Surveying For and Eradicating Phytophthora ramorum in Agricultural Commodities

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Abstract
Since 2001, Oregon nurseries, Christmas tree plantations, and other sites have been surveyed for the federally regulated pathogen Phytophthora ramorum. Host plants at each site were visually surveyed for disease symptoms and symptomatic tissues tested in the laboratory by isolation onto a selective medium and by a polymerase chain reaction (PCR) assay. In 2002 and 2003, we detected PCR-positive plants that later proved to be infected with another Phytophthora, suggesting there are limitations to the PCR assay tested. In 2003, P. ramorum was detected for the first time in Viburnum, Pieris, Rhododendron, and Camellia plants in six nurseries. All infected and neighboring plant materials were destroyed by incineration and the nurseries and surrounding environs subsequently surveyed for the pathogen. Phytophthora ramorum was not detected, indicating the pathogen was successfully eradicated.

Introduction
Phytophthora ramorum Werres, Cock, & Man in’t Veld, the sudden oak death pathogen, is established in 12 coastal counties in California and has been detected (and targeted for eradication) in Curry County, Oregon (3,7,10). The pathogen has also been reported in Europe (3,16). Phytophthora ramorum has a broad host range that includes more than 28 hosts in 12 plant families (3,9,12). These hosts are regulated by federal and international quarantines (12,14). The pathogen has also been recovered from 30 other plant species, although Koch’s Postulates have yet to be completed on these associated hosts (3,11,12). Many of the regulated and associated hosts for P. ramorum are important parts of Oregon’s $768-million horticultural industry, $149-million Christmas tree industry, and 3.91-billion-board-feet timber industry (5,8). Others are key components of Oregon’s ecosystems.

In Oregon, P. ramorum has been limited to a small area in Curry County (7). However, the potential for spread into other parts of the state is a concern. In Europe, P. ramorum is primarily found in nurseries and to a lesser extent in landscape plantings (3), suggesting the pathogen has spread through infested nursery stock. To monitor this potential pathway for introduction, the Oregon Department of Agriculture (ODA) has conducted an annual detection survey for P. ramorum in agricultural growing areas and other high-risk sites (e.g., botanical gardens) since 2001. Survey results, including the results of an eradication strategy for positive detections, are described below.

Surveys for Phytophthora ramorum

In the spring during the period of new shoot growth, at least 2% of the known susceptible hosts present at each nursery, Christmas tree plantation, or high-risk site included in the survey were visually inspected for evidence of P. ramorum infection (e.g., 3,15). Samples were collected from plants exhibiting symptoms typical of foliar infection by Phytophthora (e.g., necrotic leaf lesions with diffuse disease margins). Samples were also collected from hosts exhibiting atypical disease symptoms as symptom expression in some hosts (e.g., Camellia) may vary depending upon environmental conditions and the host’s growth stage (3; A. Wagner, Washington State Department of Agriculture, and C. Blomquist,
California Department of Food and Agriculture (CDFA), personal communication). The symptomatic leaves and/or other plant tissues were sealed in plastic bags after collection and delivered to the ODA Plant Health Laboratory for analysis. Samples were processed within 72-h of arrival. If no symptomatic plants were present, asymptomatic leaves were collected.

In the laboratory, samples were processed by isolating from disease margins and plating onto a Phytophthora-selective medium (PARP) (3,4). If samples were asymptomatic, isolations focused on the leaf tip and/or petiole. The plates were incubated in the dark at room temperature (20 to 22°C) and then examined under light microscopy after 7-day. Phytophthora isolates were transferred to corn meal agar for identification to species (4,16). Isolates identified as P. ramorum morphologically were subjected to DNA analysis with PCR (17) for further positive confirmation. Specific PCR primers that amplify part of the internal transcribed spacer (ITS) region of the nuclear ribosomal DNA were used. These primers reportedly produce a 738-bp amplicon only in the presence of P. ramorum and/or P. lateralis DNA (17). Universal internal positive control primers were added to each PCR reaction to eliminate any false negatives that would result from a failed reaction. In 2002 and 2003, in addition to plating, DNA was extracted from a randomly selected subset of the plant samples with spin column chromatography (DNeasy Plant Mini Kit and 96-well Plant Kit, Qiagen, Inc., Valencia, CA) and then tested with PCR.

