Recovery Frequency of *Phytophthora ramorum* and Other *Phytophthora* spp. in the Soil Profile of Ornamental Retail Nurseries

N. L. Dart, G. A. Chastagner, E. F. Rugarber, and K. L. Riley, Department of Plant Pathology, Washington State University Research and Extension Center, Puyallup 98371-4998

ABSTRACT

Dart, N. L., Chastagner, G. A., Rugarber, E. F., and Riley, K. L. 2007. Recovery frequency of *Phytophthora ramorum* and other *Phytophthora* spp. in the soil profile of ornamental retail nurseries. Plant Dis. 91:1419-1422.

We tested the hypothesis that inoculum of the aboveground exotic plant pathogen *Phytophthora ramorum* would be limited to the organic layer (top layer of plant debris) of soils at infested retail nurseries located outside of the area where the pathogen has become established in the landscape. To test this hypothesis and compare inoculum levels of *P. ramorum* with levels of other *Phytophthora* spp. in the soil profile, soil cores were collected and sampled from three Washington State retail nurseries at which the soil had previously tested positive for *P. ramorum*. *Phytophthora* was isolated from soil using rhododendron leaves as bait, and pure cultures were obtained and stored on V8 juice agar. Isolates were identified to species using a combination of DNA sequencing of the internal transcribed spacer (ITS) region of rDNA, real-time polymerase chain reaction (PCR) diagnostic testing, and culture morphology. Recovery frequencies were tabulated and compared by species at the organic layer, 0 to 5 cm, 5 to 10 cm, and 10 to 15 cm depth classes. The three most common *Phytophthora* spp. recovered from the soil cores were *P. citricola* (32%), *P. drechsleri* (32%), and *P. ramorum* (27%). *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 5 cm depth class (86% of recoveries). *P. ramorum* was not detected below 10 cm.

Phytophthora ramorum S. Werres & A.W.A.M. de Cock is an exotic plant pathogen responsible for the death of tan oak (*Lithocarpus densiflorus* (Hook. & Arn.) Rehd.), coast live oak (*Quercus agrifolia* Née), and California black oak (*Q. kelloggii* Newb.) in parts of coastal California, tan oak in southwestern Oregon (8,12,13), and ornamental trees such as American southern red oak (*Q. falcata* Michx.) and American northern red oak (*Q. rubra* L.) growing in urban gardens in the United Kingdom and the Netherlands (3,4). This pathogen is also found in horticultural nurseries in North America and Europe infecting a diverse group of hosts from over 16 plant families (14,16,18). *P. ramorum* is generally considered to be an aboveground plant pathogen in nursery systems, where it is known to cause leaf blight and shoot dieback (5,8,10). The pathogen produces sporangia on foliage and twigs that disperse and initiate new infections (8). *P. ramorum* chlamydospores can survive for over a year and sporangia for up to 6 months in

potting media (10), and rhododendron plants have been infected when grown in infested potting media in laboratory experiments (9,11). However, the overall significance of the soil phase in the *P. ramorum* disease cycle (or role in the epidemiology of the pathogen) in nursery systems is still unknown.

With continued detections of *P. ramorum* on ornamental nursery stock in parts of North America and Europe (8,14), there has been concern about the potential spread of this exotic pathogen throughout 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 interstate shipping of known and associated host plants (15). 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 (17). 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 (17). Positive soil tests in a number of U.S. 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 the lack of 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. These studies have demonstrated that *P. ramorum* can be recovered from inoculated leaf disks 6 months after being buried in forest soil in California (7). In a park setting in the Netherlands, the pathogen has been recovered from soil sampled 20 cm deep adjacent to the roots of infested rhododendrons (1).

In retail nursery settings, containerized plants are commonly displayed aboveground, and any inoculum entering the nursery soil presumably comes from fallen, infested leaves or inoculum originating on these plants. Moreover, the roots of containerized plants are generally confined to their pots and are therefore not as likely to provide a potential route for *P. ramorum* to colonize deep into nursery soils as in parks or forests. We hypothesized that in retail nursery settings outside of areas where the pathogen has become established in landscapes, *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 three 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* spp. in the soils and to compare the abundance and depth at which *P. ramorum* and other *Phytophthora* spp. were recovered from the soil profile.

MATERIALS AND METHODS

Collecting soil cores. Soil cores (10 × 20 cm) were collected from three Washington State retail nurseries, designated WA-1, WA-2, and WA-3, over localized

Corresponding author: N. L. Dart
E-mail: normdart@wsu.edu

Accepted for publication 1 June 2007.

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. Collection dates were all during 2006 on 6 August, 25 May, and 31 May at nurseries WA-1, WA-2, and WA-3, respectively. Soil cores were extracted from the ground using a foot extraction hole-cutter (Par Aide Products Company, Lino Lakes, MN). The cores were ejected into 10-cm-diameter, 2-liter plastic soda bottles with the tops cut off. The cores were double-bagged and wrapped with tape to stabilize the integrity of the soil profile. Soil cores were stored at 4°C for 3 to 5 months until they were processed. Soil texture at the three sites was estimated using basic hand texturing, and general notes on the soil moisture and drainage were taken in the field as well.

