How do we know if plants in our nursery have *Phytophthora*?

Detection methods and an integrated approach to monitoring.

Laura Lee Sims [1], Christa Conforti [2], Amy Mautz [2] Alisa Shore [3], Matteo Garbelotto [1]
UC Berkeley Environmental Science, Policy, and Management [1]; Presidio Trust [2]; Golden Gate National Parks Conservancy[3]

Introduction

Introduction to *Phytophthora*

The genus *Phytophthora* contains many plant pathogen species. These can cause crown and root rots in woody plants (1). An infected plant will display symptoms like chlorosis, necrosis, stunted growth, and root rot. In many cases, an infection can lead to the death of the plant.

Phytophthora species are considered water molds (oomycetes) as they require water for parts of their lifecycle (1). In the presence of water, soil inhabiting *Phytophthora* can produce swimming spores (called zoospores) that encyst and stick on root tissue, potentially leading to infection. These zoospores can be targeted for detection purposes.

The issue

In 2015, the Presidio Native Plant Nursery staff observed symptoms of root disease in a crop of the woody plant species *Ceanothus thyrsiflorus*. Samples from these plants tested positive for *Phytophthora cactorum*. In addition to *P. cactorum* , several *Phytophthora* isolates of potentially multiple species have been isolated, and continued species level identification is underway.

This finding is a serious concern because *P. cactorum* is known as a root-rotting pathogen of woody plants around the world (1), and the yet-to-be-identified species could be a significant threat to plant populations as well.

These plants were grown for habitat restoration projects in the Presidio of San Francisco. Because we do not want to jeopardize the health of the Presidio's native ecosystem, we destroyed most of this crop of *C. thyrsiflorus* in the nursery. To resolve this issue, and to explore *Phytophthora* monitoring approaches, we saved 400 of these plants for a field-trial to compare *Phytophthora* detection methods.

Objectives

- To quantify the *Phytophthora* detection level of three non-destructive sampling methods, and compare them to the actual level of detectable contamination in the crop, using destructive procedures.
- To determine the least labor intensive and most cost effective method to test a large number of plants in our nursery for *Phytophthora.*
- To develop a novel, best detection approach based on our findings, so that we will know if plants in our nursery have *Phytophthora.*

Materials & Methods

Figure 1. (A) Symptomatic *C. thyrsiflorus* (CETH), (B) nursery grower sampling plants (C) *Phytophthora* growing from bait plates, (D) pure culture of a *Phytophthora* isolated from a bait plate, (E) sequence of *P. cactorum* from trial plants.

A. Experimental Design

Four-hundred mature, *Phytophthora*-symptomatic plants (Fig. 1A) were selected from our *C. thyrsiflorus* crop, which had previously been composite sampled, and found positive for *P. cactorum*. We grouped these 400 plants into 5 blocks, each containing 80 randomly selected plants. Each block was placed in a containment tray. The plants were irrigated three days per week in two ten minute cycles, as per standard watering cycles for our nursery.

To prevent spread of *Phytophthora,* Visqueen™ containment walls were constructed around the holding bench, and bins were placed underneath the bench to collect excess water.

B. Sampling Methods for Detection

Our trial sampling methods: Drip, Scoop, ImmunoStrip and Destructive, were performed in succession (Fig 1B). All were performed using the same plants. Labor hours and material costs for each method was recorded for comparison.

Drip method. This method was loosely adapted from Vercauteren Et. al, 2013 (2). A single bait bag (3,6) was placed in the containment tray of each block of plants, and allowed to remain there, floating in effluent captured by the tray, through three irrigation events.

***Scoop method*.** Plants in each block were evaluated for necrosis, chlorosis and stunted growth. Targeting the most symptomatic plants, we used a Scoopula™ to remove 2-tablespoons of roots and soil from 22% of the plants in each block. This composite sample from each block was placed in a single gallon Ziploc bag. Each composite sample was then baited as described in Section C.

