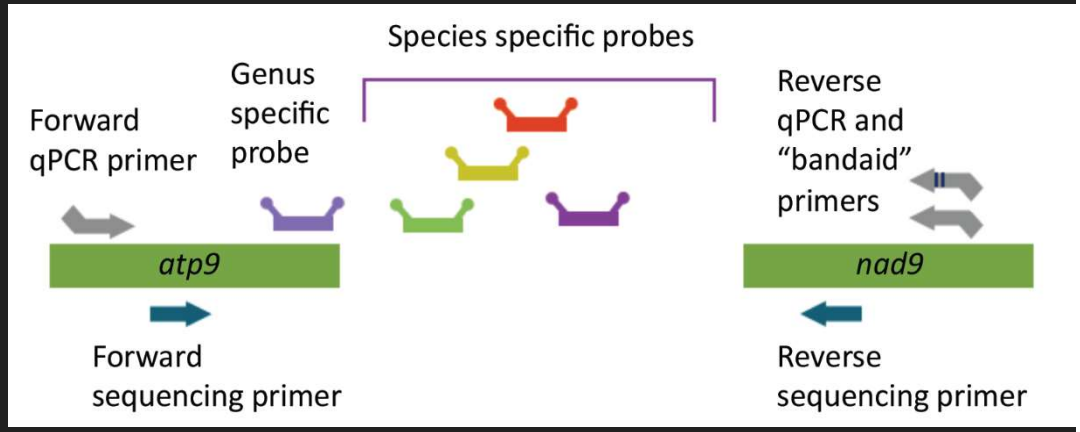


Photos: Steve Koike

Phytophthora TaqMan assay: a model system

atp9-nad9 assay based on mitochondrial gene order differences

- Amplicon size: 370-400 bps
- Genus specific detection capability (except *P. bisheri* and P. *frigida*)
- Over 50 species specific TaqMan probes validated
- Over 175 *in silico* probes predicted
- Sensitivity ~100 fg/µl
- Specificity tested on over 130 *Phytophthora* taxa and several *Pythium* and *Phytopythium* species



Bilodeau *et al.* in Phytopathology (2014)
Miles et al., in Plant Disease (2017) in press

Recombinase Polymerase Amplification of *Phytophthora* Assay

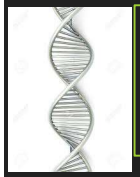
Photos: Steve Koike

What steps go into making a good assay?

- Specificity
  - Detects ONLY the pathogen of interest
  - Select region that is conserved among all isolates of the pathogen
- Sensitivity
  - Can detect very low levels
    - High copy number
- Optimization
  - Best conditions for assay
  - Works with variety of samples

Workflow for molecular assay development

Identify the cause of the disease

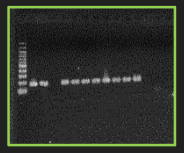


Sequence DNA from representative pathogen isolates

Identify a UNIQUE location in the genome that is present only in pathogens responsible for the disease

Identify a UNIQUE location in the genome that is present only in pathogens responsible for the disease

