

Recovering *Phytophthora ramorum* and other *Phytophthora* spp. from the soil profile of ornamental retail nurseries

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Introduction

With continued detections of *P. ramorum* on ornamental nursery stock in parts of North America and Europe, there has been concern about the potential spread of this exotic pathogen through the ornamental nursery trade. On both continents, quarantine efforts are underway to control the spread of *P. ramorum* in nursery stock. In the United States, the Department of Agriculture Animal and Plant Health Inspection Service (USDA-APHIS) has put an Emergency Federal Order into effect requiring nurseries in California, Oregon and Washington to be inspected and found free of *P. ramorum* before shipping known and associated host plants interstate. Additionally, the Emergency Federal Order requires that when *P. ramorum* is detected in a nursery, a set of procedures known as the Confirmed Nursery Protocol for *P. ramorum* be carried out to eradicate the pathogen. After the protocol has been completed, the nursery can be recertified as free of *P. ramorum*. In 2005, changes were made to the USDA-APHIS Confirmed Nursery Protocol requiring soil testing for *P. ramorum* adjacent to containerized nursery plants where the foliage tested positive during nursery inspections. Positive soil tests in a number of United States retail nurseries have confounded the mitigation process to eradicate the pathogen from these sites.

One of the obstacles in developing mitigation procedures to eradicate this pathogen from nursery soil is that there is no field data on the abundance and depth at which *P. ramorum* can be isolated from soils in nursery settings. Thus far, field research on the survival and recovery of *P. ramorum* from soil depths has focused on situations where the pathogen has established itself in a forest or park-like setting. No information has previously been collected on the distribution of *P. ramorum* in nursery soils.

We hypothesized that in retail nursery settings outside of areas where the pathogen has become established in the landscape, *P. ramorum* inoculum would be limited to the organic layer (top layer of plant debris) of nursery soils. To test this hypothesis we collected soil cores from 3 nurseries in Washington state where the pathogen is not known to be established in the natural or urban landscape, and sampled the soil profile for *P. ramorum* and other *Phytophthora* spp.. A combination of DNA sequencing, real-time polymerase chain reaction (PCR) diagnostic testing and culture morphology were used to assess the diversity of *Phytophthora* communities in the soils and to compare the abundance and depth at which *P. ramorum* and other *Phytophthora* spp. were recovered from the soil profile.

Methods

Collecting soil cores. Soil cores (10x20cm) were collected from 3 Washington state retail nurseries, designated WA-1, WA-2 and WA-3, over localized areas where the soil had tested positive for *P. ramorum* during Washington State Department of Agriculture (WSDA) nursery inspections conducted in 2005 and/or 2006. Soil cores were extracted from the ground using a foot extraction hole cutter (Par Aide Products Company, Lino Lakes, MN). The cores were then ejected into 2-liter plastic soda bottles of which the tops had been cut off, and double-bagged for transport and storage.

Sampling *Phytophthora* spp. in the soil cores. Soil was separated along the cores by the top organic layer and then in increments of 0 to 5, 5 to 10, and 10 to 15cm. The organic layer was defined as debris recognizable as plant tissues at the top of the soil profile and was placed in a separate sealable plastic bag for each core. The soil cores were then sampled by inverting the 2-liter plastic soda bottle and taking 3 sub-cores (from within the larger 10cm diameter core) using a 2cm diameter soil probe (AMS, American Falls, ID). The 3 sub-cores were sectioned at 5cm increments and bulked together in sealable plastic bags designated for the 3 different soil depths for each respective soil core.

Phytophthora was baited from the soil samples in the plastic bags by adding approximately 150ml of sterile tap water and 3 rhododendron leaves (*Rhododendron decorum* hybrid 'Caroline') that had been wounded by slicing the midrib of the leaves. The leaves in the water-soil slurry were incubated 48 hours at 16°C and incubated in a single layer pressed between trays at 17-18°C. When a lesion was observed, a section was isolated onto CARP to select for *Phytophthora*.

Identifying *Phytophthora* spp.. Cultures of *P. ramorum* were identified based on characteristic mycelium, chlamydospores, aerial mycelia and real-time PCR diagnostic testing using Taqman (2) conducted by the WSDA Plant Pathology Lab (Olympia, WA). Duplicate samples of the other *Phytophthora* cultures were sent to a commercial laboratory (Laragen Inc., Los Angeles, CA) for DNA extraction, polymerase chain reaction (PCR) amplification of the internal transcribed spacer (ITS) region of rDNA, and complete sequencing of the ITS using ITS 4 and ITS 5 primers. The sequences were then compared with published sequences. Identifications based on ITS sequences were confirmed by verifying that the colony characteristics and distinctive microscopic morphology and reproductive structures were consistent with published literature.

