

A High Throughput System for the Detection of *Phytophthora ramorum* in Susceptible Plant Species: A Preliminary Report¹

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Abstract

Phytophthora ramorum is a pathogen of regulatory concern in North America and Europe. In 2004, potentially infected plants were shipped from large, wholesale nurseries on the West Coast (California, Oregon, and Washington) throughout the U.S. This prompted a nationwide survey effort and the adoption of a federal order requiring mandatory inspection and testing of all West Coast nurseries shipping *P. ramorum* host and associated host plants (HAP) interstate. In Oregon, this required the testing of 51,645 samples from 1,034 growing areas and 79,930 samples from 1,394 growing areas in 2005 and in 2006, respectively. Because all testing must be completed before nurseries can ship HAP interstate, the Oregon Department of Agriculture developed a high throughput system using a 96-well format to enable testing of large numbers of samples in accordance with federally validated protocols (ELISA, nested PCR, and qPCR). To verify the efficacy of the system, healthy leaves from four HAP species were wounded and then artificially inoculated with *P. ramorum*; healthy control leaves were wounded and then inoculated with a sterile agar plug. Samples were collected from the inoculated and control leaves and placed into 10 X 96 collection microtubes. Subsamples from each HAP were bulked five per microtube in varying ratios of inoculated to non-inoculated tissue (5:0, 1:4, and 0:5). Sample tissues were macerated and tested with ELISA according to the manufacturer's protocol. The OD readings of all inoculated samples were consistently 5X the negative control. All non-inoculated controls were below the 2X threshold with one exception. In the second replicate, the OD reading of the *Pieris japonica* non-inoculated control was >2X the negative control. DNA was then extracted from the remaining sample tissue in the GEB2 buffer. All inoculated samples were positive using nested PCR while non-inoculated controls were negative. Sample DNA was then tested with qPCR. All inoculated samples were positive while all non-inoculated controls were negative with one exception. In the first replicate, the *Camellia* non-inoculated control had a Ct value of 40.08 for the *P. ramorum*-specific probe. According to USDA protocol, this sample would be tested with nested PCR to confirm this negative test result. The high throughput, 96-well format system was also used successfully with environmental samples. Samples from four HAP species and six HAP genera were identified as positive with ELISA and subsequently as *P. ramorum*-positive by nested PCR and/or qPCR. USDA officially confirmed these test results. These preliminary results indicate that the high throughput system successfully detected *P. ramorum* in infected plant tissue using the USDA-validated ELISA, nested PCR, and qPCR protocols.

Key words: High throughput testing, ELISA, nested PCR, qPCR.

¹ A version of this paper was presented at the Sudden Oak Death Third Science Symposium, March 5–9, 2007, Santa Rosa, California.

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Introduction

Phytophthora ramorum Werres, Man in't Veld, & de Cock is a pathogen of regulatory concern in North America and Europe. The disease is established in 14 coastal counties in California and has been detected in Curry County, Oregon (Goheen and others 2002, Rizzo and others 2002). In 2004, *P. ramorum* was detected in large, wholesale nurseries on the West Coast that shipped potentially infected plants throughout the U.S. (Tubajika and others 2006). The shipment of potentially infected plants prompted a nationwide survey effort for this pathogen in nursery stock and prompted the adoption of a Federal Order requiring the mandatory inspection of all California, Oregon, and Washington nursery stock shipped interstate (USDA 2004b). Nurseries growing host and associated plants (HAP) had to be inspected and a mandatory number of samples collected for testing. Nurseries growing non-HAP had to be inspected; samples were collected for testing if suspicious symptoms were found.

Oregon has over 2,100 wholesale nursery and greenhouse operations that generate about \$877 million in gross sales (USDA and ODA 2006). Over 80 percent of the nursery stock produced is exported. Many of these nurseries have multiple growing sites throughout the state. Each growing site with HAP present must be inspected and tested for *P. ramorum*. Since the inception of the Federal Order, this has required the testing of tens of thousands of samples from thousands of growing sites (fig. 1). Because all testing must be completed before nurseries can ship HAP interstate, the Oregon Department of Agriculture (ODA) has been developing a high throughput system that enables testing of large numbers of samples in accordance with the federally validated protocols. The system is designed to work with the ELISA and molecular detection protocols for *P. ramorum*.