***Destructive method*.** We randomly selected 30% of the plants in each block (25 plants per block), and placed the whole plant, including potting soil, in a gallon Ziploc bag. Each plant was then baited as described in Section C. This method was not being considered for wide-scale use because it requires plant destruction, but was used as our 'gold standard' to quantify the actual level of detectable pathogen in the crop.

ImmunoStrip method. Prior to Destructive baiting, the same randomly selected 25 plants per block were examined, and approximately 1 Tbsp. of the most symptomatic roots were removed from the plant. Roots were tested using Agdia ImmunoStrip™ test-kits for *Phytophthora*.

C. Baiting Materials & Procedures

Baits (3, 6) containing pear (4,5) were used as the mechanism to capture *Phytophthora* zoospores for the Drip, Scoop, and Destructive methods.

Materials:

- Mellita® Super Premium Tea Filters
- Packing peanuts
- Unripe green pear
- Knife
- Stapler w/ staples

Assembly of a bait bag:

1. Cut 5 flat blocks of pear that are 2-3 mm thick and 2 cm in length
2. Place the packing peanut, and pear pieces into the tea bag; then staple it shut (Fig. 2)

Baiting procedure:

1. Place each sample bag in a sturdy 1L container
2. Add distilled water to 1cm above sample
3. Create a control bag of distilled water (or irrigation water for Drip method)
4. Add 1 bait bag to each sample and control
5. Leave baits in place for 7 to 10 days

Figure 2. Completed bait bag

D. Plating Materials & Procedures

Place baited pear pieces into a petri plate containing *Phytophthora* selective growth medium (Fig 3).

Materials:

- Forceps
- Alcohol burner
- Autoclave bags
- 70% Alcohol
- Matches
- Scissors
- Petri plates with growth medium
- Parafilm® Tape

Plating Procedure:

1. Open the bait bag using the scissors
2. Use the forceps to remove the pear pieces
3. Place the pieces into the growth medium
4. Close the plate and seal it with Parafilm Tape
5. Between samples, clean forceps and scissors (dip in 70% alcohol and heat over the burner)
6. Discard waste in autoclave bags
7. Prepare samples for shipping

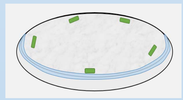

Figure 3. Pear pieces in a petri dish containing a medium for *Phytophthora* to grow in.

E. *Phytophthora* Identification

Samples are sent overnight, in a cooler with icepacks, to the UCB Forest Pathology and Mycology Lab. Plates are evaluated and if *Phytophthora* colonies are found (Fig 1C), they are transferred into a new clean petri dish with a medium for growth (Fig. 1D, pure culture). Difficult isolates are reevaluated using the ImmunoStrips. Pure cultures are set aside for extraction and storage. DNA is extracted using the Qiagen DNeasy® Plant Mini Kit, PCR amplified using ITS (internal transcribed spacer) primers for fungi (7) and oomycetes (8). The amplified product is sequenced (Fig 1E) and compared to published sequences in the GenBank database.

Results

Detection

All blocks contained *Phytophthora*-positive plants (Table 1, range: 20% to 44% using the 'gold standard' Destructive method). The Destructive and ImmunoStrip method detected *Phytophthora* in all blocks. The Drip and Scoop methods detected *Phytophthora* about 60% of the time with only one composite sample.

Table 1. Positive detections and the number of samples taken (n); within each block and in total

	Positives						n (samples)	
Method	Block A	Block B	Block C	Block D	Block E	Total	per Block	Total
Drip	1	1	0	1	0	3	1	5
Scoop	1	1	1	0	0	3	1	5
ImmunoStrip	5	9	7	4	2	27	25	125
Destructive	5	11	8	6	5	35	25	125

Cost, Labor, & Wait Time

The Scoop method was the least expensive and labor intensive (Table 2), it provides species-level *Phytophthora* detection within a standard wait time. The Drip cost, wait time and detection ability were comparable to the Scoop method, but labor time was about thirty minutes greater, which adds up quickly over several samplings. The ImmunoStrip method was by far the most expensive and labor intensive. ImmunoStrip wait time is very short, but results need validating, and the species remains unknown.