Data analysis and statistics. A record of each *Phytophthora* culture recovered was entered into a spreadsheet and sorted by nursery, core, depth class, and species. Chi-square analyses for independence were conducted with *P. ramorum* recovery frequencies and those of other *Phytophthora* spp. at the 4 soil depth classes to assess if *P. ramorum* was distributed among the soil depth classes differently than other species.

Results

A total of 69 soil cores were sampled from the 3 nursery sites (32 from WA-1, 20 from WA-2 and 17 from WA-3). The soil types at WA-1, WA-2 and WA-3 were loam, gravelly sandy loam and gravelly sand, respectively. The organic layer of the soil cores for all three nurseries ranged from 1 to 5cm. At all three nurseries the soil was moist at the time of sampling and the cores were sampled from areas with medium to poor drainage.

In all, 107 *Phytophthora* isolates were recovered that had a unique soil core, depth class, and species combination. Forty-one percent of these were from the organic layer, 28% from the 0 to 5cm depth class, 16% from the 5 to 10cm depth class, and 15% from the 10 to 15cm depth class. Forty-two percent of the *Phytophthora* isolates were recovered from WA-1, 34% from WA-2, and 26% from WA-3. On average, a higher percentage of the total *Phytophthora* isolates recovered from each nursery came from the upper depth classes (Fig. 1).

The 3 most common *Phytophthora* spp. recovered from the soil cores were *P. citricola* (32%), *P. drechsleri* (32%) and *P. ramorum* (27%). Other *Phytophthora* spp. recovered included *P. gonapodyides* and *P. cactorum*. Over 100 *Pythium* isolates were recovered from the soil cores as well. Using BLAST search on GenBank, ITS sequences from a subset of these isolates most closely matched ITS sequences of isolates previously identified as *Pythium sterilum*, *P. deliense*, *P. undulatum*, and *P. citrinum*.

P. ramorum was recovered at all three nurseries a total of 29 times from unique soil core and depth class combinations (Fig. 2). A majority (86%) of these isolates were recovered from the organic and 0 to 5cm depth class and *P. ramorum* was detected as deep as the 5 to 10cm depth class (14% of recoveries). In contrast to *P. ramorum*, *P. citricola* and *P. drechsleri* were recovered more consistently throughout the soil profile (Fig. 3). A chi-square test for independence was conducted with *P. ramorum* and *P. citricola* and a second chi-square was conducted with *P. ramorum* and *P. drechsleri*, comparing recovery frequencies at the 4 soil depth classes. The chi-square test with *P. ramorum* and *P. citricola* was significant at $P=0.01$ ($\chi^2=12.1$, $df=3$) and the chi-square test with *P. ramorum* and *P. drechsleri* was significant at $P=0.005$ ($\chi^2=8.3$, $df=3$).

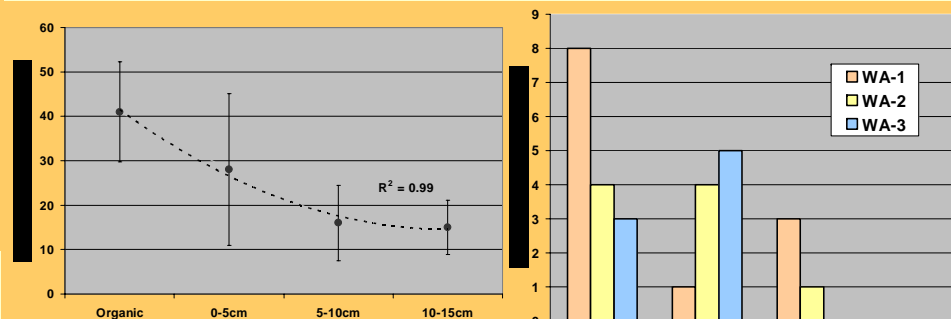


Fig. 1. Average percent of total *Phytophthora* isolates recovered at different depth classes among 3 nurseries. Polynomial trend line is shown as a solid line, the r² corresponds with the trend line and Y error bars represent standard deviation among the 3 nurseries sampled.

