Beyond *Phytophthora ramorum*: Identifying other *Phytophthora* species, searching for *P. kernoviae* and evaluating species level testing methods

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Abstract: Beginning in 2004, the Cornell University's Plant Disease Diagnostic Clinic (PDDC) has provided *Phytophthora ramorum* identification testing for numerous state and national surveys, trace forward/back events and Farm Bill projects. Since 2004, 3,695 suspect *P. ramorum* samples have been processed. Working with so many samples has triggered questions in regards to the process and our findings. Currently only the ELISA procedure is accepted by regulatory agencies for preliminary testing to the *Phytophthora* genus level. However, the ImmunoStrip® is an ideal diagnostic tool for small sample sets, therefore, a comparison of results may allow this alternative procedure. Furthermore, ELISA testing indicated that many of the samples processed contained a *Phytophthora* species but no species identification testing was done due to the additional cost of labor and supplies needed. Another significantly harmful *Phytophthora* species, *P. kernoviae*, has not yet been identified in the United States: monitoring for it is not common practice. Testing plants for *P. kernoviae* is important because this pathogen is reportedly much more damaging; it was found in Europe during their *P. ramorum* surveys. A Specialty Crop Block Grant allowed us to accomplish three objectives, using our 2014 samples: 1) sequence *Phytophthora* positive samples, 2) test samples using qPCR ITS1 and ITS2 protocols for *P. kernoviae*, and 3) compare the ELISA *Phytophthora* species procedure with the ImmunoStrip® test. This project allowed us to name 14 different *Phytophthora* species detected from 205 isolates, determine no *P. kernoviae* was present in these samples and show a few differences between results of ELISA versus ImmunoStrip®.
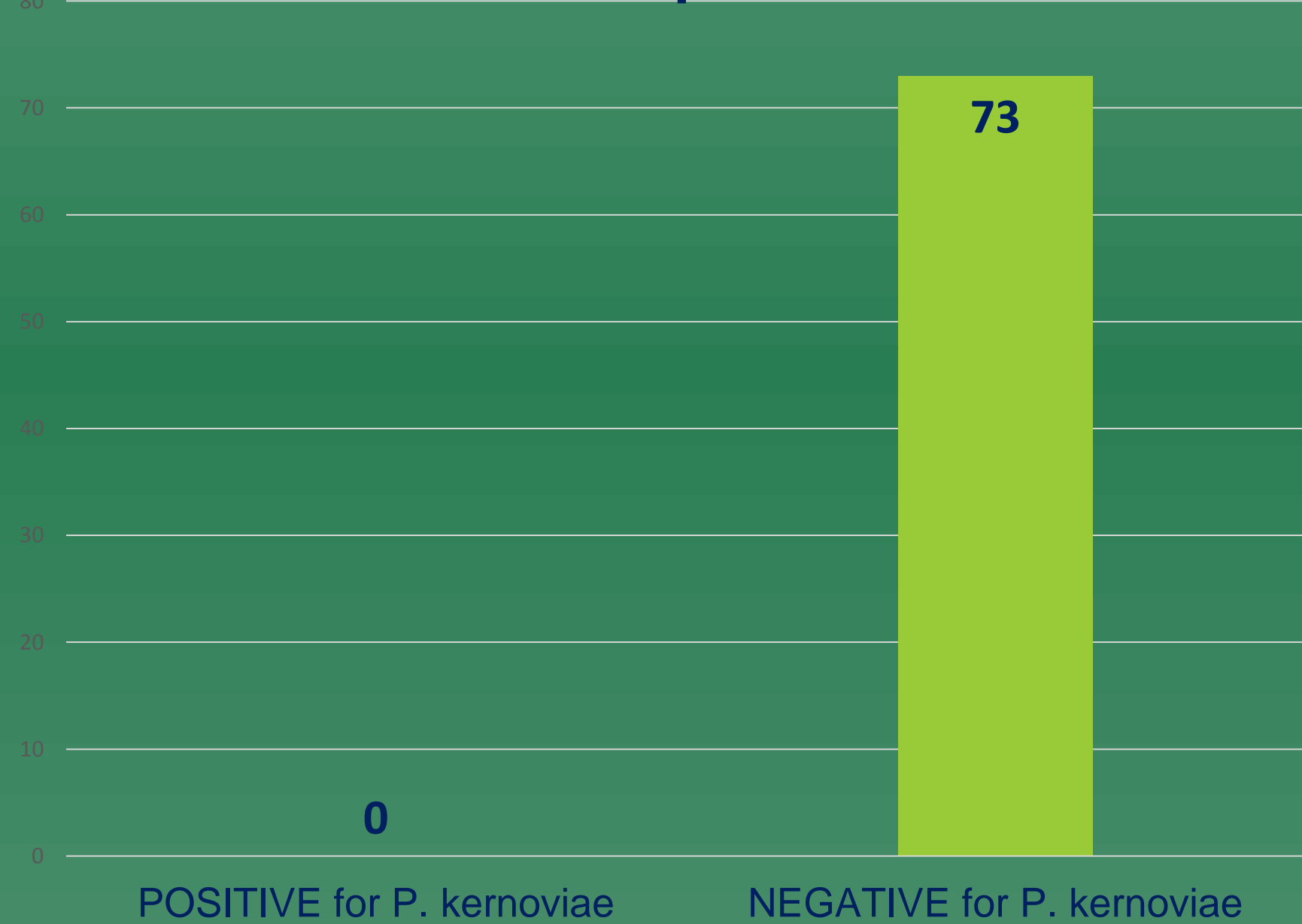
METHODS – objective #1