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 cm, 5 to 10 cm, and 10 to 15 cm. 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 three subcores (from within the

larger 10-cm-diameter core) using a 2-cm-diameter soil probe (AMS, American Falls, ID). The three subcores were sectioned at 5-cm increments and bulked together in sealable plastic bags designated for the three different soil depths for each respective soil core.

Phytophthora species were baited from the soil samples in the plastic bags by adding approximately 150 ml of sterile tap water and three rhododendron leaves (*Rhododendron decorum* Franch. hybrid Caroline) that had been wounded by slicing the midrib of the leaves. The leaves in the water-soil slurry were incubated 48 h at 16°C and then removed from the plastic bags, rinsed under tap water, blotted dry with paper towels, and incubated in a single layer pressed between trays at 17 to 18°C for up to 3 weeks. During this period, the leaves were checked periodically for developing lesions. When a lesion was observed, a section (approximately 5 × 5 mm) was isolated onto corn meal agar amended with ampicillin, rifampicin, and pimarcin (CARP) (8) to select for *Phytophthora* spp. Leaf isolations were incubated at 16°C for up to 3 weeks and were checked periodically for *Phytophthora*-like mycelia growing from the plant tissue. When *Phytophthora*-like growth was observed, pure cultures were made by sub-

culturing to V8 juice agar (3 parts filtered V8: 7 parts commercial agar) and stored at 16°C for future identification.

Identifying *Phytophthora* spp. Cultures of *P. ramorum* were identified based on characteristic mycelium, chlamydospores, aerial mycelium (18), and real-time polymerase chain reaction (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, PCR amplification of the internal transcribed spacer (ITS) region of rDNA, and complete sequencing of the ITS using ITS 4 and ITS 5 primers (19). The sequences were then compared with published sequences obtained from GenBank. 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 (6).

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 four 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 three 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 5 cm. Soil was moist at the time of sampling at all three nurseries, 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 5 cm depth class, 16% from the 5 to 10 cm depth class, and 15% from the 10 to 15 cm depth class. Forty-two percent of the *Phytophthora* isolates were recovered from WA-1, 34% from WA-2, and 26% from WA-3.

The three most common *Phytophthora* spp. recovered from the soil cores were *P. citricola* Sawada (32%), *P. drechsleri* Tucker (32%), and *P. ramorum* (27%). Other *Phytophthora* spp. recovered included *P. gonapodyides* Petersen and *P. cactorum* Leb. & Cohn. Over 100 *Pythium* isolates were recovered from the soil cores as well. Using BLAST search on GenBank, ITS sequences from a subset of

Fig. 1. Number of cores from which *Phytophthora ramorum* was recovered in the soil profiles at three nurseries.

these isolates most closely matched ITS sequences of isolates previously identified as *Pythium sterilum* Belbahri & Lefort, *P. deliense* Meurs, *P. undulatum* Petersen, and *P. citrinum* Thom.

P. ramorum was recovered 29 times from unique soil core and depth class combinations at all three nurseries (Fig. 1). A majority (86%) of these isolates were recovered from the organic and 0 to 5 cm depth class, and *P. ramorum* was detected as deep as the 5 to 10 cm depth class (14% of recoveries). In contrast to *P. ramorum*, *P. citricola* and *P. drechsleri* were recovered more consistently throughout the soil profile (Fig. 2). 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 four 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 $P = 0.005$ ($\chi^2 = 8.3$, $df = 3$).

DISCUSSION

The differing recovery trends of *P. ramorum* in the soil profile with those of *P. citricola* and *P. drechsleri* 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* (Thunb.) D. Don ex G. Don), lilac (*Syringa vulgaris* L.), California live oak, white fir (*Abies concolor* (Gord. & Glend.) Lindl. ex Hildebr.), and Douglas-fir (*Pseudotsuga menziesii* (Mirbel) Franco) (6). 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 (L.) Hull), Azalea (*Azalea indica* L.), and Douglas-fir (6). *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 (8,10), 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 (6).

Since *P. ramorum* was recovered as deep as the 5 to 10 cm depth class, 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 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 10 cm 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 10 cm. 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 (16) 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 of 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.

ACKNOWLEDGMENTS

This research was funded by the Washington State University Agricultural Research Center and the Washington Department of Agriculture (WSDA) Nursery Research Program. A special thanks to USDA-APHIS, WSDA, and the nursery owners for providing access to nursery sites and the WSDA Plant Pathology Lab for the real-time PCR confirmation of *P. ramorum* isolates. PPNS No. 0459 Department of Plant Pathology, College of Agricultural, Human, and Natural Resource Sciences Agricultural Research Center Project No. 0388, Washington State University, Pullman, WA 99164-6240.