Design PCR primers to amplify the specific region



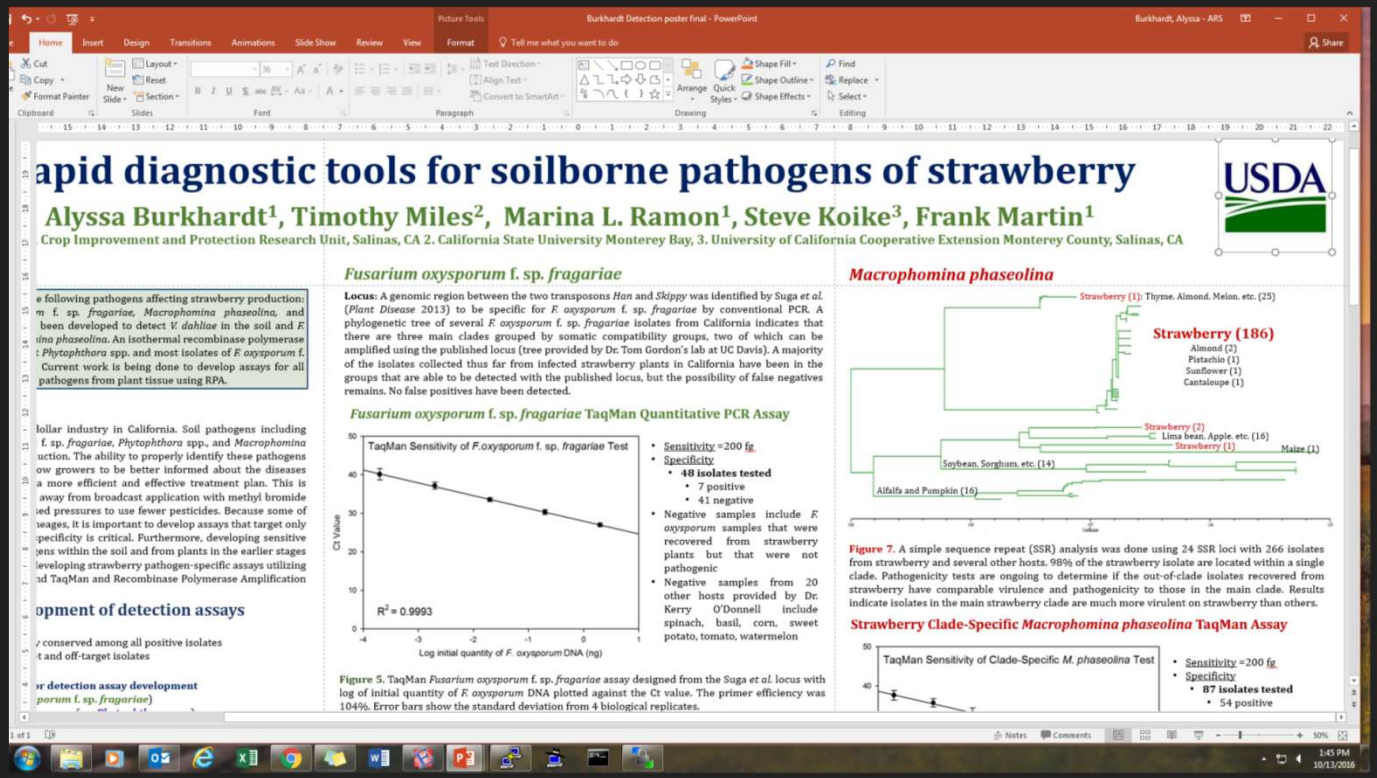
Design and optimize TaqMan qPCR assay

Design and optimize Recombinase Polymerase Amplification (RPA) assay

Validate assay with field samples

Use assay for field diagnostics

M. phaseolina isolates from strawberry hosts group in a single phylogenetic clade from SSR analysis



apid diagnostic tools for soilborne pathogens of strawberry

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USDA

e following pathogens affecting strawberry production: m f. sp. *fragariae*, *Macrophomina phaseolina*, and been developed to detect *V. dahliae* in the soil and *F ina phaseolina*. An isothermal recombinase polymerase *Phytophthora* spp. and most isolates of *F. oxysporum* f. Current work is being done to develop assays for all pathogens from plant tissue using RPA.

llar industry in California. Soil pathogens including f. sp. *fragariae*, *Phytophthora* spp., and *Macrophomina* uction. The ability to properly identify these pathogens ow growers to be better informed about the diseases a more efficient and effective treatment plan. This is away from broadcast application with methyl bromide ed pressures to use fewer pesticides. Because some of eages, it is important to develop assays that target only pecificity is critical. Furthermore, developing sensitive ens within the soil and from plants in the earlier stages leveloping strawberry pathogen-specific assays utilizing d TaqMan and Recombinase Polymerase Amplification

opment of detection assays

conserved among all positive isolates
t and off-target isolates

r detection assay development
porum f. sp. *fragariae*)

Fusarium oxysporum f. sp. *fragariae*

Locus: A genomic region between the two transposons *Han* and *Skippy* was identified by Suga *et al.* (*Plant Disease* 2013) to be specific for *F. oxysporum* f. sp. *fragariae* by conventional PCR. A phylogenetic tree of several *F. oxysporum* f. sp. *fragariae* isolates from California indicates that there are three main clades grouped by somatic compatibility groups, two of which can be amplified using the published locus (tree provided by Dr. Tom Gordon's lab at UC Davis). A majority of the isolates collected thus far from infected strawberry plants in California have been in the groups that are able to be detected with the published locus, but the possibility of false negatives remains. No false positives have been detected.

Fusarium oxysporum f. sp. *fragariae* TaqMan Quantitative PCR Assay

- Sensitivity =200 fg
- Specificity
  - **48 isolates tested**
    - 7 positive
    - 41 negative
- Negative samples include *F. oxysporum* samples that were recovered from strawberry plants but that were not pathogenic
- Negative samples from 20 other hosts provided by Dr. Kerry O'Donnell include spinach, basil, corn, sweet potato, tomato, watermelon

Figure 5. TaqMan *Fusarium oxysporum* f. sp. *fragariae* assay designed from the Suga *et al.* locus with log of initial quantity of *F. oxysporum* DNA plotted against the Ct value. The primer efficiency was 104%. Error bars show the standard deviation from 4 biological replicates.

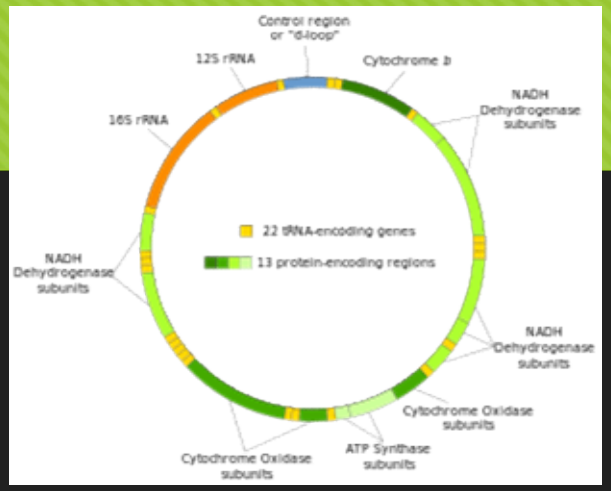
Macrophomina phaseolina

Figure 7. A simple sequence repeat (SSR) analysis was done using 24 SSR loci with 266 isolates from strawberry and several other hosts. 98% of the strawberry isolate are located within a single clade. Pathogenicity tests are ongoing to determine if the out-of-clade isolates recovered from strawberry have comparable virulence and pathogenicity to those in the main clade. Results indicate isolates in the main strawberry clade are much more virulent on strawberry than others.

