The Isolation and DNA Extraction of *Phytophthora* from Four Wildland Soil Types in Northern California, Humboldt County.

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Abstract

Phytophthora species are of global importance (Sinclair and Lyon, 2005). For most *Phytophthora* species, the natural mechanism of how these pathogens travel and spread long distances is not fully understood. They are have been shown to travel by air, water, nursery stock, and via inadvertent human transport. The purpose of this study was to identify the presence of *Phytophthora* species in four different Northern California forest/ wildland soil types. Soils from Franciscan complex parent material (riparian, mixed conifer, and oak woodland), and serpentine parent material (mixed conifer) were sampled in Humboldt County (Fig 1). *Phytophthora* and other pathogens were baited from each soil type with Rhododendron leaf disc (Fichtner et al. 2007, Reed lab) and Port Orford-cedar baits. To select for *Phytophthora* sp. we cultured baits on clarified V8 agar containing PARPNH (Reed Lab) with follow up isolation on a modified layer Ampicillin plate method to eliminate bacterial contamination. Large hyphal quantities were cultured in a clarified V8 broth, rinsed, and freeze-dried. DNA was extracted using either a NaOH method (Osmundson et al. 2012) or the Plant DNeasy kit to compare results. DNA was amplified using PCR with ITS (internal transcribed spacer) primer pairs ITS 5-4 (Ristaino et al. 1997), ITS 6-4 (Ristaino et al. 1997), and Phyto1-4 (Davidson et al. 2003). Purified PCR products from both extraction methods were submitted for sequencing. Initial results point to *Phytophthora* species including *P. cambivora and P. undulata.*

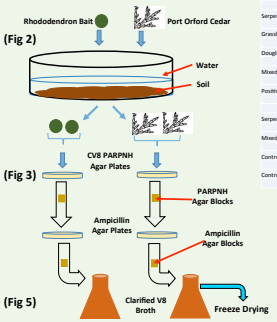
Introduction

- *Phytophthora* is a plant-parasitic oomycete (water mold; Peronosporales, Pythiaceae) pathogen that causes diverse symptoms (Sinclair and Lyon, 2005) for both agricultural plants and natural forest ecosystems.
- *Phytophthora spp.* produce hyphal structures resembling fungi, however, they are non-septate, unlike true fungi and differ from fungi in that their cell walls contain cellulose, beta glucans, and the amino acid hydroxyproline, but do not contain chitin (Rossman and Palm, 2006).
- Surveys in many forest ecosystems have shown the existence of a variety of *Phytophthora* species (Balci et al. 2007).
- *Phytophthora* can produce sexual and asexual spores (Phytophthora Basics) dependent on environmental conditions and species.
- Different types of spores can reside in the soil for long periods until conditions are suitable for germination.
- **The purpose of this study is to determine the presence or absence of *Phytophthora* species in four different forest/wildland soil types in Humboldt County, Fig 1.**

Methods and Materials

Soil sampling **-** Soils from Franciscan complex parent material (riparian, mixed conifer, and oak woodland), and serpentine parent material (mixed conifer) were sampled in Humboldt County, Northern California. The upper 15 cm of mineral soil were sampled in mid-spring and mid-summer (worst drought on record) of 2015, placed in sealed plastic bags and refrigerated.

Phytophthora Baiting Process



Soil Samples				
Spring		Bait	Sequenced	Gel
Oak	POC	Yes	no	
	Rhod.	Yes	no	
Serpentine	Rhod.	no	no	
	POC	Yes	Yes	
Grassland	Rhod.	no	no	
	POC	no	no	
Douglas fir drip-line	Rhod.	no	no	
	POC	no	no	
Mixed Conifer	Rhod.	Yes	no	
	POC	no	no	
Positive control	Rhod.	Yes	Yes	
	POC	no	no	
Summer				
Serpentine	Rhod.	no	Yes	
	POC	no	no	
Mixed Conifer	Rhod.	no	no	
	POC	no	no	
Control Dead (POC)	Rhod.	no	no	
	POC	no	no	
Control Willow Creek Channel	Rhod.	no	no	
	POC	no	no	

Table 1: Soils collected during spring and summer sampling field trips. DNA was extracted from samples that did not show bacteria, mold, or yeast contamination. Some of these samples were sequenced some were analyzed by gel (Fig 7).

