Phytophthora Populations in Nursery Irrigation Water in Relationship to Pathogenicity and Infection Frequency of *Rhododendron* and *Pieris*

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Abstract

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Phytophthora spp. are waterborne plant pathogens that are commonly found in streams, rivers, and reclaimed irrigation water. *Rhododendron* and *Pieris* trap plants at two commercial nurseries were irrigated with water naturally infested with *Phytophthora* spp. during the 2011 and 2012 growing seasons to assess the frequency of disease. *Phytophthora* spp. were consistently recovered from water samples at every collection time but detected on only 2 of the 384 trap plants during the two growing seasons. Pathogenicity assays proved that *Phytophthora* hy-

dropathica and *Phytophthora* taxon PgChlamydo, commonly recovered taxa in irrigation water at the nurseries, were foliar pathogens of *Rhododendron* and *Pieris*; however, neither species was able to cause root rot on these same hosts. Overall, *Phytophthora* spp.-infested irrigation water did not act as a primary source of infection on *Rhododendron* and *Pieris*, even though foliar pathogenic species of *Phytophthora* were present in the water.

To conserve water, preserve the surrounding ecosystems and groundwater from pesticide and nutrient runoff, and conform to stringent water regulations, growers frequently recycle or reclaim surface water for irrigation purposes. The benefits of recycling water are obvious but a major deterrent is the potential for spreading waterborne plant pathogens and pests (29). A few of the most economically important genera of plant pathogens commonly found in water are *Pythium* Pringsh. and *Phytophthora* de Bary (3). Although *Phytophthora* spp. are frequently detected in irrigation water, little is known of the risk associated with irrigating plants using water naturally infested with *Phytophthora* spp. Tjosvold (42) irrigated hybrid *Rhododendron* plants with stream water naturally infested with *Phytophthora ramorum* for four years and observed a low incidence of disease. Similarly, Werres and Wagner (45) found low to no infection of *Rhododendron* plants in simulated recirculation systems that had been infested with propagules of *P. ramorum*.

The concentration and species diversity of *Phytophthora* propagules within irrigation water fluctuates throughout a growing season (10); however, there is much to learn regarding the impact of water conditions on the dynamics of *Phytophthora* spp. Additionally, the risk of infection related to inoculum density of *Phytophthora* taxa naturally present in irrigation water has not been demonstrated. Risk can be estimated based on the frequency of infection of susceptible plants by *Phytophthora* spp. present when *Phytophthora*-infested water is used as the main source for irrigation. To evaluate the risk associated with irrigating ornamental nursery plants using water naturally infested with *Phytophthora* spp., we placed susceptible cultivars of *Rhododendron* and *Pieris japonica* as trap plants at two different commercial ornamental nurseries with a documented history of *Phytophthora*-infested water, and irrigated these plants over 5 months in the summers of 2011 and 2012. Water was sampled for *Phytophthora* spp. using a quantitative water filtration assay. In addition, plants were monitored for foliar blight and root rot symptoms and symptomatic

tissue was assayed using selective media. To confirm pathogenicity, *Phytophthora* isolates commonly recovered from irrigation water at these two nurseries were then screened for pathogenicity on two susceptible ornamental hosts.

Materials and Methods

Nursery selection. Two woody ornamental nurseries in Western North Carolina (WNC) were selected for this study based on the presence of *Phytophthora* spp. in previous water surveys at these locations. Nursery I, located in Burke County, irrigated plants from a retention basin that has been reclaiming total runoff for over 50 years, while nursery II, located in Transylvania County, pumped water directly from the French Broad River, a natural water source which drains from a forested watershed through eight counties in WNC encompassing 7,330 km².

Water sampling and filtration. Irrigation water was sampled monthly between June and October in 2011 and in May, June, August, and October during 2012 using 500-ml sterile polypropylene bottles. At each time, water samples were collected at the depth (approximately 20 to 25 cm below the surface) and nearest point of the intake pump from both the retention basin and river, and directly from sprinklers that were located within the irrigation zone used to water the trap plants. Nursery I utilized a pump with a flow rate of approximately 303 liters per minute (lpm) and impact sprinkler heads that deliver water at approximately 21 lpm. Nursery II utilized a pump with a flow rate of approximately 227 lpm and inverted wobbler sprinkler heads that emit water at approximately 4 lpm. Water samples were submerged in ice during transport and processed within 24 h of collection.