Strawberry Clade-Specific *Macrophomina phaseolina* TaqMan Assay

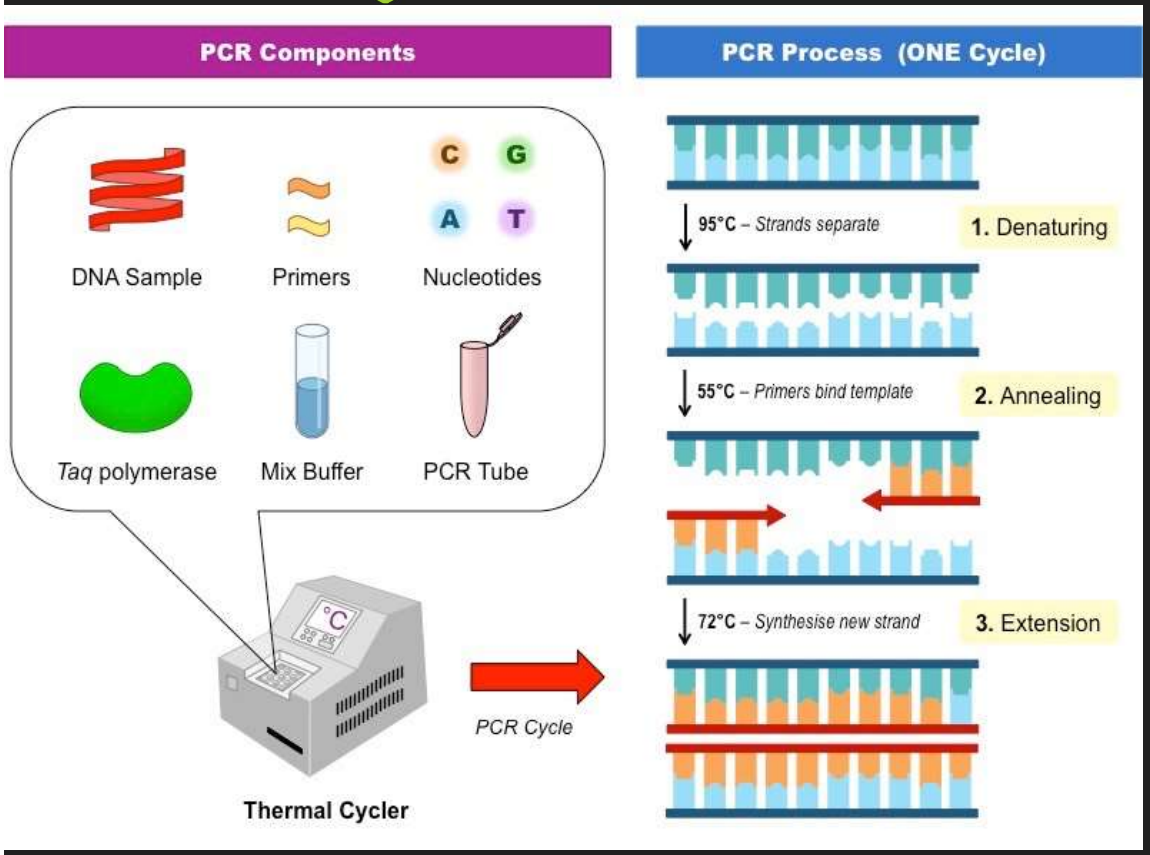
- Sensitivity =200 fg
- Specificity
  - **87 isolates tested**
    - 54 positive

Identify a Target



- Mitochondrial Genomes
- Use comparative genomics
  - Sequence and assemble multiple isolates
    - Ex: Sequenced 15 isolates for Macrophomina and 12 isolates from Fusarium to find unique loci
  - Select isolates that are closely related but have different host selection/pathogenicity

Check locus specificity with regular PCR

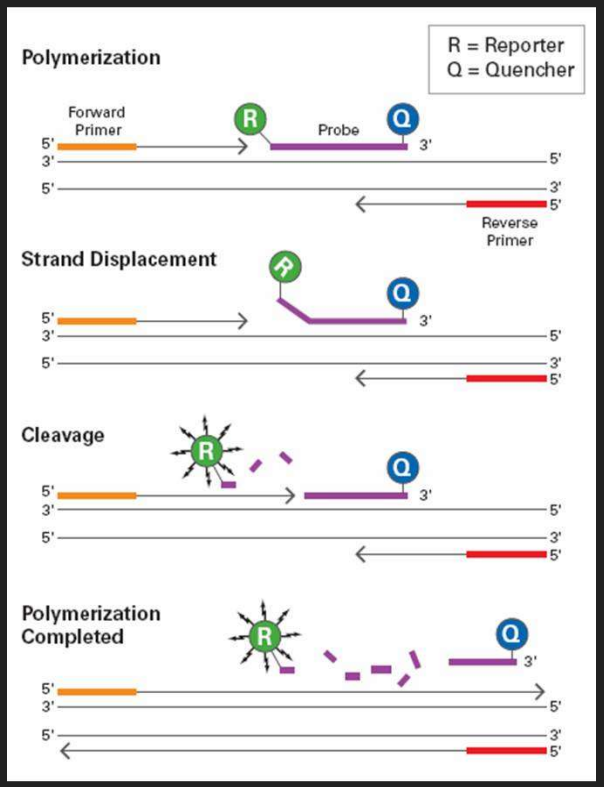


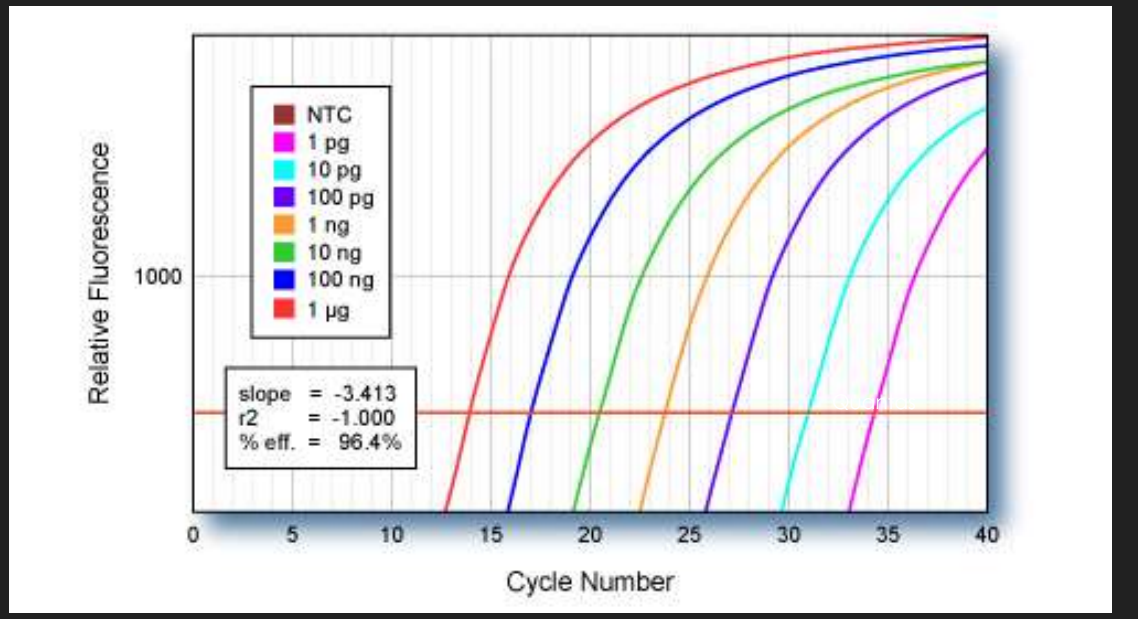
- Use primers designed for TaqMan assays to amplify a specific locus
- Test multiple +/- samples
- Takes 1-2 hours to run PCR
- Takes 1-2 hours to run gel
- Is there a faster way??
- How much pathogen is in the sample?