DNA Extraction and Sequencing **-** DNA was extracted from freeze-dried hyphae (Fig 6) using a NaOH method (Osmundson et al. 2012) and Plant DNeasy kit, amplified with the primers ITS 5/4 and 6/4 and Phyto1/4 to determine if *Phytophthora* was cultured. PCR product was purified and sent out to be sequenced.

Figure 1: Soils collected from mixed conifer, oak woodland, Port Orford cedar and serpentine soils in Humboldt County

Figure 2: Rhododendron and Port Orford Cedar baits floating in standing water above soil sample.

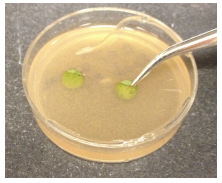


Figure 3: Rhododendron baits in a 60 mm culture plate with PARPNH agar.

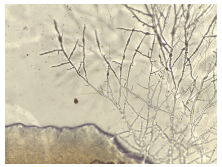


Figure 4: Non-Septate hyphae growing in the PARPNH plate on day 3.

Figure 5: Hyphae cultured in the dark with clarified V8 broth from an Ampicillin agar block inoculum.

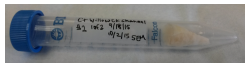


Figure 6: Freeze dried hyphae after being cultured for 7 days in clarified V8 (CV8) broth.

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Results

- Soils from both seasons produced PARPNH selective cultures during a 5 day growth period (Fig 4).
- Isolated hyphae were cultured in the dark in a clarified V8 broth (Fig 5) for up to a week in order to acquire enough material to freeze dry to perform DNA extraction.
- Agarose gel analysis of PCR product from spring soil samples indicates the ITS 6/4 amplicon length matching ~ 900 bp (Fig. 7).
- Sequencing of the Spring soil sample PCR product yielded various matches within the Phytophthora and Pythium sp. (Table 2).

1.5% TAE Agarose gel ITS 6/4 primer set amplicon

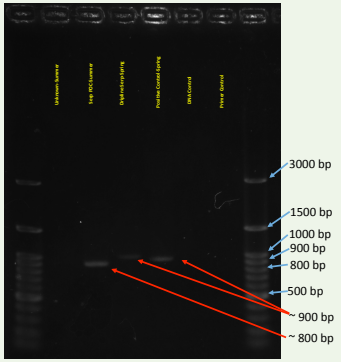

Figure 7: Agarose gel of PCR product amplified from DNA extracted from hyphae cultured from a summer soil and two spring soils.

Spring Soil Samples	Matched Species
Oak POC	P. cambivora
	P. pini
Oak Rhod. 2	Phythium sp.
	Pythium glomeratum isolate
Oak Rhod. 1	*Phytophthora* sp.
	P. undulata
	P. tentaculata
	Phytophthora sp.
	P. nicotianae
	P. alni subsp. multiformis
	P cinnamomi var parvispora
Mixed Conifer Rhod	Mortierella clone OTU117
Serpentine POC	Pythium sp.
	Phytophthora sp.
	P. idaei
	P. ramorum

Table 2: Sequences for PCR product from spring soils was BLASTed against the NCBI and checked with Phytophthora database (PDB). Various *Phytophthora* and *Pythium* species were identified at greater than 90% match.

Discussion

- Isolation of hyphae was problematic due to Bacterial/fungal contamination. Improved isolation of *Phytophthora* occurs at lower incubation temperatures (personal communication Reed lab) and that approach will be adhered to in future studies. We are keen to investigate changes in isolation success with varying seasons and soil moisture status.
- Only spring soils produced hyphae samples capable of providing a strong sequences for identification, either due to seasonal variation or an increase in culturing temperature.
- Methods of PCR product purification needs to be further tested and improved to allow for a better sequencing result.
- An alternative identification method involves digesting the PCR product to verify DNA product for better sequencing results to better identify *Phytophthora spp.*.
- The ITS 6/4 primer set yielded the most clear results indicating our ability to bait, culture, and amplify various *Phytophthora spp.* from the Spring soil samples.

Future Research

- Identifying the best conditions for baiting and propagating *P. ramorum in Humboldt county soils.*
- Does *P. ramorum* reside in arboreal histosols?
- Is there a seasonal effect, that includes temperature and moisture, on detection of *Phytophthora?*

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