Proceedings of The National Conference
On Undergraduate Research (NCUR) 2015
Eastern Washington University, Cheney, WA
April 16-18, 2015

# Molecular Analysis and Thermal Treatment of Several *Phytophthora* Species Causing Diseases of Ornamental Plants in California.

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## Abstract

The genus *Phytophthora* encompasses a range of fungal pathogens infecting and killing ornamental plant species. *P. ramorum* is known to be the causal agent of "Sudden Oak Death" (SOD), a disease that currently plagues Oak forests of coastal California and Oregon. Able to spread through water and trade of poorly monitored ornamental plants, the various strains of *Phytophthora* can spread rapidly unnoticed. Currently no known treatments efficiently terminate the spread of SOD in forests. Other *Phytophthora* species such as *P. cf fallax, P. syringae,* and *P. tentaculata,* have been observed recently in nurseries causing symptoms which mimic *P. ramorum.* To better understand the facets that govern the spread of *Phytophthora*, researchers at the National Ornamentals Research Site at Dominican University (NORS-DUC) analyzed and developed various techniques to control the proliferation and spread of this pathogen. Results from past experiments have demonstrated that *P. ramorum* can be neutralized through the use of high-temperature soil steaming. In this battery of experiments, we tested the effects of both wet and dry heat at a temperature of 50°C for 30, 60, and 120 minutes on isolate strains *P. cf fallax, P. syringae,* and *P. tentaculata* in the lab. Post-steamed samples were plated on selective media to test for survival rates. In addition, a steaming experiment was performed at the NORS-DUC nursery. The inoculum was placed at soil depths of 5cm, 15cm, and 30cm, and steamed for neutralization. Analysis of the *Phytophthora* phylogeny would contribute to molecular species identification and understanding of the genetic traits leading to symptom development. Phylogenetic relationships of the nursery pathogens were studied using sequence information of the genes for β-tubulin, elongation factor 1 α, and cytochrome oxidase I and II. The markers were evaluated through PCR and sequence analyses, followed by phylogenetic tree construction using the distance based neighbor-joining method with BLAST2 and ClustalW programs. With this research, we hope to identify and develop a safe, simplistic, and more efficient way of protecting native plants against *P. ramorum* and other plant pathogens.

**Keywords: Heat Treatment, Steaming, Sudden Oak Death**

## 1. Introduction

The etymology of the word genus name *Phytophthora* is derived from the Greek word *phytón*, meaning "plant" and *phthorá*, meaning "destruction", translating to "plant-destroyer". *Phytophthora* is a group of microbial pathogens that cause disease in a wide array of plants. *P. ramorum* is an oomycete that was discovered approximately 20 years ago, with very little information known about its origin and background [1]. It is the causal agent of Sudden Oak Death (SOD), a tree disease that kills Coast Live Oaks (*Quercus agrifolia*) and Tanoaks (*Notholithocarpus densiflorus*). Symptoms include dieback of foliage, wilting, and bleeding cankers on the trunk of the tree. First recorded in Marin

and Santa Cruz Counties around 1995, the pathogen has advanced along the coast of California and southern Oregon, afflicting 17 counties with SOD [2]. In 2004, over one million ornamental plants (mainly *Camellia* and *Rhododendron*) possibly infected with SOD were inadvertently shipped to nurseries across the country. As a result, 176 nurseries in 21 states suffered from ramorum blight [3]. Several other species of *Phytophthora* can be present in nurseries, exhibiting symptoms that mimic that of *P. ramorum*. For example, *P. tentaculata*, a species new to North America, has been discovered in native plant nurseries and restoration sites [4]. Similarly, *P. cf fallax* and *P. syringae* were isolated at the NORS-DUC nursery recently on exterior sentinel plants (T. Pastalka, personal communication). Members of the genus *Phytophthora* infect over 60 different genera and 100 host plant species. Presently, very little to no control methods are in place to manage or control *Phytophthora* in infected nursery soils. Chemical control shows only limited results, and also raises environmental concern. Biological control agents can inhibit *Phytophthora* growth, but rarely eliminate the pathogens completely (W. Schweigkofler, personal communication) Researchers at the National Ornamentals Research Site at Dominican University of California (NORS-DUC) are focusing on establishing effective methods to eradicate the spread of *Phytophthora*. *P. ramorum* can be inactivated by heat treatment at 50° C for 30 minutes [5]. NORS-DUC developed a steaming method to eradicate *Phytophthora* from infected soils. Our experiments seek to i) study the temperature effects on nursery-related *Phytophthora*species in the lab, ii) test effects of steaming on survival of selected species, and iii) use molecular analysis of selected strains in order to achieve proper strain identification.