Water samples were mixed by inverting several times. Electrical conductivity and pH were measured with a solu-bridge (YSI 35, Yellow Springs Instrument Co.) and pH meter (Accumet 910; Fisher Scientific) prior to filtration (*data not shown*). A filtration assay adapted from MacDonald et al. (31) was used to estimate the CFU of pythiaceae propagules present in each sample. Aliquots of 200 ml were passed through a 1- to 5- μ m P2 filter (Fisher Scientific) using a Büchner funnel attached to a vacuum pump. An additional 200-ml aliquot was passed through a 4- to 8- μ m P4 filter (Fisher Scientific) with the same vacuum apparatus. Filters were removed and placed top-side down on V8-PARPH, a *Phytophthora*-selective medium previously described by Jeffers and Martin (26). Plates with filters were incubated in the dark at 21°C (room temperature [RT]) for 24 h. After initial incubation, filter papers were removed and plates were incubated in the dark at

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RT for an additional 48 h. CFU were counted after the second incubation and converted to CFU per liter. The CFU per liter between filters were averaged together to represent the mean CFU per liter per sample time. Isolations were made from a subset of colonies with visually distinctive morphological characteristics and hyphal branching typical of *Phytophthora* spp. Subcultures were obtained from each colony by transferring a hyphal tip to V8-PARPH. Pure culture isolates were maintained on corn meal agar (CMA) for further characterization and for the preparation of select isolates for pathogenicity assays.

In situ plant bioassays. During the summer of 2011 and 2012, containerized hybrid *Rhododendron* sp. 'Nova Zembla' (2011), *Rhododendron* sp. 'English Roseum' (2012), and *P. japonica* 'Mountain Fire' (2011 and 2012) were used as susceptible trap hosts and placed within the irrigation zones of both nurseries under 55% shade. Plants were purchased from commercial sources and maintained at the Mountain Horticultural Crops Research Station (MHCRC) under shade on a container pad for 2 months prior to initiating the study to avoid the risk of any remaining fungicide residues at the start of the study. Plants were potted into pine bark substrate consisting of 1.8 kg of lime and 965 g of MicroMax (Scotts) per cubic meter of substrate. Osmocote Plus (15-9-12) was surface applied using the recommended middle rate (52 and 21 g/pot for 11.4- and 3.8-liter pots, respectively). Plants were potted into 11.4- and 3.8-liter containers in 2011 and 2012, respectively. Plants were arranged in a randomized complete block design with eight blocks of six plants each for both *Rhododendron* and *Pieris* spp. Blocks were spaced 1 m apart, and pots were placed adjacent to each other within each block. Plants were watered as necessary; daily water input (rain plus irrigation) was measured on site with a HOBO Data Logging Rain Gauge (Onset) using BoxCar Pro 4.0 software.

Root plating. Root cores from one of the six plants in each block were taken with a 2.5-cm-diameter soil probe each month and transported to the lab in a cooler on ice. Samples were stored at 4°C and assayed within 1 week. Roots were rinsed free of debris, cut into 1-cm-long sections, and placed just under the surface of V8-PARPH. Plates were incubated in the dark at RT for 24 to 96 h and monitored daily for any growth. Colonies representing typical morphology of *Phytophthora* spp. were further cultured by hyphal-tip transfers onto V8-PARPH. Pure culture isolates were maintained on CMA for further characterization.

Root flooding. Plants were flooded to simulate a conducive environment for *Phytophthora* root rot and to stress test plants to increase the likelihood of foliar infections. In the summer of 2012, one plant from each of the eight blocks of both hosts was flooded on 25 June, 13 August, and 9 October at both nurseries. Plants were placed into 19-liter buckets and well water was filled up to the crown level of each plant. Root zones of plants were submerged for a period of 48 h, then removed and monitored for symptoms over the rest of the growing season. On 11 October, all plants subjected to flooding from both nurseries (48 plants of each host), were destructively harvested and roots and leaves with disease symptoms were rinsed, plated on V8-PARPH, and monitored for *Phytophthora* spp. If *Phytophthora* spp. were observed growing from the roots on V8-PARPH, pure cultures were obtained, as previously described.

Root baiting. One plant from each block from all plots was subject to a baiting assay to detect *Phytophthora* spp. present in the substrate and roots. In 2011, root balls of each cultivar were standardized by weight based on the weight of the lightest root ball. Standardized root balls were placed in plastic crisper boxes (30 by 22 cm) and flooded with 1 liter of distilled water. Symptomless leaves of native *Rhododendron maximum* were collected, and 10 leaf discs (3-mm each) were floated on the water surface in each container and incubated for 48 h in the dark at RT. After incubation, six leaf discs were randomly selected from each box, placed just under the surface of V8-PARPH, incubated in the dark at RT for 48 to 96 h, and monitored for growth of *Phytophthora* spp. In 2012, a method developed to detect asymptomatic infections of

Phytophthora ramorum (43) was used to bait roots. For this method, two *Rhododendron* leaves that had no history of fungicide exposure were wounded by cutting six 2-cm slits evenly spaced along the edges of the entire leaf, and placed directly under the root ball within the pot of one plant from each block of both host type at both locations, totaling 32 plants. Pots were placed in clay saucers filled with distilled water and incubated in situ for 48 h. Leaves were collected and rinsed free of debris, and wounded areas of the leaves were excised and submerged in V8-PARPH. In both baiting assays, leaf pieces placed just under the surface of V8-PARPH were monitored daily for growth, and colonies representative of *Phytophthora* spp. were transferred as mentioned previously for identification and long term storage.