Quantitative PCR

- Time from start to finish – 1.5 hours
- Like conventional PCR but uses fluorescent dye
- Quantitative – signal timing can correspond to the amount of target DNA
- Typically more sensitive than conventional PCR
- Specificity based on primer location AND on a probe (in the case of TaqMan qPCR)
- Can detect multiple targets in the SAME reaction

Real-time PCR (TaqMan method)

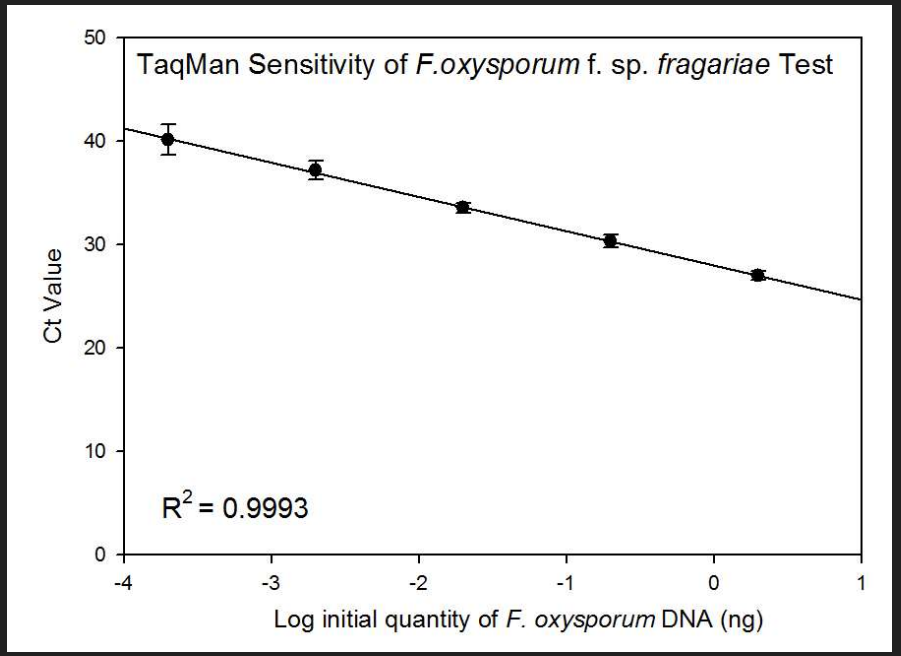




Idaho State University

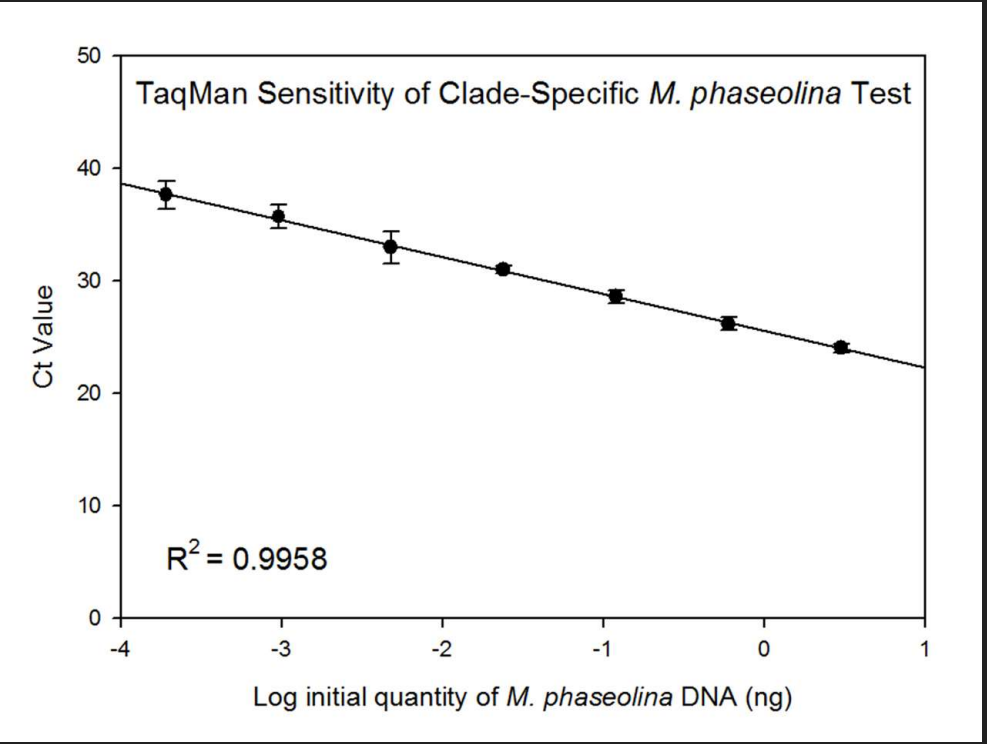
Fusarium oxysporum f. sp. *fragariae* TaqMan Assay

- Using locus published by Suga *et al.* Plant Disease 2013
- Sensitivity =200 fg
- Specificity
  - **48 isolates tested**
    - 7 positive
    - 41 negative
- Negative samples from 20 other hosts including spinach, basil, corn, sweet potato, tomato, watermelon