## 2. Materials and Methods

### 2.1. Microbial Strains

The following *Phytophthora* strains were used for the experiments: *P. ramorum* 1418886 and 1418983-2 (both strains isolated from nurseries in Marin County), *P. tentaculata* 010P06220159-5 (isolated in Alameda County), *P. cf fallax* 6 and *P. syringae* 7 (both isolated at NORS-DUC, Marin County). Because the ITS-sequence of strain *P. cf fallax* 6 showed a limited number of basepair substitutions compared to the typestrain (data not shown), the definitive taxonomic position of the strain is still unknown.

### 2.2. Heat Experiment In The Laboratory

Each of the *Phytophthora* strains were grown on CV8 media plates for two weeks. Cultures were checked microscopically for presence of chlamydospores, sporangia, and oogonia. Twenty plugs of 5 mm diameter were placed into 1.5 mL centrifuge tubes. Ten of these tubes were filled with 1 mL ultrapure water to simulate the wet conditions. No water was added to the ten remaining tubes to simulate dry conditions.
These twenty tubes were then heat treated in an Eppendorf Thermomixer R for 30, 60, or 120 minutes at 50 °C. Treated plugs were plated on fresh selective media plates and incubated for two weeks at 20 °C. Afterwards, cultures were observed microscopically for any growth. Control groups for both wet and dry conditions were incubated at room temperature for 30, 60, or 120 minutes before plating on fresh media plates. The experiment was done in duplicate.

### 2.3. Steaming Experiment In The Research Nursery

Microbial isolates were grown on pieces of Whatman filter paper no. 1 (diameter: 5 mm) for two weeks on selective media before being placed in permeable sachets (tea bags). Sachets containing ten filter disks each were placed into the soil. Holes were dug to a depth of about 30 cm and sachets were placed at 5 cm, 15 cm, and 30 cm. One sachet inoculated with each strain was placed at each depth. Three holes were dug to simulate three separate replicates, resulting in a total number of 45 sachets used. As a control, sachets containing microbial cultures on filter papers were kept at room temperature in the lab for three days, before transferring the filter papers on PARPH-V8 plates.
The research plot with an area of 3.7 m x 4.6 m was lined with waterproof liners and filled with a 30cm-deep layer of gravelly loam soil. It was treated using a steamer unit SIOUX Steam-Flo SF-11 (Sioux Corp., Beresford, SD) with a boiler horsepower of 10.67 and a steaming output of 168 liters/h (Fig. 1). It was equipped with an attached soaker hose 31m in length, which was laid out on the soil surface. The plot was covered with a waterproof, pond liner-type tarp. Temperature sensors (Onset Corp., Cape Cod, MA) were placed at 5, 15, and 30 cm soil depths to monitor temperatures.

Figure1. Thermal treatment of *Phytophthora* samples at the NORS-DUC research nursery using a steaming unit SIOUX Steam-Flo SF-11.

The entire research plot was steamed at a minimum temperature of 50 °C for 30 minutes before turning off the steamer. The sachets were collected when soil temperatures had cooled down to ambient temperature again. The sachets were then cleaned using ultra-pure water and the filter disks were re-plated on selective media PARPH-V8 and incubated for two weeks at 20 °C. Afterwards, plates were observed microscopically for any microbial growth.