LITERATURE CITED

1. Aveskamp, M. M., van Baal, P. J. M., and de Gruyter, J. 2005. Effect of sanitary measures on the survival of *P. ramorum* in soil. Page 279 in: Sudden Oak Death Science Symposium II, 18-21 January 2005, Monterey, CA.
2. Bilodeau, G. J., and Hamelin, R. C. 2005. Molecular detection of *Phytophthora ramorum* by real-time PCR using Taqman, SYBRGreen and molecular beacons with three genes. Page 139 in: Sudden Oak Death Science Symposium II, 18-21 January 2005, Monterey, CA.
3. Brasier, C. 2003. *Phytophthoras* in European forests: Their rising significance. Sudden Oak Death Online Symposium. American Phytopa-

Fig. 2. Total number of soil cores from which three *Phytophthora* spp. were recovered in the soil profiles at three nurseries.

- thological Society, St. Paul, MN. Online publication. doi:10.1094/SOD-2003-CB.
4. Brasier, C. M., Denman, S., Rose, J., Kirk, S. A., Hughes, K. J. D., Griffin, R. L., Lane, C. R., Inman, A. J., and Webber, J. F. 2004. First report of ramorum bleeding canker on *Quercus falcata*, caused by *Phytophthora ramorum*. Plant Pathol. 53:804.
 5. Davidson, J. M., Wickland, A. C., Patterson, H. A., Falk, K. R., and Rizzo, D. M. 2005. Transmission of *Phytophthora ramorum* in mixed-evergreen forest in California. Phytopathology 95:587-596.
 6. Erwin, D. C., and Ribeiro, O. K. 1996. *Phytophthora* Diseases Worldwide. American Phytopathological Society, St Paul, MN.
 7. Fichtner, E. S., Lynch, and Rizzo, D. 2005. Summer survival of *Phytophthora ramorum* in forest soils. Page 165 in: Sudden Oak Death Science Symposium II, 18-21 January 2005, Monterey, CA.
 8. Hansen, E. M., Parke, J. L., and Sutton, W. 2005. Susceptibility of Oregon forest trees and shrubs to *Phytophthora ramorum*: A comparison of artificial inoculation and natural infections. Plant Dis. 89:63-70.
 9. Lewis, C. D., Roth, M. L., Choquette, C. J., and Parke, J. L. 2004. Root infection of rhododendron by *Phytophthora ramorum*. (Abstr.) Phytopathology 94:S60.
 10. Linderman, R. G., and Davis, E. A. 2006. Survival of *Phytophthora ramorum* compared to other species of *Phytophthora* in potting media components, compost, and soil. Hort-Technology 16:502-507.
 11. Parke, J. L., Roth, M., and Choquette, C. 2005. *Phytophthora ramorum* disease transmission from infested potting media. Page 291 in: Sudden Oak Death Science Symposium II, 18-21 January 2005, Monterey, CA.
 12. Rizzo, D. M., and Garbelotto, M. 2003. Sudden oak death: Endangering California and Oregon forest ecosystems. Front. Ecol. Environ. 1:197-204.
 13. Rizzo, D. M., Garbelotto, M., Davidson, J. M., Slaughter, G. W., and Koike, S. T. 2002. *Phytophthora ramorum* as the cause of extensive mortality of *Quercus* spp. and *Lithocarpus densiflorus* in California. Plant Dis. 86:205-214.
 14. Tubajika, K. M., Bulluck, R., Shiel, P. J., Scott, S. E., and Sawyer, A. J. 2006. The occurrence of *Phytophthora ramorum* in nursery stock in California, Oregon, and Washington states. Online publication. Plant Health Progress doi:10.1094/PHP-2006-0315-02-RS.
 15. U.S. Dep. Agric. APHIS 2004. *Phytophthora ramorum* emergency federal order restricting movement of nursery stock from California, Oregon, and Washington nurseries. <http://programs.cphst.org/pram/files/FEDERAL%20ORDER%2012-21-04-FINAL.pdf>.
 16. U.S. Dep. Agric. APHIS. 2005. List of plants regulated and associated with *Phytophthora ramorum*. http://www.aphis.usda.gov/plant_health/plant_pest_info/pram/downloads/pdf_files/usdaprlst.pdf.
 17. U.S. Dep. Agric. APHIS. 2005. Official regulatory protocol for nurseries containing plants infected with *Phytophthora ramorum*. http://www.aphis.usda.gov/plant_health/plant_pest_info/pram/downloads/pdf_files/CNPv7.pdf.
 18. Werres, S., Marwitz, R., Man in 't Veld, W. A., De Cock, A. W. A. M., Bonants, P. J. M., De Weerd, M., Themann, K., Ilieva, E., and Baayen, R. P. 2001. *Phytophthora ramorum* sp. nov., a new pathogen on *Rhododendron* and *Viburnum*. Mycol. Res. 105:1155-1165.
 19. White, T. J., Bruns, T., Lee, S., and Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. Pages 315-321 in: PCR Protocols: A Guide to Methods and Applications. M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White, eds. Academic Press, San Diego, CA.