2006 Pilot Survey for *Phytophthora ramorum* in Forest Streams in the USA[1]

S.W. Oak,[2] J. Hwang,[3] S.N. Jeffers,[3] and B.M. Tkacz[4]

Abstract

Methods for detecting *Phytophthora ramorum* and other *Phytophthora* species with rhododendron leaf baits were pilot tested in high-risk watersheds in 11 states for the purpose of recommending a national survey protocol. Ninety streams, including 14 draining *P. ramorum*-endemic areas, yielded 587 baiting chances. Molecular diagnostic assays detected the pathogen in all known *P. ramorum*-endemic streams at least once in five baiting periods, and in nearly 50 percent of all bait leaf sets. Isolation using selective media detected the pathogen in all but one known positive stream in California and Oregon, and for the first time in one Washington stream draining a confirmed positive ornamental nursery. Overall, *Phytophthora* spp. were detected by isolation in all but one stream over the course of the pilot survey, and in over 80 percent of the leaf bait sets. The national *P. ramorum* early detection survey for U.S. forests will recommend rhododendron leaf baiting for five baiting periods in high-risk watersheds with redundant molecular and selective media isolation assays for 2007.

Key words: *Phytophthora ramorum*, survey, *Phytophthora* baiting.

Introduction

Phytophthora species are well adapted to aquatic environments. The occurrence and distribution of *Phytophthora* in waterways in various environmental settings worldwide has been studied using filtration and plant tissue baits from a wide variety of plants species. Recently, monitoring of *Phytophthora ramorum* Werres, De Cock, & Man in't Veld in forest streams has been shown to be effective using pear fruits and foliage of tanoak and rhododendron in California (CA) and Oregon (OR). The success of these methods for detecting *P. ramorum* in water, even before symptoms are visible in aerial surveys, has resulted in early detection and treatment of known infested sites (Murphy and others 2006, Hansen and others 2006). Given that previously unknown infection centers have been detected up to 8 km downstream from the inoculum source, monitoring forest streams for *P. ramorum* in high-risk regions should afford an opportunity to survey larger land areas with greater efficiency and at lower cost than is possible with current terrestrial survey techniques. The objective of this pilot survey was to evaluate at a large geographic scale, existing

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[2] USDA Forest Service, Southern Region FHP 200 W.T. Weaver Blvd, Asheville, NC 28804.
[3] Clemson University Department of Entomology, Soils, and Plant Sciences, 120 Long Hall, Clemson, SC 29634.
[4] USDA Forest Service, Washington Office FHP, 1601 N Kent St RPC, Arlington, VA 22209.

stream baiting and lab diagnostic methods for the purpose of recommending protocols for the national *P. ramorum* early detection survey for U.S. forests.

Methods

Stream Selection and Bait Deployment

Pilot survey protocols were developed by consensus of researchers experienced in *Phytophthora* spp. early detection by baiting in nursery and forest environments. To achieve the desired scale of the evaluation, we targeted 11 states that contain a wide diversity of oak forest ecosystems that have been projected as high risk for *P. ramorum* establishment and damage (Oak and others 2006). Included were states with endemic areas (CA and OR); those where the pathogen had been confirmed only in woody ornamental nursery stock [Georgia (GA), Maryland (MD), North Carolina (NC), Pennsylvania (PA), Tennessee (TN), Virginia (VA), and Washington (WA)]; and those that had only received unconfirmed, but potentially infected nursery stock [Kentucky (KY) and West Virginia (WV)]. We recommended that 5 to 10 watersheds in high-risk areas of 2,000 to 4,000 ha each be surveyed, including some known *P. ramorum* positive streams in endemic areas. At a minimum, areas selected required high-risk host type and suitable climate. Where possible, a confirmed trace forward nursery inside the limits of the watershed was preferred.

Each stream was baited with four detached but otherwise unwounded *Rhododendron* spp. leaves contained in a mesh bag made of plastic window screen. Bags were tethered to the stream banks and floated in the current for one to two weeks (exposure period depended on symptom development). After retrieval, bait leaves were wrapped in a paper towel moistened with stream water, sealed in plastic bag, and placed on ice for transport to diagnostic laboratories.

