# Evaluation of a Novel Diagnostic Procedure to Detect the Presence of *Phytophthora ramorum* by Sampling Ooze from Infected Cankers

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## Introduction

*Phytophthora ramorum,* the causal agent of the disease commonly known as Sudden Oak Death, is a prevalent pathogen in California with its effects evident in 12 counties and found on 14 different oak, tanoak and non-oak hosts. On oak and tanoak, cankers are formed along the lower trunk and often ooze seeps from them. The traditional field diagnostic technique to confirm the presence of the pathogen in these species involves shaving away the outer bark near the perimeter of the canker, collecting pieces of inner bark at the perimeter of the canker, and placing these pieces on selective PARP media. This sampling technique is often very destructive, leaving large wounds on the lower trunk of the tree. Also, this technique is not very dependable; at best, it may require multiple samples to get an affirmative result on known diseased trees.

Since diseased trees commonly have cankers where ooze seeps to the surface of the bark- and *P. ramorum* sporangia have been observed in this ooze- it is possible that sporangia or other fungal components could be detected with the right detection technique. This study evaluates the reliability of using PCR (polymerase chain reaction) and traditional PARP selective media to confirm the pathogen's presence in canker ooze.

*In a characteristic infection of SOD, cankers are formed on oak (left) and tanoak (right) along the lower trunk and often ooze seeps from them.*

## Materials and Methods

- For 9 months (August 2001 to April 2002) ooze samples were taken weekly from naturally infected, seeping cankers of 10 coast live oaks *(Quercus agrifolia)* and 4 tanoaks *(Lithocarpus densiflora)* in several locations in north Santa Cruz County.
- When present, ooze was tested for the presence of *P. ramorum* with PCR (polymerase chain reaction) or by plating on PARP selective media.
- Ooze was periodically examined for fungal particles under a light microscope.
- Cankers were sampled using traditional diagnostic techniques, where small inner bark samples are placed into selective media.



*Ooze samples for PCR analysis were collected from the freshest appearing exudates on each tree. A sample ranging in volume from 0.25 ml to 1 ml (dependent on amount available at the time of sampling) was collected with a stainless steel laboratory spatula and placed into an Eppindorf micro centrifuge tube. Samples were labeled and stored under refrigeration until PCR analysis was performed. The spatula was disinfected with 70% ethanol and flamed between each sample.*

*A sample of ooze from each tree was collected with a stainless steel laboratory spatula and plated onto PARP selective medium filled petri plates. The spatula was disinfected with 70% ethanol and flamed between each sample.Plates were sealed with Parafilm® and incubated at 20$^0$ C for 48-72 hrs, then initially inspected for characteristic Phytophthora ramorum colonies. Plates were then repeatedly inspected for colony formation for up to two weeks following sampling.*

*Samples of bark from the margin of suspected P. ramorum lesions were taken from each tree and plated on PARP selective medium. Efforts were made to minimize the size of the wound to allow for repeated sampling in the following weeks. A small sample, approximately 1-3 mm in size, of the canker margin including both necrotic and healthy tissue was removed with stainless steel forceps that had been disinfected with 70% ethanol and flamed. Up to 5 bark samples were then plated into each plate of PARP selective medium. The plates were incubated at 20$^0$ C for 48-96 hours, and then inspected for characteristic P. ramorum colonies.*

## Results

The three detection techniques, 1) inner bark plated on PARP, 2) ooze tested with PCR, and 3) ooze plated on PARP resulted in 24.3, 16.7, and 2.1 % with positive findings of *P. ramorum* respectively. See Table 1.

Detection of *P. ramorum* was less successful on tanoak than on live oak. See Table 2.

No fungal structures of *Phytophthora* were observed in ooze samples.

Ooze was not always present for sampling on some trees during some months, particularly following exceptionally warm weather. Fall had more ooze present than the other seasons (data not shown). Winter is the best time to sample for detection. See Table 3.

Table 1

***Effect of Treatments on Success of Isolation of Phytophthora ramorum***

| *Treatment* | *N* | *% Actual Positive* | *% Expected Positive* |
|---|---|---|---|
| Inner bark plated on PARP | 403 | 24.3 | 14.7 |
| Ooze plated on PARP | 340 | 2.1 | 14.7 |
| Ooze tested with PCR | 216 | 16.7 | 14.7 |

Chi-square =73.7 P< 0.001, Positive = successful isolation of *P. ramorum*
N = number of total samples

Table 2

***Effect of Species on Success of Isolation of Phytophthora ramorum***

| *Species* | *N* | *% Actual Positive* | *% Expected Positive* |
|---|---|---|---|
| Live oak | 607 | 16.7 | 14.7 |
| Tanoak | 122 | 8.3 | 14.7 |

Chi-square = 73.7 , P = 0.002, Positive = successful isolation of *P. ramorum*
N = number of total samples

## Discussion

PCR proved to be an effective technique to detect *P. ramorum* in canker ooze. This technique was nearly as effective as the traditional detection technique, plating bark samples on selective PARP media. However, plating ooze on selective PARP media was relatively ineffective.

No *Phytophthora* fungal structures were observed during periodic examination of the ooze, and therefore the plating would understandably be relatively ineffective since viable inoculum structures would be necessary for detection on selective media. Apparently enough DNA was extruded from cankers for the PCR analysis to effectively detect *P. ramorum*.

Winter (Nov., Dec., and Jan.) may be the most productive months of sampling, possibly because of the cool weather favoring the pathogen.

The limiting factor for the ooze technique is that ooze is not always present on active cankers. Sometimes ooze dries, especially in warm weather. In this case a traditional detection technique would be required.

The clear advantage of the ooze / PCR detection technique is that it is a non destructive technique.

Table 3

***Effect of Season on Success of Isolation of Phytophthora ramorum***

| *Season* | *N* | *% Actual Positive* | *% Expected Positive* |
|---|---|---|---|
| Fall | 538 | 12.3 | 14.7 |
| Winter | 260 | 21.9 | 14.7 |
| Spring | 161 | 11.2 | 14.7 |

Chi-square =14.95 P= 0.001, Positive = successful isolation of *P. ramorum*
N = number of total samples. Fall= Aug., Sept., Oct. ; Winter = Nov., Dec., Jan. ; Spring = Feb., March, Apr.

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