In 2001, 69 nurseries and high-risk sites were visually surveyed for P. ramorum. A total of 2,657 samples were collected for an average of 39 samples per site. All samples were taken from rhododendron (Rhododendron). No P. ramorum was found from isolation onto PARP, although other Phytophthora spp. (P. cactorum, P. cambivora, P. cinnamomi, P. citricola, P. heveae, P. nicotianae, and P. syringae) were recovered (Fig. 1).

In 2002, 98 nurseries, Christmas tree plantations, and high-risk sites were surveyed. A total of 3,927 samples were collected for an average of 40 samples per site with the majority of samples coming from rhododendron (Rhododendron). Phytophthora ramorum was not recovered by isolation. Other Phytophthora spp. were recovered; P. cinnamomi and P. citricola from manzanita (Arctostaphylos manzanita) and rhododendron, P. nicotianae and P. syringae from tanoak (Lithocarpus densiflorus) and rhododendron, P. cactorum and P. citrophthora from rhododendron, and P. heveae from rhododendron and viburnum.
Viburnum). Of the 3,927 samples, a subset of 717 randomly selected samples was also tested with PCR. One rhododendron sample produced the 738-bp amplicon. The amplicon was purified (QIAquick Gel Extraction Kit, Qiagen, Inc., Valencia, CA) and submitted to the Center for Gene Research and Biotechnology Central Services Laboratory, Oregon State University, for sequencing using an ABI 377 automated fluorescence sequencer. Partial ITS1 and ITS2 sequences of the isolate were compared to sequences of *P. lateralis* (GenBank Accession No. AF287256), *P. ramorum* (GenBank Accession Nos. AF429774 and AF429773), and *P. hibernalis* (C. Blomquist, CDFA, unpublished data). In the ITS1 region, the isolate differed from *P. lateralis* and *P. ramorum* by 5.0% and 6.7%, respectively, but was identical to *P. hibernalis* (Fig. 3). In the ITS2 region, the isolate differed from *P. lateralis* and *P. ramorum* by 5.2% and 7.1%, respectively, but was identical to *P. hibernalis* (data not shown).

*Phytophthora hibernalis* was found in the nursery that supplied the rhododendron (C. Blomquist, CDFA, personal communication). This *Phytophthora* was also detected by the PCR assay.

In 2003, 147 nurseries, Christmas tree plantations, and high-risk sites were surveyed. A total of 6,142 samples were collected for an average of 42 samples per site with the majority of samples coming from rhododendron (Fig. 2). *Phytophthora ramorum* was detected in six nurseries (Fig. 1, Table 1). Morphological identification of the *P. ramorum* isolates was further confirmed by PCR. Other *Phytophthora* spp. were also recovered: *P. cambivora*, and *P. nicotianae* from andromeda (*Pieris*) and rhododendron; *P. gonapodyides* and *P. cactorum* from rhododendron; *Phytophthora* sp. from rhododendron, andromeda, huckleberry (*Vaccinium*), *Arbutus*, and camellia (*Camellia*); *P. syringae* from *Kalmia*, andromeda, *Arbutus* and rhododendron; *P. citrophthora* from andromeda; and *P. heveae* from rhododendron, andromeda, camellia and viburnum. Of the 6,142 samples, a subset of 1,303 randomly selected samples was also tested with PCR. Samples from six rhododendrons, two andromeda, three viburnum, and one camellia produced the 738-bp amplicon. *Phytophthora ramorum* was isolated from the andromeda, viburnum, and camellia samples; another *Phytophthora* from one rhododendron sample; and no *Phytophthora* from five rhododendron samples. PCR amplicons from all six rhododendron samples were purified and sequenced as described above. As in 2002, the partial ITS1 and ITS2 sequences of the isolates were compared to those of *P. lateralis*, *P. ramorum*, and *P. hibernalis*. In the ITS1 region, the isolates differed from *P. lateralis* and *P. ramorum* by 5.0% and 6.7%, respectively, but were identical to *P. hibernalis* (Fig. 3). In the ITS2 region, the isolates differed from *P. lateralis* and *P. ramorum* by 5.2% and 7.1%, respectively, but were identical to *P. hibernalis* (data not shown). Once again, the PCR assay detected *P. hibernalis*. 
Table 1. Oregon nurseries where *Phytophthora ramorum* was detected.