Diagnostics

Bait leaves were washed under running tap water to remove silt and organic debris and blotted dry. Symptomatic leaf pieces were then excised without surface disinfestation for primary and secondary diagnostics. The primary diagnostic tested for the presence of *P. ramorum* using nested or real-time PCR according to United States Department of Agriculture-Animal and Plant Health Inspection Service (USDA-APHIS) protocols (Levy and others 2004, DeVries and others 2006). The secondary diagnostic tested for the presence of any *Phytophthora* species, with the exact method left to the discretion of the diagnostician. Choices were isolation on selective medium (PARPH-V8 preferred; Jeffers and others 1987); an independent molecular diagnostic (for example Bonants and others 1997); or commercially available ELISA kits (Agdia® Elkhart, IN). After excision of bait leaf pieces, leftover leaf tissues were shipped to a separate laboratory for isolation on PARPH-V8 as validation of the *Phytophthora* spp. diagnosis in the case that diagnosticians did not choose isolation. This validation was performed only for baits from non-*P. ramorum* endemic watersheds in eastern states to prevent the inadvertent transport of the pathogen in baits.

Results and Discussion

Results for NC and TN are combined since all watersheds were within the boundary of the Great Smoky Mountains National Park, and the same field crew and laboratory handled field sampling and diagnostics. Overall, 90 watersheds were sampled for the

requisite five baiting periods outlined in the protocol, or more. These 90 watersheds afforded 587 baiting chances, of which fewer than 7 percent were lost due to high storm flows, vandalism or other causes (table 1). The highest rate of loss was in WV, where a single storm event in June disrupted all bait stations. The average number of baiting chances per stream overall was about six, with a range of four (VA) to 11 (WA).

Table 1–Pre-Survey *P. ramorum* status and bait retrieval results

	Number of Streams			
State	Total	*P. ramorum* Positive	Baiting Chances	Lost Chances[1]
CA	10	8	50	0 (0.0)[2]
OR	12	6	121	4 (3.3)
WA	11	0	129	10 (7.8)
GA	10	0	50	0 (0.0)
MD	9	0	48	6 (12.5)
NC-TN	10	0	50	7 (14)
PA	10	0	49	3 (6.1)
VA	7	0	27	0 (0.0)
KY	6	0	33	3 (9.1)
WV	5	0	30	6 (20)
Total	90	14	587	39 (6.6)

[1]Lost bait sets not available for diagnosis due to high storm flows or vandalism.
[2]Numbers in parentheses are percentage of total baiting chances for each state.

Previous sampling showed that *P. ramorum* was present in 14 of 22 watersheds in CA and OR. Nested PCR applied in CA detected *P. ramorum* in over 75 percent of the bait leaf sets from known positive streams, while real-time PCR applied in OR detected the pathogen in less than one-third (table 2). This could be due to differences in the relative sensitivity of the assays, or to lower rates of bait colonization in OR where inoculum density in streams draining *P. ramorum*-endemic areas undergoing eradication treatments may be lower than in infested streams in CA. The pathogen was detected in baits from all known positive streams at least once over the prescribed five baiting periods in both states. Real time PCR did not detect the pathogen in OR streams thought to be free of the pathogen, but nested PCR detected the pathogen in two CA streams where it was not previously known to occur. These are either instances of first detections in a previously negative stream, or false positives.

Isolation detected *P. ramorum* in all positive streams in CA at a frequency comparable to nested PCR (table 3). However, isolation failed to detect the pathogen in one known positive stream in OR, and detection rates were lower overall than for real-time PCR. The difference in detection success may reflect greater relative

Table 2—Molecular diagnosis results for *P. ramorum* in OR and CA, by pre-survey pathogen status [1]

	P. ramorum Positive Streams			*P. ramorum* Negative Streams		
State Method	No.	Diagnosis Chances	Pram+ Diagnosis	No.	Diagnosis Chances	Pram+ Diagnosis
CA Nested	8	40	31 (77.5)[2]	2	10	3 (30)
OR Real-Time	6	55	16 (29.1)	6	52	0 (0.0)
Total	14	95	47 (49.5)	9	62	3 (4.8)

