Development of molecular diagnostic tools for the invasive oomycete pathogen *Phytophthora tentaculata*

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Abstract

Phytophthora basal rot (PBR) of plants is a disease recently observed in Central California Native plant nurseries and restoration locations. The primary causal agent of this disease is the oomycete pathogen *Phytophthora tentaculata*. Prior to 2012, *P. tentaculata* was among the ~30 *Phytophthora* species not known to be present in the United States. Previous research had developed an isothermal diagnostic tool known as recombinase polymerase amplification (RPA), which had *Phytophthora* genus specific detection capability with results obtainable within as little as 15 minutes directly in the field without conventional DNA extraction. A *P. tentaculata* species-specific RPA assay was developed and specificity validated against pure DNA from 135 *Phytophthora* taxa. To test this technique for detection of the pathogen in PBR, 113 symptomatic samples were collected and evaluated with the new RPA assay as well as the previously validated TaqMan assay, Immunostrip and conventional culturing and baiting techniques. Results were similar across the various amplification platforms, with qPCR being the most sensitive in general. Finally, spatial models were created to test if elevation correlated with the presence of the pathogen. These results indicate that *P. tentaculata* is often present in low-lying areas when infected plant material is used for restoration. This information will assist in more efficient, rapid, sensitive, specific pathogen detection for management decisions in a field and nursery setting as well as understanding pathogen distribution and spread within a field.

Introduction

Main Objectives:

Utilize four different methods of detection to identify *P. tentaculata* at a restoration site:

1. 1) Determine the amount of time required for each method: qPCR, genus/species specific RPA assays and Agdia Immunostrips.
2. 2) investigate the ease and feasibility of each method
3. 3) correlate detection results of each method across different platforms.

In 2012 the fungal plant pathogen, *Phytophthora tentaculata, known to cause basal rot* was detected in California native plant nurseries. Native plant Nurseries are generally nonprofit organizations with main focus on community based restoration.

P. tentaculata currently has a known host range of five plants species, but the number is expanding and extensive host range studies are being conducted (Latham *et al*., personal communication).

Cal State Monterey Bay's Watershed Institute recently detected this pathogen on native plant species at the nursery, Return of the Natives (RON), and within a restoration site. The spread of this pathogen in a natural setting could negatively affect many of California's plant communities.

Best Management Practices (BMPs) have been recommended but are often more expensive and laborious which can prose issues for nonprofit organization

Many native plant nurseries do not have access to testing methods and obtaining results from outside laboratories can be tedious. Current methods of detection can be cumbersome and inaccurate. However, the recent development of a rapid molecular detection technique, Recombinase Polymerase Amplification (RPA), has been developed for the *Phytophthora* genus (Miles *et al*., 2015)

A

B

Fig. 1. A) *Diplacus auranticus (*sticky monkey flower) symptomatic plant (Left) and healthy individual (Right). **B)** A section of the nursery in which highly symptomatic plants are found on the ground in an area know for pooling of water

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Fig. 2. *Diplacus Auranticus* (Sticky Monkey Flower) is featured in this picture in order to demonstrate what the plant looks like and its root system. Circled and labeled **A**) is the main root stalk where the soil line is found. **B**) represents the taproot which we have found experimentally is the targeted region for testing for a pathogen. It is found generally where the main root system has a notch and begins to turn "hooking" into soil. **C**) The sub root system is the other part of the root system tested. It is advised not to test from this portion of the plant as it is less likely the pathogen will be present.

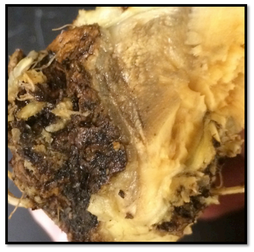

Fig. 3. Necrosis on sticky monkey flower root crown with leading line sampled.

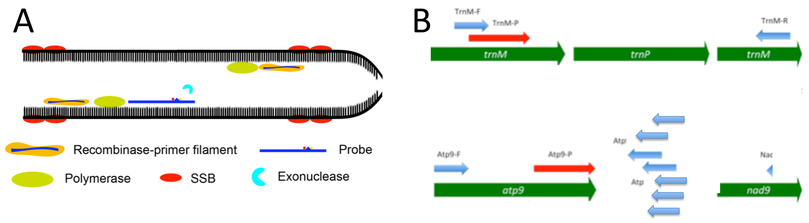

Fig. 4. A) Schematic of the primers and probes required for the development of recombinase polymerase amplification assays. **B)** RPA species specific primers for several *Phytophthora* species creating specificity by location within previously identified gene order differences in mitochondrial regions (Miles et al., 2015).

Table 1. Methods used to detect *Phytophthora tentaculata* in field samples in this study. Table includes a variety of attributes such as number of positive samples, approximate processing time, cost per sample and detection target.