## 2.4. Molecular Analysis

DNA was isolated from all selected *Phytophthora* strains, according to FastDNA kit protocol. A total of four reference genes were selected for molecular analysis of the *Phytophthora* strains: Elongation Factor 1-α (EF1-α), β-tubulin, and Cytochrome c Oxidase I (COX I). Forward and reverse primers (included in Table 1) were selected to amplify selected DNA fragments with Polymerase Chain Reaction (PCR). Samples were denatured for 30 seconds at 94 °C, annealing took place at 58 °C for 30 seconds, and elongation occurred at 72 °C for 10 minutes. Amplified PCR products were purified using QIAGEN Kit protocol. Amplification of desired fragments were confirmed and checked for purity using gel electrophoresis on 2 % agarose gels. DNA fragments were sequenced at UC Berkeley DNA Sequence Facility. Chromatogram sequences produced were manually reviewed and analyzed. BLAST2 search engine (www.ncbi.blast.com) was used to compare against sequences with existing data in GenBank [6]. Phylogenetic analysis was conducted using ClustalW software (www.clustal.org) [7].

Table 1. Primer Sequences

| Gene | Primer name (Fwd and Rev) | Forward Sequence | Reverse Sequence |
|---|---|---|---|
| *Translation Elongation factor 1-α* | ELONGF1<br>ELONGR1 | 5' TCACGATCGA CATTGCCCTG 3' | 5' ACGGCTCGAG GATGACCATG 3' |
| *β-tubulin* | TUBUF2<br>TUBUR1 | 5' CGGTAACAAC TGGGCCAAGG 3' | 5' CCTGGTACTG CTGGTACTCAG 3' |
| *Cytochrome c oxidase subunit 1* | COXF4N<br>COXR4N | 5' GTATTTCTTC TTTATTAGGTGC 3' | 5' CGTGAACTAA TGTTACATATAC 3' |

## 3. Results

### 3.1. Thermal Treatment Effect On The Survival Of *Phytophthora* Strains

Heat treatment of five *Phytophthora* strains in the laboratory at 50 °C for 30, 60, and 120 minutes resulted in complete inactivation of the cultures (survival rate: 0 %). The heating method ('wet' vs. 'dry' heat) had no influence on treatment efficacy. All mycelial plugs used as controls and incubated at room temperature showed post-treatment growth (survival rate: 100%). Results are shown in Fig. 2 for the treatments for 30 minutes alone.

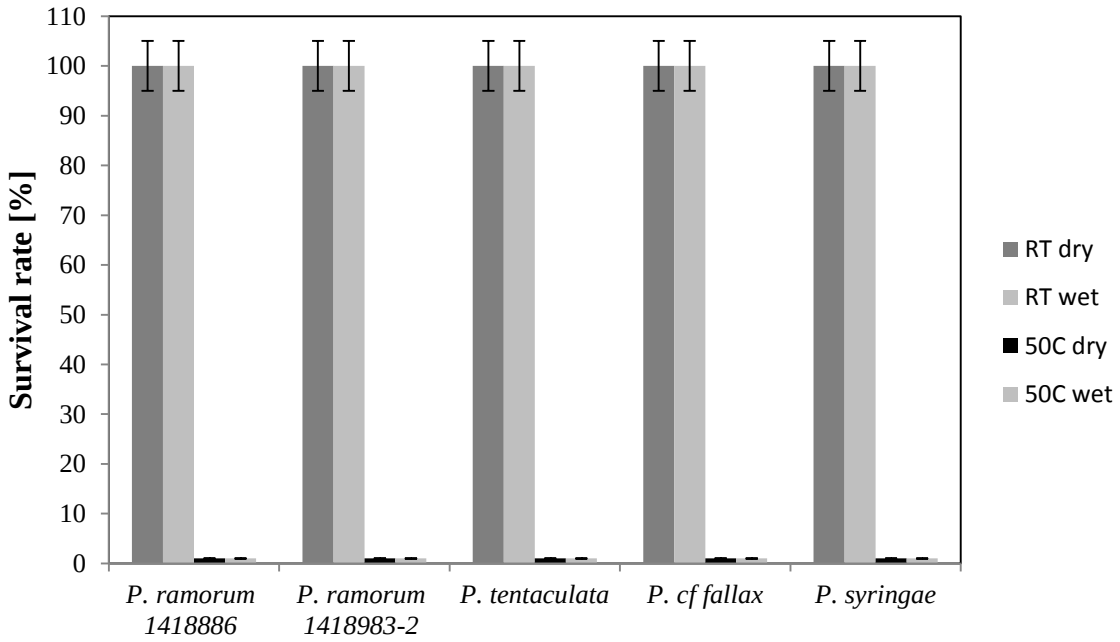


Figure 2. Thermal treatment effect on the growth of five *Phytophthora* isolates. Mycelial plugs were incubated at 50 °C for 30 minutes at 'dry' (dark gray) and 'wet' (light gray) conditions. Control plugs were incubated at room temperature (RT, approx. 20°C). Error bars indicate 95 % confidence intervals.