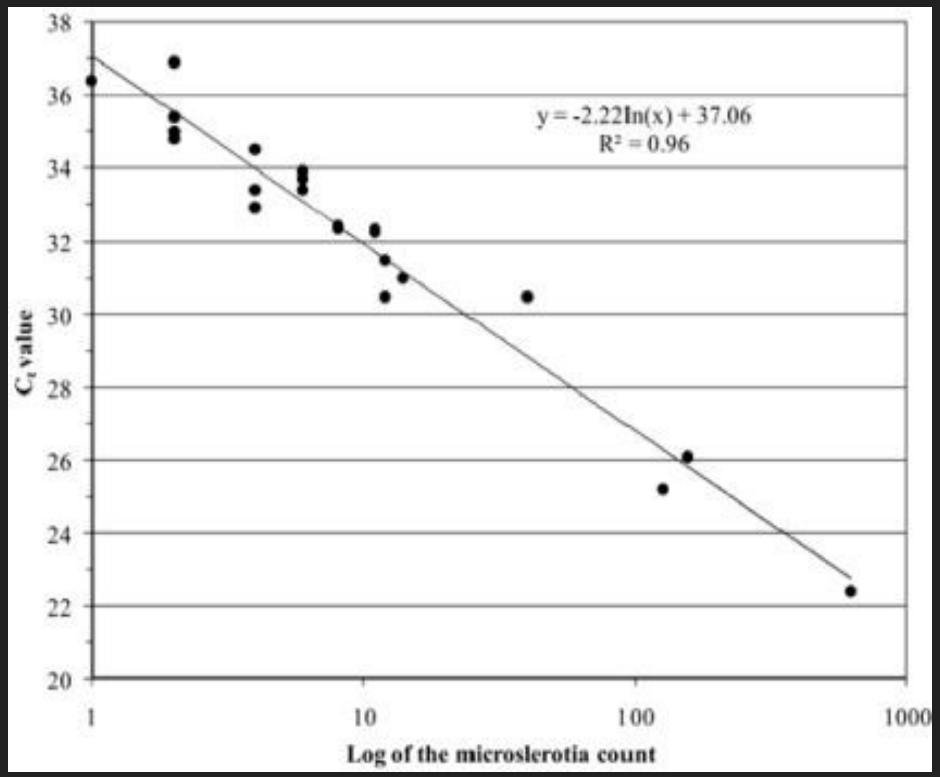


Macrophomina phaseolina Taqman assay

- Unique locus identified through comparative genomics
- Sensitivity: 200 fg
- Specificity: 87 isolates tested
  - 54 positive (in strawberry clade)
  - 33 negative (out of strawberry clade)
- Nested PCR in development to detect microsclerotia in SOIL



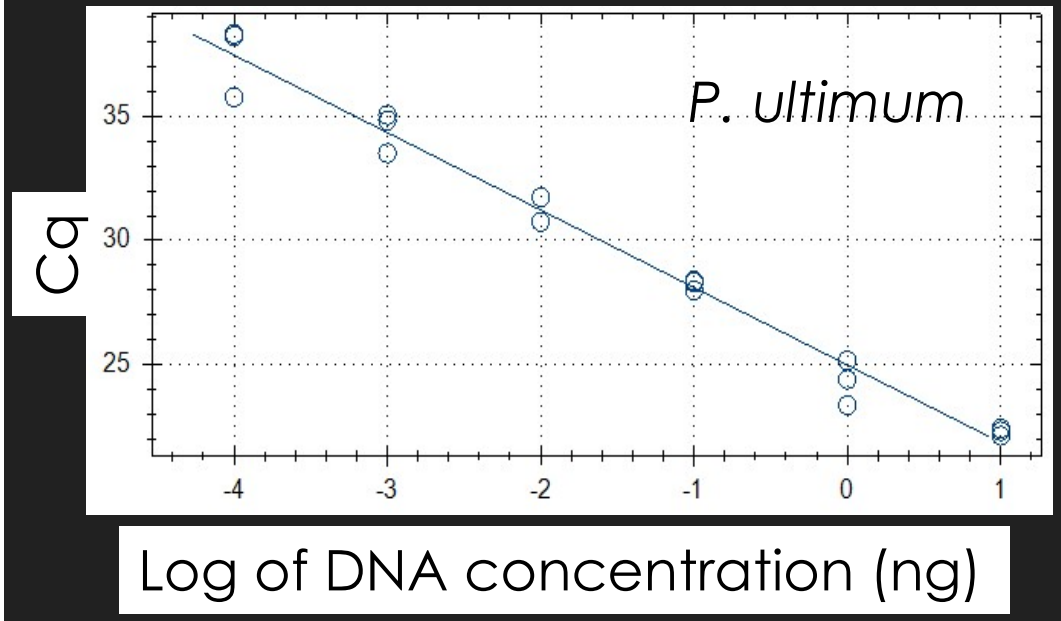
Verticillium dahliae TaqMan Assay



Published by Bilodeau *et al.* in Phytopathology (2012)

- Sensitivity
  - 1-2 microsclerotia/g of soil
  - **31 soil samples** tested to evaluate sensitivity
- Specificity
  - Tested with fungal DNA from multiple *Verticillium* species
  - **70 isolates tested** (40 positive)

Pythium genus specific TaqMan assay



Assay currently in development to compliment the *Phytophthora* genus specific assay

- Sensitivity
  - ~100 fg
  - Validated on 175 environmental samples
- Specificity
  - Tested with 81 different *Pythium* species
  - Tested against 145 *Phytophthora* taxa

Making these assays rapid and portable

Phytophthora cactorum
Photo: F. J. Louws

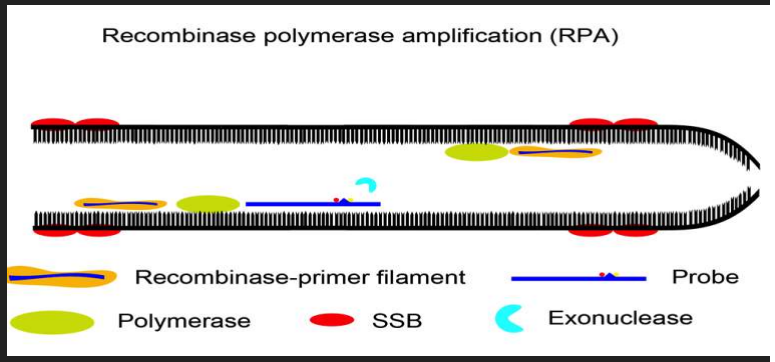
Verticillium dahliae (wilt)