Each sample was tested twice using the ITS1 & ITS2 protocols for *P. kernoviae* identification. The ITS1 protocol uses primers Pkern 60F and Pkern 121R, and the Pkern 84T probe. It also contains an internal control 5.8S primer and probe which indicates if a *Phytophthora* species is present. The ITS2 protocol uses primers Pkern 615F and Pkern 722R and the Pkern 606T probe. It also contains an internal control COX primer and probe which indicates if plant DNA is present and, therefore, shows whether the DNA extraction step worked properly.

Objectives were satisfied using samples originally submitted to the Clinic for *P. ramorum* testing. These samples all tested positive for a *Phytophthora* species using the ELISA test.

METHODS – objective #2

Because these were environmental samples, each sample was molecularly cloned in hopes of isolating different species present. The sequencing preparation steps are listed below:

- **Nested PCR (round 1):** primers: 18Ph2F, 5.8-1R
- **Nested PCR (round 2):** primers: ITS6, 5.8-1R
- **Molecular Cloning:** Promega pGEM®-T Easy Vector System II
- **Conventional PCR:** universal primers: pUC/M13F, pUC/M13R
- **Quantify DNA & prep for sequencing:** prepared 2 samples per clone (forward & reserve priemrs)
- **Sequence:** Cornell University Genomics Core facility
- **Sequence alignment:** Geneious Software by Biomatters
- **BLAST Sequences:** NCBI database

OBJECTIVE #1

Survey for *P. kernoviae*

OBJECTIVE #2

Sequence unknown *Phytophthora* species samples

RESULTS – objective #1

The results for all 73 samples for ITS1 & ITS2 were NEGATIVE; NO *P. kernoviae* detected

P. kernoviae qPCR Results

POSITIVE for P. kernoviae: 0
NEGATIVE for P. kernoviae: 73

RESULTS – objective #2

Approximately 7 clones were chosen from each sample. In sum, 204 clones were generated from 30 samples and sequenced. The *Phytophthora* species identified are displayed on the chart below.

Sequencing Results

Phytophthora species	# of clones
P. gonapodyides	2
P. pini	5
P. plurivora	11
P. parsiana	1
P. hydropathica	7
P. hibernalis	6
P. cactorum	14
P. borealis	2
P. lacustris	4
P. riparia	1
P. citricola	55
P. megasperma	1
P. citrophthora	44
P. cryptogea	51

CONCLUSION – objective #1

Although *Phytophthora kernoviae* has not been detected in the US, it has been detected in Europe and was reported to be a more damaging pathogen than *P. ramorum*. In Europe, *P. kernoviae* was first discovered accidently during *P. ramorum* testing. For that reason and because we have not searched for this pathogen in the past, we tested all of our 2014 *P. ramorum* samples using the molecular protocols validated by the USDA-APHIS-PPQ-S&T-Beltsville Laboratory facility.