## 3.2. Steaming Effect On The Survival Of *Phytophthora* Strains

The top-down heating approach using steam produced by a commercial steamer resulted in the temperature profile shown in Fig. 3 for the three soil layers at 5, 15, and 30 cm below surface. The steaming experiment was carried out on February 20, 2015 with an ambient temperature of approximately 14 °C (no significant differences were measured at the three soil depths before steaming). The target temperature of 50 °C at 5 cm soil depth was reached after 3 h, at 15 cm after 8 h, and at 30 cm after 16.5 h. The maximum temperatures at 5 cm were 85.9 °C, at 15 cm 70.1 °C, and at 30 cm 53.0 °C, respectively. The steamer was switched off 30 minutes after the deepest soil depth reached the target temperature. Whereas the temperatures at the upper soil layers started to drop quickly, the temperature at 30 cm rose for several degrees Celsius because of continuing heat transfer from the upper soil layers. The total time above 50 °C was 22 h 15 m for the top soil layer (5 cm); 20 h 45 m for the middle soil layer (15 cm), and 9 h 45 m for the deepest soil layer (30 cm), respectively. The filter disks with *Phytophthora* inoculum were recovered from the treated soil when the temperatures had cooled down to approximately 25 °C and transferred on fresh media plates. After two weeks incubation at 20 °C no *Phytophthora* growth was observed on any plate from the three soil depths (5, 15, and 30 cm), corresponding to a survival rate of 0 %. All filter papers kept for three days at room temperature as a control developed cultures of the *Phytophthora* strains.