Macrophomina phaseolina (charcoal rot)

Fusarium oxysporum (Wilt)

Recombinase polymerase amplification (RPA)

- Utilizes 3-4 enzymes to produce an amplicon
- ***Fast:*** Results within 5-25 minutes
- Can be multiplexed with multiple dyes, similar to TaqMan
- ***Very limited*** sample prep required
- Not quite as sensitive as TaqMan PCR



Advantages of RPA over other technologies

- Fast!
- No traditional DNA extraction is required and can use very crude samples (see right)
- Tolerant of PCR inhibitors
  - Multiple ways to read results (fluorometric or lateral flow), some are portable
- Can perform nested PCR to confirm a product

Crude root sample

Reading a RPA reaction fluorometrically

A) Twista/ESEQuant (Twistdx/Qiagen)

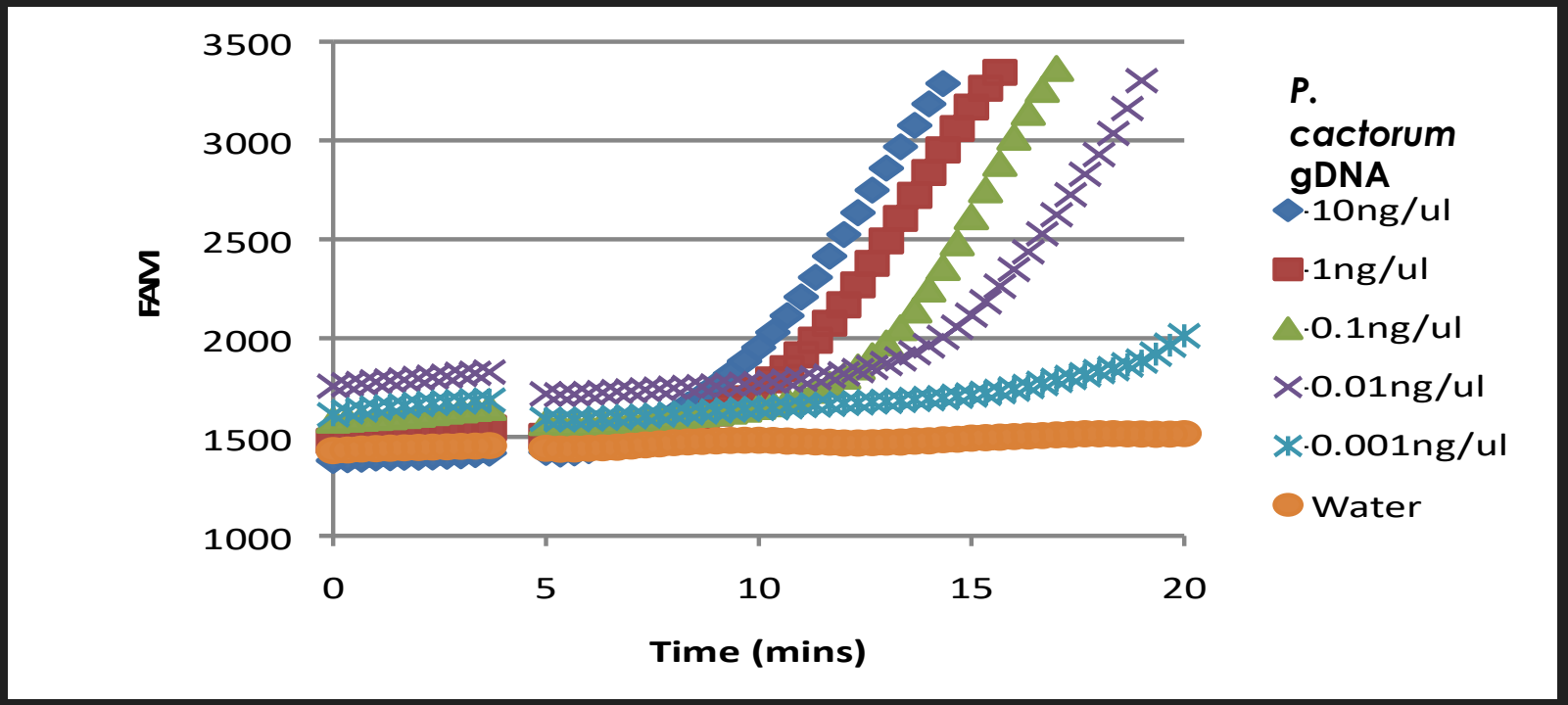
B) T16 isothermal/Axxin (Twistdx/Axxin)

C) Genie II/III (Optigene)

D) SMART-Dart (Diagenetix)

Any qPCR machine

What does an amplification look like?