<table>
<thead>
<tr>
<th>County</th>
<th>Nursery</th>
<th>Infected plant species and cultivars</th>
<th>Mating type</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clackamas</td>
<td>16</td>
<td><em>Pieris floribunda</em> × <em>japonica</em> cv. Bouwer's Beauty, <em>V. plicatum</em> var. tomentosum cv. Mariesii</td>
<td>A1</td>
<td>European</td>
</tr>
<tr>
<td>Clackamas</td>
<td>11</td>
<td><em>Camellia japonica</em></td>
<td>A2</td>
<td>North American</td>
</tr>
<tr>
<td>Jackson</td>
<td>18</td>
<td><em>C. sasanqua</em> cv. Bonanza</td>
<td>A2</td>
<td>North American</td>
</tr>
<tr>
<td>Jackson</td>
<td>15</td>
<td><em>C. sasanqua</em> cv. Bonanza</td>
<td>A2</td>
<td>North American</td>
</tr>
<tr>
<td>Washington</td>
<td>13</td>
<td><em>Camellia</em> sp., <em>C. sasanqua</em> cv. Bonanza</td>
<td>A2</td>
<td>North American</td>
</tr>
</tbody>
</table>

**Treatment of Infected Plant Materials at Nurseries**

At each nursery, all plant blocks containing infected plants and other plants located within 2 m of the affected blocks were confiscated and destroyed per interim federal requirements (Fig. 4) (13). Other hosts located within 10 m of the affected blocks were placed under quarantine until pathogen-free status could be determined as required (13). In addition to the federally required testing, all regulated and associated host genera on site were surveyed three to five times within 90 d of destroying the infected plants and again 5 to 6 months later. Samples were collected from symptomatic plants and processed in the laboratory as described above. Due to cultural practices at three of the nurseries, we also tested the irrigation water and growing beds (soil) at those sites. The irrigation water was baited three times at 2-week intervals with *Rhododendron* and/or *viburnum* leaves and the leaves processed as described above (4). The soil was tested by baiting 500-ml (volume) soil samples with *Rhododendron* leaves and the leaves processed as described above (4).

Fig. 4. Destruction of plant blocks containing *Phytophthora ramorum*-infected plant materials at Nursery 02.
A total of 2,767 samples were collected and tested from the affected nurseries during the subsequent surveys (Fig. 5). *Phytophthora ramorum* was not found, although other *Phytophthora* species were detected. *Phytophthora cinnamomi*, *P. citrophthora*, *Phytophthora* sp., *P. nicotianae*, and *P. gonapodyides* were recovered from rhododendron; *P. nicotianae*, *P. syringae*, and *P. heveae* from soil; *Phytophthora* sp. and *P. syringae* from maple (*Acer*); *P. syringae* from *Paeonia*; and *P. nicotianae* and *P. syringae* from irrigation water. No samples were positive for the presence of *P. ramorum* using PCR.

Delimitation surveys were conducted around each nursery to determine if the pathogen had spread into the natural environment. Plants on all properties within 0.25-mile radius of each nursery were visually surveyed for disease symptoms. A total of 3,298 samples (0.5% maple, 24.3% camellia, 1.2% *Lonicera*, 1.9% andromeda, 0.3% *Pseudotsuga*, 63.2% rhododendron, 1.9% huckleberry, 3.2% viburnum, and 3.5% other species) from 328 properties were collected and analyzed in the laboratory as described above. No *P. ramorum* was detected.