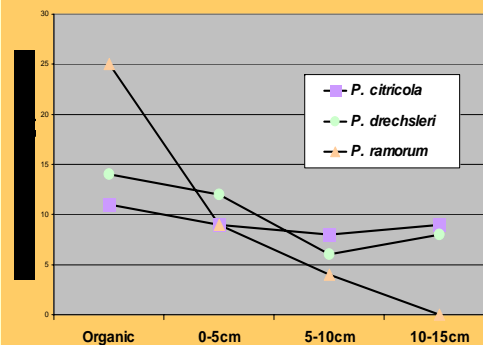


Fig. 3. The total number of soil cores from which 3 different *Phytophthora* spp. were recovered in the soil profiles at 3 nurseries

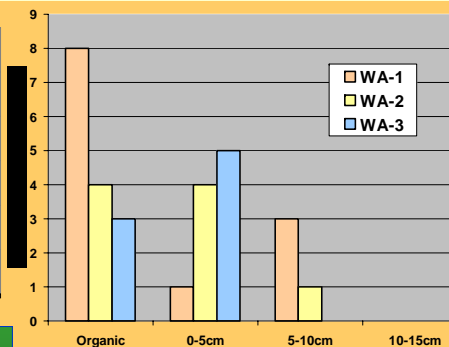
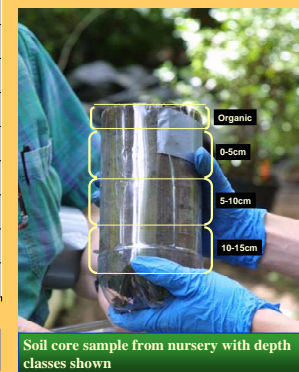


Fig. 2. Number of cores from which *Phytophthora ramorum* was recovered in the soil profiles at 3 nurseries.



Soil core sample from nursery with depth classes shown

Discussion

Overall, more *Phytophthora* recoveries were observed from samples taken at the upper levels of the soil profile (Fig. 1). When broken down to the species level, differences (chi-square tests significant to a value of $P \leq 0.01$) were observed in the recovery frequency of *P. ramorum* with those of *P. citricola* and *P. drechsleri* at different soil depth classes. Recoveries of *P. citricola* and *P. drechsleri* were more evenly distributed throughout the soil profile whereas *P. ramorum* was primarily recovered from the organic and 0 to 5cm depth class (Fig. 3). These differing trends may be explained by differences in how long these species have been at the nursery sites. Compared to the recent introduction of *P. ramorum*, *P. citricola* was described in the United States as early as 1932 and has been reported on a diversity of hosts sold as ornamental nursery stock including *Rhododendron* spp., Japanese andromeda (*Pieris japonica*), lilac (*Syringa vulgaris*), California live oak, white fir (*Abies concolor*) and Douglas-fir (*Pseudotsuga menziesii*). Likewise, *P. drechsleri* was reported in the United States as early as 1929 and has been reported on common ornamental hosts such as heather (*Calluna vulgaris*), Azalea (*Azalea indica*), and Douglas-fir. *P. ramorum* was first reported in North American nursery stock in 2003 which may explain why soil inoculum levels have not built up at deeper levels in the soil profile. Additionally, *P. ramorum* is known as an aboveground pathogen and therefore its inoculum may not be as well adapted to permeating and surviving deep within the soil profile as *P. citricola* and *P. drechsleri* which are primarily root rot pathogens on a number of hosts.

Since *P. ramorum* was recovered as deep as the 5 to 10cm depth class (Fig. 2), we reject the hypothesis that this pathogen is limited to the organic layer of nursery soil. It is likely that sporangia produced on the foliage of nursery stock have percolated into the soil matrix during precipitation events or that chlamydospores embedded within decomposing organic debris on the soil surface have been worked into the soil profile by foot traffic or invertebrates. The recovery of *P. ramorum* as deep as the 5 to 10cm depth class indicates that soil treatments at nurseries where the pathogen is detected in soil should aim to remove or kill inoculum to a depth of at least 10cm. To minimize the potential for *P. ramorum* soil positives at retail nurseries, nursery personnel should consider storing and displaying known and associated host containerized nursery stock on concrete, blacktop or well-drained gravel surfaces. Since 52% of the *P. ramorum* recoveries were from the organic layer, aggressive sanitation programs aimed at cleaning up and disposing plant debris of known and associated hosts may drastically reduce inoculum levels on the soil surface thereby reducing the potential for *P. ramorum* to become established at lower levels of the soil profile.

Acknowledgements

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Collecting soil cores at retail nursery sites that tested positive for *P. ramorum* during WSDA nursery inspections