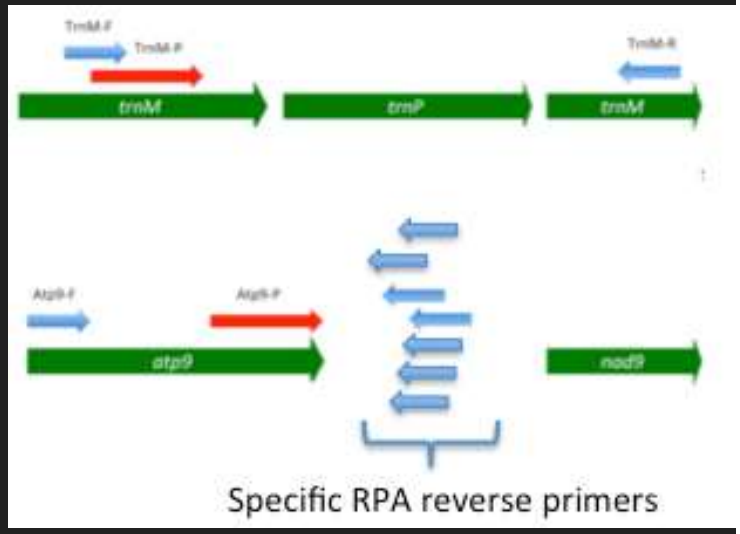
Currently developed RPA assays for *Phytophthora* spp.

trnM-trnP-trnM

- *Phytophthora* genus specific (Miles et al., 2015)

atp9-nad9

- *P. cactorum**
- *P. cinnamomi**
- *P. fragariae**
- *P. kernoviae* (Miles et al., 2015)
- *P. rubi**
- *P. sansomeana* (Rojas et al., 2017 in press)
- *P. sojae* (Rojas et al. 2017 in press)
- *P. ramorum* (Miles et al., 2015)



*Miles and Martin, in preparation

Laboratory validation of recent *Phytophthora* RPA assays

- Validated tests on over 96 *Phytophthora* species, 22 *Pythium* species and a wide range of plant species
- Field validation on a variety of samples
- RPA results agreed with CMA plating
  - *P. cactorum* (9 positive out of 21 samples)
  - *P. cinnamomi* (21 positive out of 57 samples)
  - *P. rubi* (8 positive out of 11 samples)
- TaqMan detected 2 more positives in *P. cinnamomi* samples
  - Evidence that avocado tissue can inhibit RPA at extremely low pathogen titer

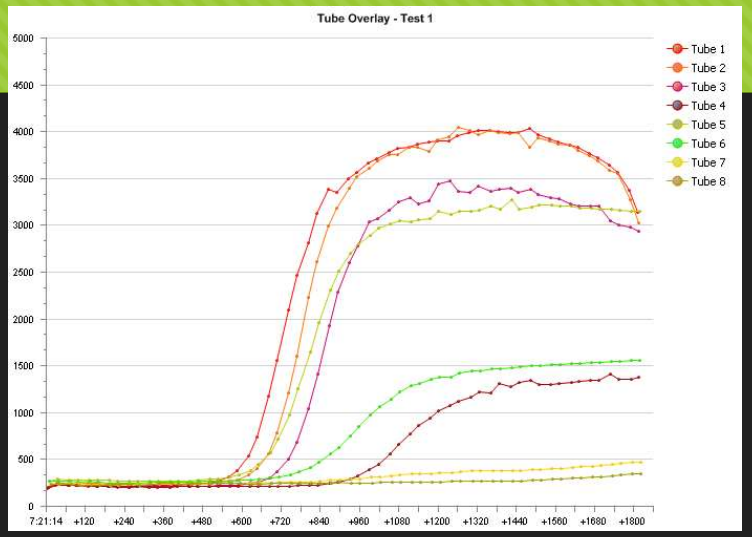
P. cactorum

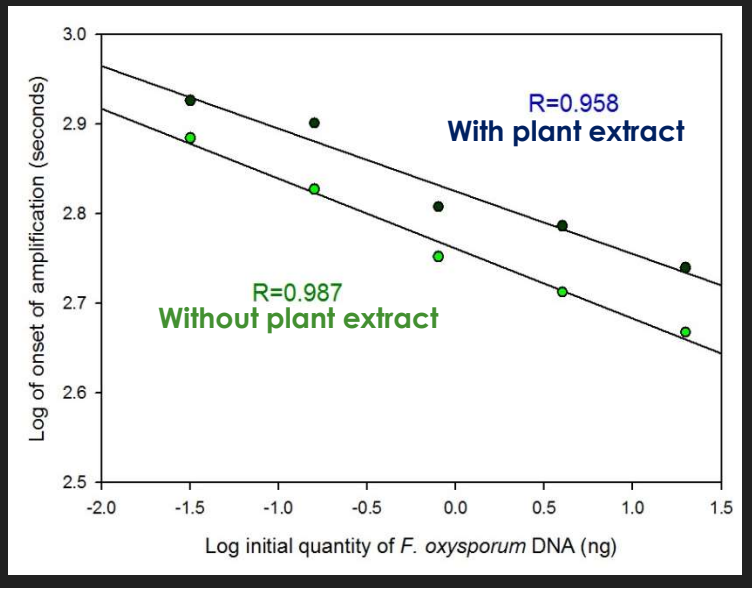
P. cinnamomi

P. rubi

Fusarium oxysporum f. sp. *fragariae* RPA

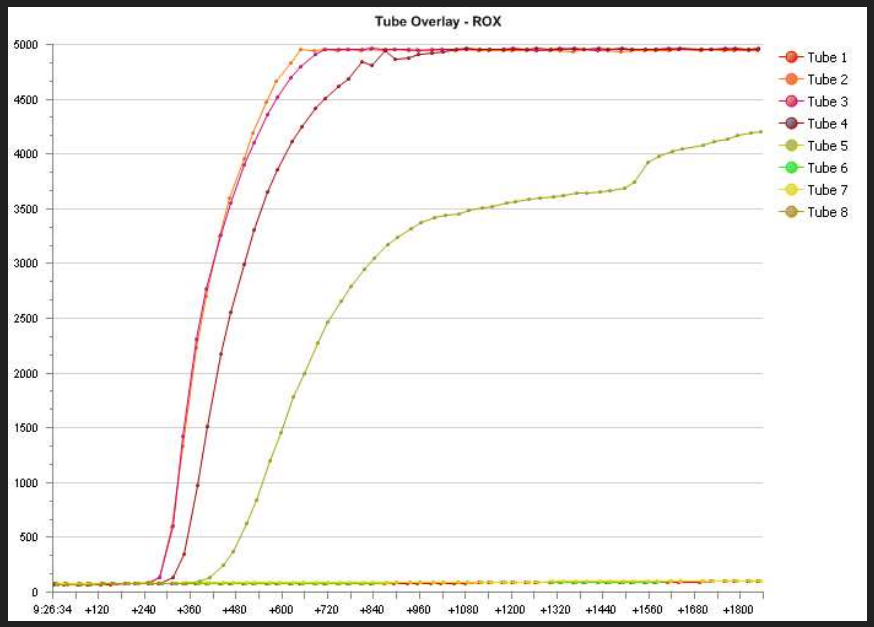
- DNA From Fungal Tissue
- Pure DNA extracted
- *Specificity*: **48 isolates tested**
- 7 positive, 41 negative
- *Sensitivity*: 32 pg
- Crude Tissue Extract
- *Specificity*: **22 plants tested**
  - 13 positive, 7 negative * corroborates with diagnostic lab testing
  - 2 negative plants don't match lab positive