Based on this survey, our findings indicated that *P. kernoviae* was not detected in sampled nurseries.
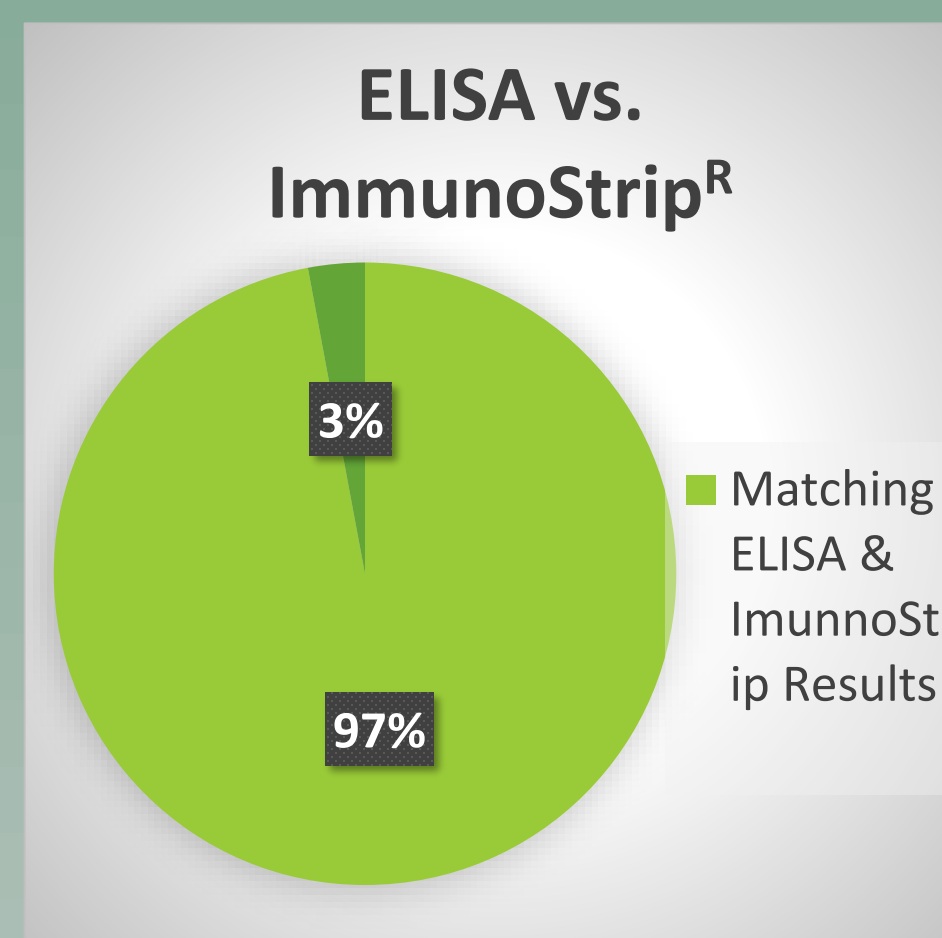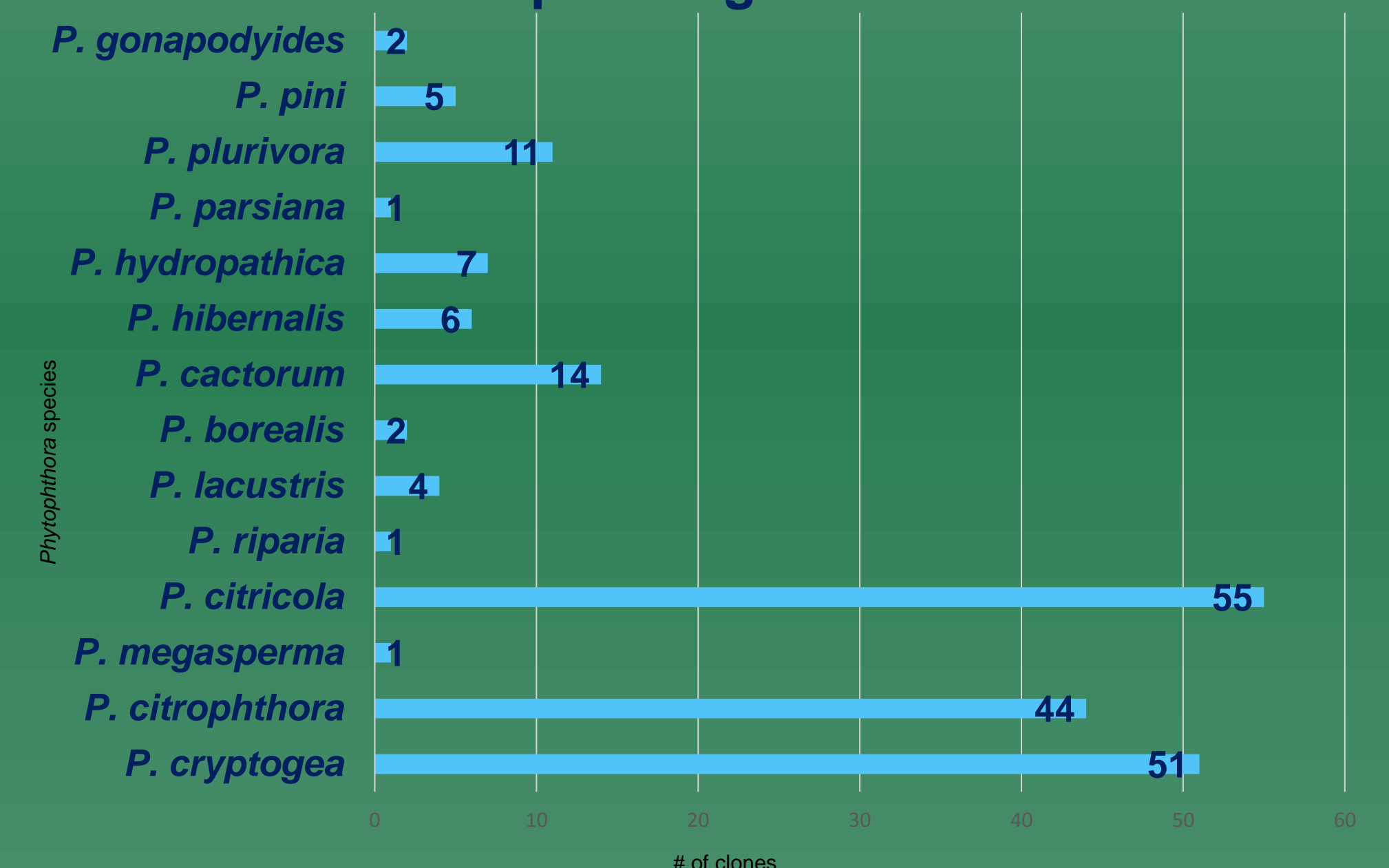
OBJECTIVE #3