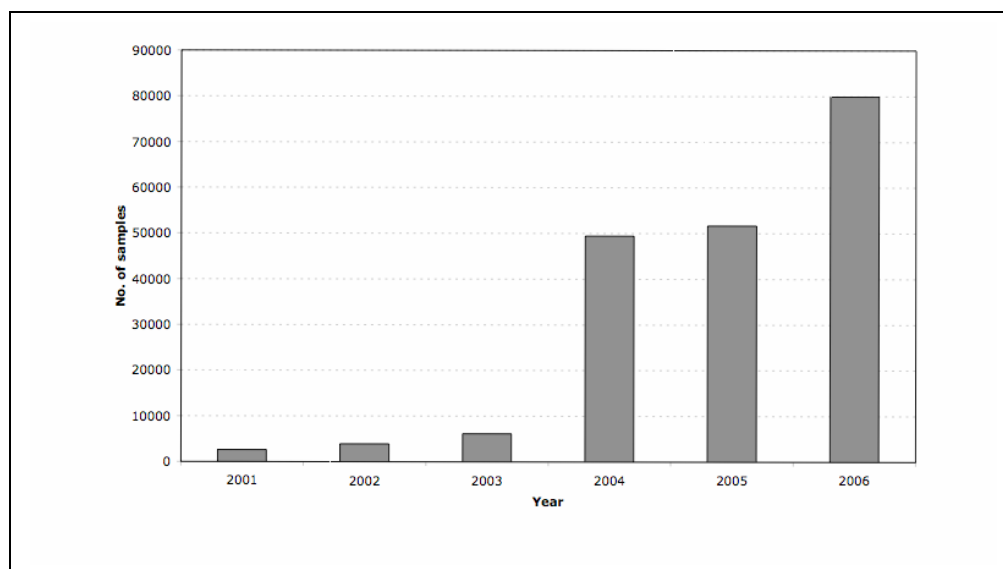


Figure 1—Number of nursery samples tested for *P. ramorum* since 2001.

A sterile hole punch was used to remove a 6 mm diameter disk of infected leaf tissue from each leaf. The leaf disk was taken from the disease margin whenever possible. For all leaves, the area where the mycelial or V8 agar plug was placed on the leaf was avoided.

The leaf disks were placed in a 96-well extraction plate (Qiagen, Inc., Valencia, CA, Cat. No. 19560). For each host, the leaf disks were placed in the microtubes (test wells) in the following combinations of inoculated to non-inoculated tissue: 5:0, 1:4, and 0:5. Two to five replicates of each treatment combination were tested. Eight hundred microliters of sodium azide-free GEB2 buffer (Agdia, Inc., Elkhart, IN, Cat. No. SRA 92600) was added to each test well along with a single tungsten carbide bead. The test wells were sealed to ensure sample integrity and then agitated for 1.5-minutes at 30 MHz in a Mixer Mill. The extraction plates were rotated and the agitation repeated. The extraction plates were centrifuged for approximately 10 seconds at 6000 rpm. The resulting plant extract was then subjected to ELISA testing.

The USDA-validated ELISA testing was performed according to the manufacturer's directions (AgDia Inc., *Phytophthora* instructions for the *Phytophthora* reagent set, m394). Three controls were included with each plate: *P. ramorum* from mycelium (positive), the manufacturer's *Phytophthora*-positive, and GEB2 buffer (negative). After 1 hour incubation in the dark, the optical density of the alkaline phosphatase label was read at 405 nm. Per the manufacturer's directions, the positive/negative threshold was set at 2x the optical density (OD) of the negative (buffer) control. Plant extracts were frozen at -20 °C before proceeding to DNA testing.