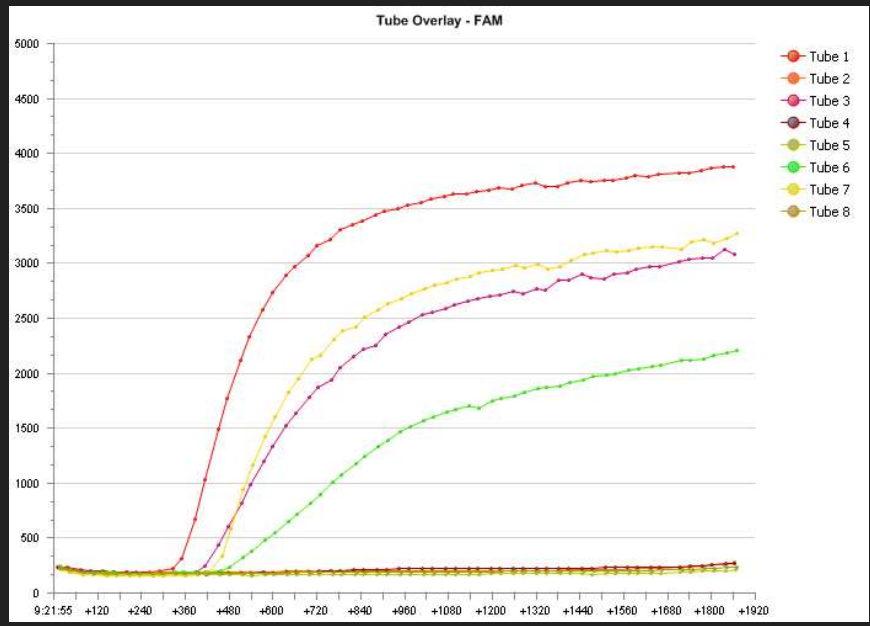
Macrophomina phaseolina RPA

- Macrophomina assay in early stages of development
- Using specific locus from TaqMan assay
- Sensitive to 1 pg
- No false positives for Fusarium or Verticillium
- Positive result for 20 crown tissues lab-tested positive for Macrophomina
  - 2 plants don't match lab culture positive



Verticillium dahliae RPA

- RPA assay being developed from same loci used for TaqMan assay published by Bilodeau *et al.* 2012
- Current problems with specificity within *Verticillium* in the RPA assay need additional troubleshooting
- Is able to detect all tested *V. dahliae* isolates and does not detect crown samples infected with *Macrophomina* or *Fusarium*



Outreach with RPA assays

- Currently we have worked on transferring these assays to a number of collaborators scientists at the follow institutions:
  - Michigan State University
  - UC Riverside
  - Oregon State University
  - USDA-Animal Plant Health Inspection Service
  - California Department of Food and Agriculture
  - Canadian Plant Health Inspection Service
  - Agdia Inc.
  - UC Cooperative Extension
- RPA assays have been incorporated as a Mycology lab at CSUMB
- Our most active collaboration has been with Steve Koike's lab at UCCE in transferring the *Phytophthora* assays for routine diagnoses

Technology transfer of RPA: Molecular laboratory diagnostic at UCCE in Salinas

Molecular diagnostic equipment at
UC Cooperative Extension in Monterey County

Phytophthora assays at UCCE on strawberries

- Due to rains in early 2016 many *Phytophthora* samples were received at UCCE in Steve Koike's laboratory.
- A total of 27 strawberry samples were tested using the RPA method; 25 of these samples were simultaneously tested with the culture medium isolation method
- For 20 of the 25 total samples, the RPA and isolation methods were in agreement regarding *Phytophthora*;
  - 8 times RPA and isolations agreed that the sample was positive for *Phytophthora*
  - 12 times RPA and isolations agreed that the sample was negative for this pathogen
- For the 5 remaining:
  - 2 were positive for RPA but negative for isolation***
  - 3 were negative for RPA but positive for isolation
- ***These two samples tested positive for the red stele pathogen (*P. fragariae*) using the species-specific primers for that species. For these two particular cases, we were unable to find or obtain *P. fragariae* using isolation methods

Future directions of this work

- Complete assay development and validation in the laboratory
  - Goal is to validate assays with at least 40 infected plants per assay
  - Improve extraction techniques for better sensitivity and specificity of RPA reactions
- Expand RPA assays to study other items outside of plant tissue into soil diagnostics
- Transfer assays to Steve Koike's laboratory at UCCE and validate on field samples

Thank you!

Acknowledgements

- UCCE: Steve Koike and Stacy Mauzey
- USDA-ARS: Dr. Frank Martin, Dr. Marina Ramon, Dr. Guillaume Bilodeau, Brett Smith, Noah Luecke, Julia Schrandt
- Dr. Kerry O'Donnell: Providing DNA samples from multiple isolates of *Fusarium oxysporum*
- Dr. Tom Gordon and Peter Henry: Phylogenetic tree of *F. oxysporum* f. sp. *fragariae,* cataloging Suga negative and positive isolates in CA, providing *F. oxysporum cultures*

Funding

- California Strawberry Commission,
- USDA/CDFA Specialty Crop Block Grant Program
- California Avocado Commission
- CSU-Agricultural Research Institute