Compare test methods: ELISA vs. ImmunoStrip®

CONCLUSION – objective #2

The goal of this portion of the project was to learn more about specific *Phytophthora* species present in New York State nurseries. Sequencing the *Phytophthora* species enabled us to learn which species were present in samples that tested negative for *P. ramorum*. Rather than stopping at a negative test result for *P. ramorum* with the survey samples as in the past (due to insufficient funding), this state-funded project allowed for the additional analysis of samples that contained pathogens related to *P. ramorum*. Through further analysis of nursery samples, we are beginning to increase our knowledge of the *Phytophthora* species present in New York State nurseries, which may lead to a better understanding of *Phytophthora*-related disease damage on nursery plants.

Numerous species of *Phytophthora* are being identified in the survey samples collected as part of the *P. ramorum* survey. The ability to clone and sequence the other *Phytophthora* species is providing useful information that may help us better understand *Phytophthora*-related plant damage.

METHODS – objective #3

All samples received from New York State sites for *P. ramorum* survey processing were tested following the approved protocol using Agdia's PathoScreen *Phyt (*ELISA for *Phytophthora)* kit to determine if a *Phytophthora* species was present and if additional testing (i.e. molecular analysis) was needed. Further, this study required each sample to gbe tested with Agdia's ImmunoStrip® test strip to determine if consistent results would be found.

RESULTS – objective #3

The results of the ImmunoStrip® matched the results of the ELISA testing 132 times out of the 136 samples. Therefore there were 4 samples which produced conflicting results using these two tests.

For these 4 samples, the ELISA test produced a positive result while the ImmunoStrip® results were negative. During sequencing analysis (objective #2) of the 4 samples, 2 were sequenced. One sample was identified as *P. citrophthora* and the other was *P. citricola*.

ELISA vs. ImmunoStrip^R

- Matching ELISA & ImunnoStrip Results: 97%, 3%

CONCLUSION – objective #3

There are times when it is more convenient to use the ImmunoStrip® test strip rather than the ELISA. This study was important because the Cornell PDDC often receives single samples and the ImmunoStrip® is the ideal test method in this situation. The ELISA kit can be used for any number of samples, but repeated use with a low number of samples uses up the reagents quickly and the kit's testing capacity is drastically reduced. Because of this it would be ideal to use the ImmunoStrip® for single samples and the ELISA for processing larger groups of samples to minimize waste. Since there was variation between the two different test methods (ELISA vs. ImmunoStrip®), such that the ImmunoStrip® missed four (4) samples that were positive in the ELISA testing, the risk of not finding a positive result in the *Phytophthora* screening is too high when processing regulatory samples.

One hypothesis is that the ImmunoStrip ® is less sensitive detecting specific *Phytophthora* species while the ELISA test can detect a larger range of species. To test this hypothesis we would like to research the different levels of detection each test provides. If funding is procured, we plan to use Elicitin qPCR, paired with sequencing analysis, to formulate beneficial data that could be used to better prepare for detecting *Phytophthora* species in the future and provide more insight into the validity of these tests.