Total DNA was extracted from the sample extract using the DNeasy Plant Mini and DNeasy 96 Plant kits (Qiagen, Inc., Valencia, CA, Cat. Nos. 69106 and 69181, respectively). The DNA was then subjected to testing with the USDA-validated nested PCR protocol (USDA 2004a) and the real-time PCR (qPCR) protocol developed by the United Kingdom (Tomlinson and others 2005).

The experiments were replicated twice.

Environmental samples were collected from nurseries according to the requirements of the Federal Order and/or the USDA National Survey Protocol for *P. ramorum*. Samples were collected and bulked using the following criteria: HAP genus or species, cultivar, and location (for example, block) within the nursery. At least 40 samples were tested per nursery; five samples were bulked per test well. Samples were processed as described above with the following exceptions: 1) The volume of GEB2 buffer used for the ELISA pre-screen was lowered to 600 µL per test well; and 2) The USDA-validated qPCR protocol was used (USDA 2005).

Results

The OD readings of all samples containing inoculated plant tissues were consistently five times the negative control (table 1). All non-inoculated controls were less than two times the negative control with one exception. In the second experiment, the OD reading for the *P. japonica* non-inoculated control was 0.093 greater than the negative control.

All inoculated samples were positive for *P. ramorum* using the USDA-validated nested PCR protocol and all non-inoculated controls were negative (table 1). Results were similar upon testing with the qPCR protocol with one exception (table 1). In the first experiment, the *Camellia* non-inoculated control had a Ct value of 40.08 with the *P. ramorum*-specific probe.

Table 1—ELISA, nested PCR, and qPCR test results for plant species inoculated with *P. ramorum*

Sample	Ratio ^a	No. of replicates per experiment	Mass (g) (SE) ^b	OD (405 nm) (SE)	Nested PCR	qPCR (Ct)	
						Pram (SE)	COX (SE)
<i>Pieris japonica</i>	5:0	2	0.022 (±0.001)	1.562 (±0.290)	Positive	28.7 (±1.7)	38.2 (±3.6)
<i>P. japonica</i>	1:4	5	0.027 (±0.003)	0.739 (±0.188)	Positive	31.7 (±2.5)	30.8 (±1.7)
<i>P. japonica</i>	0:5	2	0.034 (±0.004)	0.216 (±0.077)	Negative	NA ^c	27.0 (±1.5)
<i>Kalmia</i> sp.	5:0	2	0.037 (±0.000)	2.178 (±0.027)	Positive	31.5 (±5.9)	NA
<i>Kalmia</i> sp.	1:4	5	0.037 (±0.001)	1.487 (±0.406)	Positive	29.2 (±2.2)	29.6 (±1.1)
<i>Kalmia</i> sp.	0:5	2	0.048 (±0.001)	0.155 (±0.023)	Negative	NA	30.4 (±0.3)
<i>Camellia</i> sp.	5:0	2	0.056 (±0.002)	2.163 (±0.348)	Positive	30.4 (±1.6)	37.6 ^d
<i>Camellia</i> sp.	1:4	5	0.049 (±0.004)	1.213 (±0.586)	Positive	31.4 (±2.2)	31.0 (±0.9)
<i>Camellia</i> sp.	0:5	2	0.061 (±0.002)	0.103 (±0.004)	Negative	40.1 ^d	31.7 (±3.1)
<i>Syringa vulgaris</i>	5:0	2	0.043 (±0.000)	2.542 (±0.097)	Positive	24.2 (±0.3)	NA
<i>S. vulgaris</i>	1:4	5	0.034 (±0.003)	1.804 (±0.483)	Positive	24.8 (±2.8)	33.9 (±2.6)
<i>S. vulgaris</i>	0:5	2	0.043 (±0.001)	0.114 (±0.003)	Negative	NA	25.4 (±2.5)
<i>P. ramorum</i>	Mycelial plug	2	0.096 (±0.000)	1.586 (±0.014)	Positive	28.34 (±1.1)	NA
AgDia positive	— ^e	2	—	1.226 (±0.006)	—	—	—
Negative (buffer)	—	2	—	0.089 (±0.001)	Negative	NA	NA

^a Ratio of inoculated to non-inoculated plant tissue.