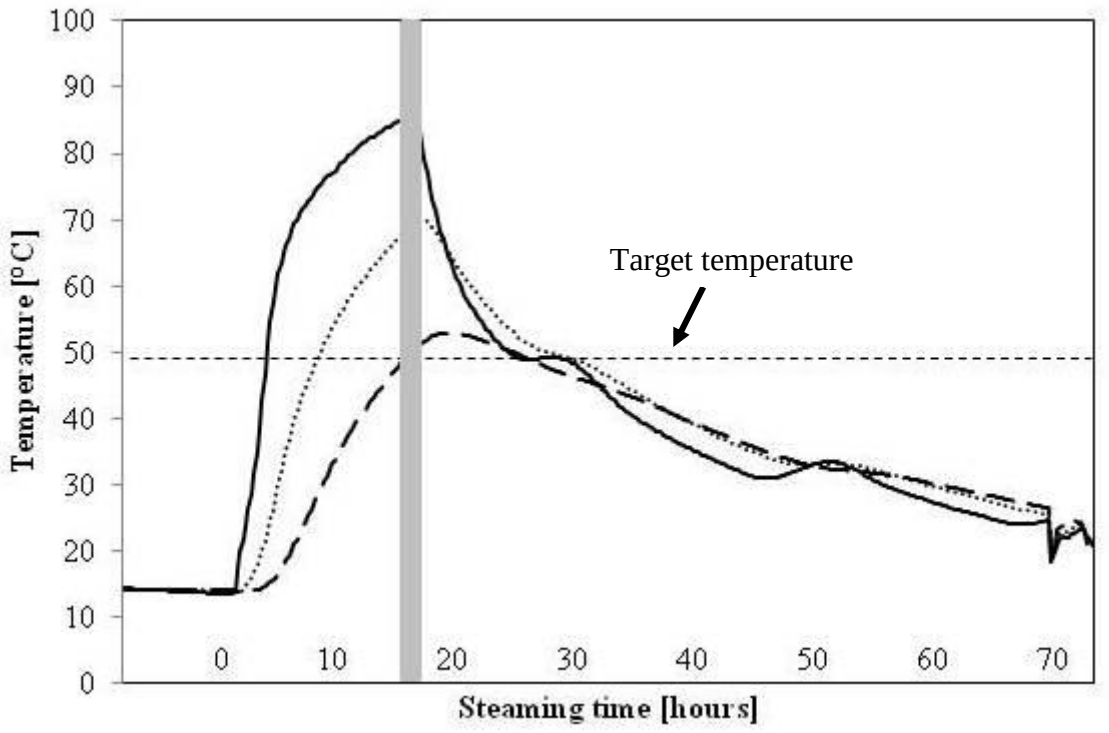


Figure 3. Temperature profile of the steaming event at the research nursery at NORS-DUC. Temperatures were measured at depths of 5 cm (solid line), 15 cm (dotted line), and 30 cm (dashed line). The horizontal line shows the target temperature of 50 °C. The 'inactivation period' (defined as the time between reaching the target temperature and turning off the steamer) is shown in light gray.

## 3.3. Molecular Identification Of *Phytophthora* Strains

DNA of the five *Phytophthora* strains was isolated and three genes (Translation Elongation factor 1-α, β-tubulin and Cytochrome c oxidase subunit 1) amplified using PCR. The purified DNA fragments were sequenced by the UC Berkeley Sequence facility. The sequences were inspected visually and then compared to sequences stored in the GenBank. The results of the BLAST-search are shown in Table 2. With the exception of the β-tubulin gene of *P.*

*tentaculata*, all *Phytophthora* strains showed the best match with genes from the corresponding species already stored in the GenBank. Cladograms created for each separate gene also clustered the five *Phytophthora* strains with the corresponding co-specific species from GenBank (data not shown).

Table 2. Result of Blast Search for *Phytophthora* strains using three different genes. The best match from GenBank and its accession number is listed. The similarity between the two is expressed by the identity percentage of shared basepairs.

2.1 Gene amplified: *Translation Elongation factor 1-α*

| **NORS strain** | **Best GenBank Match** | **Accession No.** | **Identity (%)** |
|---|---|---|---|
| *P. tentaculata* | *Phytophthora tentaculata* isolate PD_00180 (P8496) | EU080155 | 97 % |
| *P. ramorum 886* | *Phytophthora ramorum* isolate PD_00048 (P10467) | EU080587 | 99 % |
| *P. cf fallax* | *Phytophthora fallax* isolate PD_00162 (P10725) | EU080036 | 98 % |
| *P. ramorum 83.2* | *Phytophthora ramorum* isolate PD_00048 (P10467) | EU080587 | 99 % |
| *P. syringae* | *Phytophthora syringae* isolate PSY-06-099 | HQ917891 | 99 % |

2.2. Gene Amplified: B-*Tubulin*

| **NORS strain** | **Best GenBank Match** | **Accession No.** | **Identity (%)** |
|---|---|---|---|
| *P. tentaculata* | *Phytophthora syringae* isolate GW-12-087 | KM501463 | 98 % |
| *P. ramorum 886* | *Phytophthora* sp. P10800 | EU080724 | 99 % |
| *P. cf fallax* | *Phytophthora fallax* isolate P10725 | HM534902 | 99 % |
| *P. ramorum 83.2* | *Phytophthora ramorum* isolate PD_00048 (P10467) | EU080586 | 99 % |
| *P. syringae* | *Phytophthora syringae* isolate PSY-06-099 | HQ917875 | 99 % |

2.3. Gene Amplified: *Cytochrome C Oxidase Subunit 1**

| **NORS strain** | **Best GenBank Match** | **Accession No.** | **Identity (%)** |
|---|---|---|---|
| *P. ramorum 886* | *Phytophthora ramorum* mitochondrion, complete genome | DQ832718 | 97 % |
| *P. cf fallax* | *Phytophthora fallax* isolate 46J2 | KC733451 | 96 % |
| *P. ramorum 83.2* | *Phytophthora ramorum* mitochondrion, complete genome | DQ832718 | 97 % |
| *P. syringae* | *Phytophthora syringae* isolate PD4987850 | JX667780 | 99 % |

*the COX gene of *P. tentaculata* has yet to be analyzed

## 4. Discussion

Heat treatment of infected soils has previously been shown to be an effective method to neutralize invasive plant pathogens such as *P. ramorum* [5]. However, it has not yet been determined whether or not steaming would be as useful on other closely related *Phytophthor*a strains. The first goal of our research was to determine the effects of heat treatment on *P. tentaculata*, *P. cf fallax* and *P. syringae* in the lab. Two strains of *P. ramorum* were included in the study to compare them with previous findings. Schweigkofler et al. [5] used *P. ramorum* growing on *Rhododendron* leaf disks for their experiments. Because *P. tentaculata* does not grow on *Rhododendron*, we had to adapt our methodology and use mycelial agar plugs instead.