Destructive sampling and disease assessments. Plants were monitored by visual observation for foliar disease and root rot symptoms over the entire 5-month growing season for both 2011 and 2012. At the end of each season, the aboveground portion of one plant from each block was abscised and weighed. Roots from each plant were rated for root rot symptoms on a scale of 1 to 5, where 1 = healthy roots, 2 = <25% root necrosis, 3 = 25 to 49% root necrosis, 4 = 50 to 75% root necrosis, and 5 = 76 to 100% root necrosis or dead plant. Means of the visual root rot rating and aboveground plant weight were analyzed by analysis of variance (ANOVA; Proc GLM, SAS 9.1; SAS Institute) and separated by Waller-Duncan *k*-ratio test.

Identification of isolates. Isolates collected in this study were identified to species with standard molecular and morphological methods, including DNA sequencing of the internal transcribed spacer (ITS) region, colony morphology, and characterization of sporangia, chlamydospores, and oospores (11,12,19–21). Isolates were grouped based on colony morphology and hyphal branching, and a subset of isolates (>20%) from each group was identified based on sequence analysis of the ITS region of the ribosomal DNA (rDNA). DNA was extracted from lyophilized mycelia with the Puregene kit (Qiagen). Amplification was performed with primers ITS4 and ITS6 (46) with thermocycling conditions of 1 cycle of 94°C for 5 min; 35 cycles of 94°C for 1 min, 48°C for 1 min, and 72°C for 1 min; and a final cycle of 72°C for 5 min, resulting in an approximately 900-bp fragment. Amplicons were cleaned with the QIAquick PCR purification kit (Qiagen) and sequenced with ABI sequencing technology (MCLab). Raw sequence reads were imported and aligned in Sequencher v.4.9 and visually edited, including accounting for heterozygous sites if present. Edited sequences were compared with the sequences deposited in the *Phytophthora* Database (www.phytophthoradb.org) (34) and the nonredundant nucleotide database GenBank (National Center for Biotechnology Information) using the BLAST algorithm (2). Isolates were identified based on highest percent similarity within the ITS region. Sequences of isolates generated in this study were deposited in GenBank.

Morphological features of chlamydospores, oospores, and sporangia for at least one isolate representing each unique ITS taxon were examined by microscopy. On average, 10 propagules per isolate per propagule type were measured for characterization of each taxon. Sporangia were produced by growing each isolate in 5% V8 broth and incubating at RT in the dark for 3 to 4 days. After incubation, V8 broth was removed and mycelial mats were rinsed with sterile, distilled water, flooded with 2% nonsterile soil extract (NSSE; 25), and incubated under continual fluorescent light at a luminous intensity of 181.4 candelas at RT for 2 to 4 days (25).

Pathogenicity assays of isolated *Phytophthora* taxa. During the summer of 2012, isolates representing four unique taxa of *Phytophthora* most commonly recovered from irrigation water from the nurseries during 2011 were tested for pathogenicity to both roots and leaves of *Rhododendron* sp. English Roseum and *Pieris japonica* Mountain Fire. The four isolates were representatives of *Phytophthora pini* Leonian, *P. hydropathica* C. X. Hong & Gallegly, and undescribed *Phytophthora* sp. BF4 and *Phytophthora* taxon PgChlamydo. Leaf and root pathogenicity tests were performed in Raleigh at the North Carolina State Horticultural Field

Lab (HFL) and in Mills River at the MHCRS. Average mean temperature throughout the duration of these tests was 24.8°C at the HFL and 22.4°C at the MHCRS; the average high and low temperatures were 31.0 and 18.5°C, respectively, at the HFL and 28.0 and 16.8°C at the MHCRS. Locally sourced plants were repotted into pine bark substrate amended with lime at 2.4 kg/m³ and of slow release 18-6-12 fertilizer Multicote 8 (HAIFA NutriTech) at 4.2 kg/m³. Plants at the HFL were purchased as first-year rooted liners, whereas the plants utilized at MHCRS were purchased as second-year container plants. The treatments were arranged in randomized complete block design with four replications and two observations per host plant per treatment. Plants were placed on top of inverted plastic trays to avoid interplot interference from runoff and irrigated twice daily for 45 min/irrigation event (0.2 cm/day). Water was sourced from a manmade lake stocked with city water (HFL) and a retention pond which underwent chlorine treatment at 1.5 ppm (Accu-Tab) (MHCRS).