^b Standard error.

^c NA means no amplification occurred.

^d Ct from one replicate. There was no amplification in the second replicate.

^e Not tested.

Environmental samples were also tested with the high throughput system (partial dataset shown in table 2). The high throughput system was used with ELISA to successfully screen multiple plant species for *Phytophthora* infection. Subsequent DNA testing with the USDA-validated nested PCR and/or qPCR protocols successfully detected *P. ramorum* in the following HAP: *Abies concolor* (Gord. & Glend.) Lincl. ex Hildebr., *Acer macrophyllum* Pursh, *Camellia* sp., *Gaultheria shallon* Pursh, *Kalmia* sp., *Magnolia* sp., *Pieris* sp., *Rhododendron* sp., *S. vulgaris*, and *Viburnum* sp.. *P. ramorum* was also detected from soil and potting media bait leaves using the high throughput system. These results were officially confirmed by the USDA-APHIS at their testing laboratory in Beltsville, MD (data not shown).

Table 2—*P. ramorum* testing results for samples collected from a subset of Oregon nurseries

Nursery	Host	No. of samples	OD (405 nm) (SE) ^a	Nested PCR (ODA)	USDA Confirmation
---034	<i>Camellia</i> sp.	10	0.102 (±0.002)		
---034	<i>Pieris japonica</i>	15	1.890 (±0.265)	Positive	Negative
---034	<i>P. japonica</i>	15	0.394 (±0.226)	Negative	
---610	<i>Camellia</i> sp.	15	0.102 (±0.002)		
---610	<i>P. japonica</i>	10	0.106 (±0.004)		
---610	<i>Viburnum davidii</i>	15	0.422 (±0.264)	Negative	
---065	<i>Abies grandis</i>	20	0.107 (±0.004)		
---065	<i>Pseudotsuga menziesii</i>	20	0.119 (±0.006)		
---415	<i>Pseudotsuga menziesii</i>	10	0.425 (±0.217)	Negative	
---415	<i>P. menziesii</i>	10	0.136 (±0.034)		
---415	<i>P. menziesii</i>	10	0.152 (±0.006)		
---415	<i>P. menziesii</i>	10	0.194 (±0.033)		
---734	<i>Arbutus menziesii</i>	10	0.112 (±0.000)		
---734	<i>Rhododendron</i>	10	0.107 (±0.004)		
---734	<i>Taxus brevifolia</i>	10	0.104 (±0.001)		
---734	<i>Vaccinium ovatum</i>	10	0.115 (±0.005)		
---327	<i>Kalmia latifolia</i>	10	0.873 (±0.118)	Negative	
---327	<i>Pieris japonica</i>	10	0.786 (±0.014)	Positive	Negative
---327	<i>Rhododendron</i>	10	0.133 (±0.010)		
---327	<i>Rhododendron</i>	10	0.745 (±0.048)	Positive	Positive
---327	<i>Rhododendron</i>	10	0.159 (±0.032)		
---327	<i>Viburnum tinus</i>	10	0.110 (±0.004)		
	Positive control		1.041 (±0.018)	Positive ^b	
	Negative control		0.100 (±0.001)	Negative ^c	

^a Standard error.

^b A *P. ramorum* positive control was used for nested PCR.

^c A no template control was used for nested PCR.