A minimum incubation time of 30 minutes at 50 °C inactivated all *Phytophthora* strains tested (Fig. 1). When incubated at room temperature, all *Phytophthora* strains developed cultures. There were no notable variances between strains incubated in either wet or dry conditions. Many microbes are better suited to survive dry heat than wet heat [8]. Under our conditions, we could not observe any significant differences.

Schweigkofler et al. [5] showed that incubation at 40 °C decreases survival rates of *P. ramorum* without complete inactivation. While the effects of temperatures below 50 °C were not the focus of this study, it should be further analyzed in the future. Our results are in agreement with environmental data that showing that *P. ramorum* and many related *Phytophthora* strains inhabit areas with cool climates and relatively high humidity [1]. Because our lab experiments showed that *Phytophthora* strains can be inactivated at 50 °C, we wanted to test if heat treatment is an efficient method to treat infected nursery soil. The NORS-DUC research site contains research plots that mimic characteristics of a commercial nursery and therefore is well suited for semi-field experiments on quarantine plant pathogens [9].Using a steamer (SIOUX Steam-Flo SF-11), inoculated filter disk paper was heated to a minimum of 50 °C for 30 minutes. *Phytophthora* cultures grown on filter disk paper were used instead of *Rhododendron* disks because *P. tentaculata* does not grow on *Rhododendron* leaves [5].Important structures that characterize *Phytophthora* (chlamydospores, sporangia, and oogonia) were developed on the filter disks. The integrity of the filter disk remained intact throughout the steaming process, showing the effectiveness of this method. Results from our steaming experiment confirmed our lab heating experiments. After plating the treated filter disks, no *Phytophthora* growth was observed after being heated at 50 °C for a minimum of 30 minutes. Temperature sensors were placed at depths of 5 cm, 15 cm, and 30 cm, where *Phytophthora* has been found in the soil. Due to the top-down heating method of the steamer, three varying depths were used. The target temperature of 50 °C was reached quickly in the uppermost layer (5 cm), compared to the bottom layer (30 cm). Results from the steaming and lab experiments showed the protocol developed by Schweigkofler et al. [5] to eliminate *P. ramorum* from infested soil can also be used to control other stains of *Phytophthora*.

Molecular identification of *Phytophthora* strains using variable genes is a useful method to characterize species which are not otherwise easily distinguishable using morphology. Sequence analysis of Elongation Factor 1-α (EF1-α), β-tubulin, and Cytochrome c Oxidase I (COX I) and comparison with sequences stored in the GenBank allowed us to identify all five *Phytophthora* strains on the species-level. In general, sequence identities of 97 % or more are considered to be co-specific. The only exception was the β-tubulin gene of *P. tentaculata*, which showed a match with *P. syringae*. The remaining small genetic differences between our *Phytophthora* strains and the ones stored in GenBank (1-3 % mismatch) could be explained by inter-specific variability. Further analysis is needed to confirm our findings.

Accurate identification of invasive microbes is a necessary first step for developing control options. We think that our research will contribute to establish an efficient and sustainable method for controlling plant pathogenic *Phytophthoras* in nurseries and similar environments.

## 5. Acknowledgements

NORS-DUC is funded by a grant from the 2008 Farm Bill, Section 10201, and administrated through the United States Department of Agriculture (USDA) Animal and Plant Health Inspection Service (APHIS) Plant Protection and Quarantine (PPQ) Center for Plant Health Science and Technology (CPHST). We would also like to extend our thanks to both Dr. Wolfgang Schweigkofler and Dr. Supriya Sharma for their undying support and drive for NORS-DUC's success. They have served not only as faculty advisors, but mentors and family as well.

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