	ImmunoStrip	qPCR	qPCR	RPA	RPA
Assay intended target	All *Phytophthora* spp. (known to cross react with *Pythium* spp.)	All *Phytophthora* spp.	*Phytophthora tentaculata* only	All *Phytophthora* spp.	*Phytophthora tentaculata* only
Detection/Number of samples tested	22/113	15/113	12/113	15/113	6/113
Time per sample	40 minutes	6.5 hours	6.5 hours	1.1 hours	1.1 hours
Cost per sample ($)	12	4	4	4	4
Primer Region	ELISA based	*atp9-nad9*	*atp9-nad9*	*trnM-trnP-trnM*	*atp9-atp9/ nad9* spacer

Methods

- Samples were collected at a restoration site adjacent to Cal State Monterey Bay property.
- Symptomatic *Diplacus aurantiacus* root crowns were collected under sterile conditions.
-DNA was extracted from the root crown using Plant DNA extraction kit (Qiagen, 3 hours)
-Assembled qPCR master mix contained (30 min)
-Reactions were ran in the BioRad CFX96 qPCR machine (3 hours)
-Ground a section of the root crown in 5mL of General Extraction Buffer 2 (Agdia Inc.) (10 min)
-Assemble a master mix (with RPA primers/probe) and run reaction reaction (25 min)
-5mL of Simple Extraction Buffer was added to root crown section in Extraction pouch (Agdia)
-Sample was emulsified until a homogenous mixture was achieved (10 min)
-Immunostrip added to emulsified liquid (30 min)
-Positive Identification: presence of two lines

Results

DNA Extraction

DNA was confirmed in all methods of detection, with qPCR family specific primers were used and with RPA and Immunostips DNA was visible (macerated tissue)

qPCR Probe Detection

The qPCR method was accurate and was the method most commonly used even though this was the most tedious process, 6.5 hours. (see Table 1)

Genus and Species Specific RPA

The RPA assays were accurate in *Phytophthora* detection, aligning with the qPCR technique. This method was extremely more time efficient with results achieved in 1.1 hours.

Immunostrip Detection

Although results were obtained in a minimal amount of time (40 minutes), immunostrips were inaccurate due to cross reactions with *Pythium spp.* that provided false positives for non-*Phytophthora* samples.

Fig. 5. Agdia Immunostrip with positive detection of *Phytophthora* at a genus level (2 Lines)

Discussion

After undergoing four separate tests results would indicate the RPA is the best option for rapid detection of a pathogen in a wild setting or in the case of a nursery. The Immunostrips were useful for general pathogen detection they were unspecific (see Table 1). qPCR provides a great amount of information and should be used for validation as well as quantification but is time consuming and requires a considerable upfront investment. Validation using pear baiting and classic culturing technique however proved to be difficult during dry season. These types of assays are necessary for making decisions to save time and money as well as assist with thorough understanding of the pathogen and prevent it from spreading outside of nurseries and restoration sites.

Future Work

The use of additional RPA assays will identify the epidemiology of the pathogen and will in turn be used to map projected spread of disease in correlation to elevation. This will predict the movement patterns of the pathogen and determine if there is a correlation between pathogen presence, specified elevations and areas of tarning. Furthermore, additional studies about sampling strategies on plant roots will be conducted using these techniques

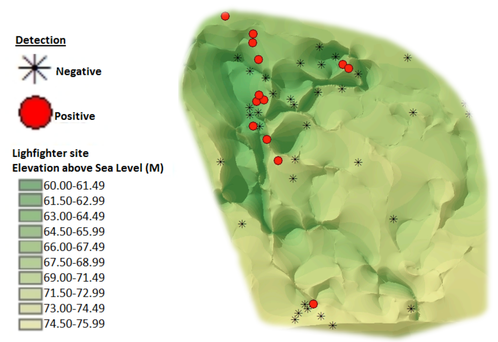

Fig. 6. Map of the *Lightfighter site* that has the positive samples (Red) tested with qPCR and all other samples collected (Black). Development of these models assists in understanding the pathogens movement in certain terrain. This site mainly consists of a basin about three square acres with different depressions at different elevations.

References

1. S. Rooney-Latham and C. L. Blomquist. First report of root and stem rot caused by *Phytophthora tentaculata* on *Mimulus auranticus* in North America. Plant Disease 2014 98:7, 996-996
2. Timothy D. Miles, Frank N. Martin, and Michael D. Coffey. Development of Rapid Isothermal Amplification Assays for detection of *Phytophthora* spp. in Plant Tissue. Phytopathology 2015 105:2, 265-278
3. Bongsoo Park, Frank Martin, David M. Geiser, Hye-Seon Kim, Michele A. Mansfield, Ekaterina Nikolaeva, Sook-Young Park, Michael D. Coffey, Joseph Russo, Seong H. Kim, Yilmaz Balci, Gloria Abad, Treena Burgess, Niklaus J. Grünwald, Kyeongchae Cheong, Jaeyoung Choi, Yong-Hwan Lee, and Seogchan Kang. *Phytophthora* Database 2.0 Update and new direction. Phytopathology 2013 103:12, 1204-1208 http://www.phytophthoradb.org/species