Conclusions

The high throughput system was used successfully to detect *P. ramorum* in four known host species that were artificially inoculated with the pathogen. The system worked well with all three USDA-validated protocols (ELISA, nested PCR, and qPCR). Because of the large number of samples that needed to be tested (Fig. 2), we investigated the possibility of bulking samples. In an initial, internal study (data not shown), we tried bulking 10 samples per test well and were able to successfully detect *P. ramorum* infections. However, ELISA is known to be less sensitive than PCR for detecting plant pathogens (for example, Lee and others 2001) and we became concerned about the potential for false negatives in samples with low levels of infection. Because of this concern, we reduced the number of samples bulked per test well to five in this study. This allowed for consistent detection of a low infection level (one of five samples infected) with the ELISA pre-screen. When environmental samples were tested with the high throughput system, *Phytophthora* was successfully detected in multiple HAP species including conifers at 15 percent of the nursery sites surveyed in both 2005 and 2006 (Oregon Department of Agriculture, 2005 Plant Division Annual Report and 2006 Plant Division Annual Report available at <http://oregon.gov/ODA/PLANT>). Subsequent testing with nested PCR and qPCR successfully detected *P. ramorum* in four plant species including the conifer *Abies concolor* and in six plant genera. All PCR results were officially confirmed by USDA, indicating *P. ramorum* could be detected in bulked environmental samples. To our knowledge, ODA is the only regulatory laboratory at this time that has the equipment necessary for this type of high throughput system. Replication of this work in one or more other laboratories will be needed to validate the system.



Figure 2—Inoculated *Kalmia* leaves.

Because samples were bulked in a 96-well format for testing, our laboratory was able to process tens of thousands of samples in a timely and accurate manner. In terms of manual labor costs for sample processing, this translated into a savings of about \$150,000 over a 2 year period. The 96-well format could also result in long-term savings by automating the testing procedures. Automation could also decrease the risks of cross-contamination or environmental contamination of samples, potentially reducing the chance of false positives.

The USDA-validated ELISA test requires a 1:10 (w:v) sample to extraction buffer ratio for protein extraction. In our artificial inoculation study, the average mass of the plant species tested was 0.041 mg, which would require an extraction buffer volume of 410 µL. Because there are more than 100 plant species susceptible to natural infection by *P. ramorum*, we adjusted the volume to 600 µL of the extraction buffer GEB2 per sample tested to account for any species differences in mass. This volume was sufficient to meet the 1:10 (w:v) requirement for all HAP species tested and also worked well with the DNeasy 96 Plant Kit for DNA extraction. Based on the Ct values for COX in the qPCR test and the results from the nested PCR test, the DNA extracted from the ELISA sample extract was of sufficient quality for PCR amplification. This showed that DNA was successfully extracted from sample tissue that was initially macerated in GEB2 buffer. Thus, sample integrity was maintained as the exact same sample tissue was tested with all three USDA-validated protocols. This is important because *Phytophthora* species, like other plant pathogens, may grow unevenly along the disease margin. By testing the exact same ELISA-positive tissue, diagnosticians can be assured that a *Phytophthora* is present in the sample for DNA extraction and testing.

In a study performed by USDA on a cultivar of *C. japonica* L., researchers determined that a positive/negative threshold of 2X a negative (healthy plant) control was necessary to reduce the rate of false-negatives detected with ELISA (Bulluck and others 2006). In this study, we chose to use the extraction buffer as the negative control for ELISA testing. Using the manufacturer's recommended positive/negative threshold of 2X the negative (buffer) control's OD reading resulted in a false ELISA positive from healthy *P. japonica* plant tissue in the controlled inoculation study (Table 1). If a healthy plant control produced such a high OD reading during the testing of environmental samples, it could result in false ELISA negatives for infected environmental samples because the OD reading of the infected environmental sample would be less than 2X the healthy plant control. In our study, The OD readings for infected plant tissue were consistently 5X the negative control for all artificially inoculated HAP species tested and consistently $\geq 3X$ the negative (buffer) control for environmental samples tested. In a previous study that examined the sensitivity of *Phytophthora*-specific immunoassay kits, a positive/negative threshold of 0.3 was used with limited success (Pscheidt and others 1991). Our data suggest that there is a need for a comprehensive ELISA analysis of additional HAP species to determine an appropriate positive/negative threshold that eliminates false positives from healthy plant tissue while minimizing the rate of false-negatives. Such a study could be used to identify an appropriate healthy plant tissue control for the testing of multiple susceptible species for *Phytophthora*.

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