Table 2. Cost, Labor and Wait Time

	Non-Destructive Sampling Method			Destructive Sampling
	Drip	Scoop	ImmunoStrip	
Cost of supplies (per block of 80 plants)	$55.20	$51.30	$139.40	--
Labor time (per block of 80 plants)	70 minutes	43 minutes	192 minutes	--
Wait time for results (post sampling)	17 days	17 days	30 minutes	17 days
Phylogenetic level of detection	species	species	genus (needs validating)	species

Conclusions

Integrated Approach to Monitoring

We feel confident we will know if plants in our nursery have *Phytophthora* by using an integrated monitoring approach. This approach combines the non-destructive Scoop and the Destructive sampling methods (Table 3). The Scoop method requires the least labor and cost, and can evaluate many plants with one sample, but lacks the strength that the Destructive method has in evaluating whole plants. Table 3 shows how we sample a non-symptomatic plant lot. For Presidio Native Plant Nursery, this monitoring approach is both powerful in detection strength and feasible terms of labor and cost.

Table 3. The Scoop and Destructive methods were combined, and this is the sampling combination currently used for a non-symptomatic plant lot.

Plant Lot Size	Sampling Combination
10-75	3 Destructive sampled
76-300	4 Destructive sampled
301-750	4 Destructive + 1 Scoop composite sample from 25 plants
751-1500	5 Destructive + 1 Scoop composite sample from 25 plants
1501-2000	5 Destructive + 2 Scoop composite samples each from 25 plants

Overview of Methods

Scoop. The least labor intensive and most cost effective method to test a large number of plants at our nursery for *Phytophthora*. With a detection level of 60%, it should be combined with another method.
Destructive. Gold Standard for detection, but impractical on a large-scale. Should be done on small-scale and combined with another method.
Drip. Detected *Phytophthora* in equal number of blocks as Scoop. However, Drip took more labor time due to management of containment trays and a control tray to capture non-effluent irrigation water.
ImmunoStrip. The cost and labor is prohibitive, and results are limited (genus). Can give a false-positive in the presence of *Pythium,* resulting in rejection of *Phytophthora*-free crops, which is wasteful and expensive. Can be good for other applications such as evaluating leaf symptoms.

Acknowledgements

Sharon Farrell, Lew Stringer, Terri Thomas, Alison Forrestel, and Jennifer Parke provided input to shape this trial. John Doyle, Eliza Lasky, Alison Pollack, Hillary Kato, Will Lide, and Kim Horrell assisted in performing sampling, baiting, plating, and running ImmunoStrip tests. The Presidio Trust (Steve Potts), and the Golden Gate National Parks Conservancy (Sharon Farrell) provided budget support.

References

1.Erwin & Ribeiro. *Phytophthora* Diseases Worldwide. *APS Press* (1996).
2. Vercauteren et al. Survival of *Phytophthora ramorum* in Rhododendron root balls and in rootless substrates. *Plant Pathology* (2013).
3. Reeser et al. *Phytophthora* species in tanoak trees, canopy-drip, soil, and streams in the sudden oak death epidemic area of south-western Oregon, USA. New *Zealand Journal of Forest Science* (2011).
4. McIntosh. *Phytophthora* spp. in soils of the Okanagan and Similkameen valleys of British Columbia. *Canadian Journal of Botany* (1964).
5. Mircetich et al. *Phytophthora* root and crown rot of cherry trees. *Phytopathology* (1976).
6. Sims et al. The *Phytophthora* species assemblage and diversity in riparian alder ecosystems of western Oregon, USA. *Mycologia* (2015).
7. White et al. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *PCR Protocols: A Guide to Methods and Applications* (1990).
8. Cooke et al. A molecular phylogeny of *Phytophthora* and related oomycetes. *Fungal Gen & Bio* (2000).