[1]Streams must have had at least five bait sets with the diagnostic to be included.
[2]Numbers in parentheses are percentage of total diagnosis chances for the state/method combination.

sensitivity of the molecular assay than isolation, but could also have been due to a smaller sample size (only one-third the number of isolation chances versus real-time PCR) and low inoculum density. Isolation on selective media detected *P. ramorum* for the first time in one intermittent WA stream draining a previously confirmed positive ornamental nursery. Subsequent vegetation surveys up- and downstream did not detect any infection centers outside the nursery, and the inoculum source appears to be associated with an area where infected plants were held prior to being destroyed. The pathogen was recovered in over half of the isolation attempts overall, but these included mid- and late summer baiting periods inhospitable for the pathogen. Isolation success was over 80 percent during optimal conditions.

Isolation recovered *Phytophthora* spp. in over 80 percent of the attempts, and only one WV stream failed to yield a *Phytophthora* spp. at least once in five baiting periods (table 3). Recovery rates among states ranged from 55.8 to 100 percent

demonstrating the ubiquitous distribution of *Phytophthora* spp. in streams. ELISA assays were used only in GA and PA, and in concert with isolation in both cases. All positive ELISA results obtained in these two states were substantiated by isolation. Though unreported here in detail, molecular tools for detection of *Phytophthora* spp. in this survey were inadequate. At times, samples known positive from isolation yielded a negative molecular diagnosis. Other complications arose in PCR protocol parameters and the availability of some reagents.

2007 Early Detection Protocol

The results of this pilot survey support our recommendation of the following protocol for the 2007 national *P. ramorum* early detection survey for U.S. forests:

1. Survey up to 10 high-risk watersheds per cooperating state, as funding permits.
2. Deploy two mesh bags containing four intact, symptom-free leaves of *Rhododendron* spp.
3. Use native or naturalized source plants, if available, and ensure that they have been free of pesticides for six weeks before use.
4. Expose bait leaves in the stream current for one to two weeks, depending on symptom development.

5. Deploy bait leaves once per month for five months beginning when average daily temperature exceeds 15°C.
6. Pool baits from both bags and sort into four leaf sets to ensure that the range of symptom types is represented in each set.
7. Diagnose the presence of *P. ramorum* and any other *Phytophthora* spp. using isolation on selective medium (PARPH-V8 preferred) from one set.
8. In an independent laboratory, diagnose the presence of *P. ramorum* using PCR (real-time preferred) in the other set. Redundant *P. ramorum* diagnostics limit potential false negatives from less sensitive isolation, and false positives from very sensitive molecular assays.

Table 3—Isolation results for *P. ramorum* and any *Phytophthora* spp. for *P. ramorum* positive and all streams [1]

	***P. ramorum* Positive Streams**			**All Streams**		
State	**No.**	**Isolation Chances**	***P. ramorum* Positive**	**No.**	**Isolation Chances**	**Any *Phytophthora* spp.**
CA	8	40	33 (82.5)	10	50	40 (80.0)
OR	3	17	2 (11.8)	12	74	68 (91.9)
WA	1	13	7 (53.8)	11	109	68 (62.4)
GA	0	50	0 (0.0)	10	50	50 (100)
MD	0	42	0 (0.0)	9	42	42 (100)
NC-TN	0	43	0 (0.0)	10	43	24 (55.8)
PA	0	49	0 (0.0)	10	49	35 (79.5)
VA	0	27	0 (0.0)	7	27	26 (96.3)
KY	0	30	0 (0.0)	6	30	30 (100)
WV	0	24	0 (0.0)	5	24	17 (70.8)
Total	12	335	42 (60.0)[3]	90	498	400 (80.3)

[1]Only streams with at least five isolation attempts are included.
[2]Number in parenthesis is the percentage of isolation chances for the state/*P. ramorum* stream status combination.
[3]Percentage calculated from isolation chances in *P. ramorum* positive streams only.

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