**Origin of the Infected Plants**

Mating type and genetic analysis showed the *P. ramorum* isolates recovered from five plant species at Nursery 02 were of European origin (Table 1) (6), although isolates from two infected plants in a single block of rhododendron were of North American origin. Oospores formed when these two rhododendron isolates were crossed in vitro with a viburnum isolate (mating type A1, European genotype) from the same nursery (Fig. 6). Genetic analysis confirmed these isolates have a genotype identical to the predominant North American clone (E. M. Hansen, Oregon State University (OSU), *unpublished data*). This suggests the pathogen may have been introduced twice into the nursery or the nursery received a shipment with mixed infections. Investigations by the ODA suggest *P. ramorum* was introduced to Nursery 02 on the rhododendrons, although the original propagation site for the rhododendrons was never determined.
The *P. ramorum* isolates recovered from the remaining nurseries, with the exception of Nursery 16 which received infected plants from Nursery 02, are apparently of North American origin (Table 1). All isolates have the A2 mating type and have genetic patterns consistent with the predominant North American clone (6; E. M. Hansen, OSU, *unpublished data*). According to nursery invoices, all four nurseries received camellias from a Stanislaus County, California, nursery that is reportedly infected with the North American type of *P. ramorum* (1,2). We isolated *P. ramorum* directly from the imported camellias. The pathogen was not detected in the delimitation surveys, indicating there was no local source of inoculum. These results show that the infected camellias originated from California.

**Conclusions**

The survey protocol as described above successfully detected *P. ramorum* in nursery stock during our annual survey of Oregon nurseries, Christmas tree plantations, and other sites at risk for the introduction of this pathogen. *Phytophthora ramorum* was isolated from the known hosts *V. bodnantense*, *P. japonica*, *C. japonica*, and *Rhododendron*. However, the pathogen was also isolated from *Pieris* hybrids, *C. sasanqua*, and *V. plicatum* var. *tomentosum* -- species not known to be hosts for the pathogen at the time of the survey (3,9,12,15). This suggests host genera should be inspected rather than host species when surveying for *P. ramorum* in nurseries, both locally and nationally. *Phytophthora ramorum* was also recovered from an asymptomatic *C. sasanqua* cv. Bonanza by isolation and PCR. This detection illustrates the need to sample *Camellia* even if no symptoms are present and suggests that visual inspection alone may not be adequate in preventing dispersal of this pathogen through nursery stock.

*Phytophthora ramorum* was detected by both isolation onto PARP and PCR. In 2002 and 2003, the PCR assay detected another *Phytophthora*, indicating this assay has limitations as a diagnostic tool for *P. ramorum*. Because symptoms on hosts like camellia can be subtle and the pathogen sometimes difficult to recover by isolation (3,9), a DNA-based assay is very useful for detecting the pathogen’s presence (3,17). However, DNA-based assays such as the PCR assay we used may unexpectedly detect other *Phytophthoras*. This suggests that putative positives detected by ITS-based PCR assays must be carefully evaluated.

The federal interim infected nursery protocol (13) appears to be an effective means of eradicating the pathogen in imported infected nursery stock. This interim protocol outlines the destruction of infected nursery stock, safe disposal of contaminated containers and growing media, and subsequent testing of the remaining plants on-site. *Phytophthora ramorum* was not detected in plants at the nurseries after the destruction of the originally infected plant material. Due to cultural practices (e.g., irrigation system), we also tested the irrigation water and soil at three nurseries. Again, no *P. ramorum* was found. However, the federal interim protocol does not address the possibility of soil or water
becoming infested at a site (13). Testing procedures and/or treatments may be needed in the future should the pathogen infest the water and/or soil at an introduction site.

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**Literature Cited**