Root assays. Rice grain inoculum of all four isolates was prepared as described by Holmes and Benson (18). On 19 July and 26 July at MHCRS and HFL, respectively, three holes approximately 4 cm deep were made in the soilless medium of each container, and three colonized rice grains of the corresponding treatment were placed into each hole, for a total of nine colonized rice grains per plant. Noninfested rice grains were used for the negative control treatment and rice grains colonized with isolate 2386 of *P. cinnamomi* served as the positive control. Plants were monitored weekly for 6 weeks and rated for visual aboveground symptoms (*data not shown*). At the end of each experiment, one observation per host from each treatment replication was baited with wounded *Rhododendron* leaves for a period of 48 h (43) and recovered leaves were washed in running water prior to culturing on V8-PARPH. Plants were destructively harvested on 11 and 25 September at HFL and MHCRS, respectively. Aboveground weight and root rot ratings were recorded using the rating system mentioned earlier and analyzed by ANOVA (Proc GLM; SAS Institute).

Leaf assays. Two different inoculation methods were used to evaluate foliar pathogenicity of the four most commonly recovered *Phytophthora* taxa on *Rhododendron* and *Pieris* spp. The two leaf assays consisted of a zoospore or mycelial suspension spray and a detached leaf assay. Both inoculation assays were replicated.

Zoospore or mycelial spray. To produce zoospores for inoculation, sporangia of each isolate were produced as described above. To induce the release of zoospores from sporangia, cultures were incubated at 4°C for 1 h, then placed on the bench top at RT for 1 to 2 h. Zoospores were quantified by taking a 1-ml aliquot from the original zoospore suspension and mixing with 1 ml of lactophenol cotton blue (Becton, Dickinson and Co.) to encyst and stain zoospores for counting using a hemacytometer. Each suspension was diluted to a final concentration of 2.5×10^3 zoospores/ml if zoospores were observed (*P. pini* and *Phytophthora* taxon PgChlamydo). If sporangia did not form or did not release zoospores (*P. hydropathica* and *Phytophthora* sp. BF4), mycelial mats were agitated with a sterile glass rod and the suspensions diluted with distilled water at a dilution of one part *Phytophthora* suspension to four parts deionized water for a total volume of 200 ml.

Forty *Rhododendron* and *Pieris* plants each were inoculated in vivo with the zoospore or mycelium inoculum on 25 May at the HFL and 1 August at the MHCRS. Inoculum was applied with 200-ml spray bottles set to a stream setting. Individual plants were sprayed with the respective treatment (*Phytophthora* isolate) until inoculum ran off the leaves. The negative control treatment consisted of 2% NSSE and four parts distilled water (1:4, vol/vol). After application, plants were covered with white plastic bags to create a temporary humidity chamber. To avoid heat damage, treatments were applied at dusk and bags were removed at sunrise the following day (12 h postinoculation). Plants were rated weekly after inoculation on a scale of 1 to 5, with 1 = completely healthy leaves, 2 = foliage with ≤ 5 foliar lesions, 3 = foliage with ≥ 6 foliar lesions, 4 = plants with stem lesions or bud lesions, and 5 = dead plants. Foliar disease ratings taken on the last date (12 July and 24

August at HFL and MHCRS, respectively) were recorded and analyzed by ANOVA (Proc GLM; SAS Institute). Necrotic leaf or stem tissue collected from one plant of each host species within each treatment replication was plated on V8-PARPH to demonstrate infection.

Detached leaf assay. The second inoculation method used detached *Rhododendron* leaves with agar inoculum to evaluate pathogenicity of the four isolates. Detached leaves from one *Rhododendron* sp. 'Roseum Elegans' plant were placed in plastic crisper boxes lined with three layers of moistened paper towels to create a humidity chamber. Each leaf was inoculated on two sites, which were separated by the midrib; one site was wounded by piercing the leaf with a sterile inoculation needle and the other site was not wounded. Agar plugs (7 mm in diameter) were cut from the leading edge of a 7- to 10 day-old culture of all four isolates and placed on both the wounded and unwounded sites within each leaf. A positive control of *P. heveae* A. W. Thomps. (4) and a negative control of noncolonized CMA were included. Eight inoculated leaves per isolate were incubated in humidity chambers for 10 days at RT, and misted to restore moisture every other day. Treatments were set up in a randomized complete block design with two observations per treatment replication; this test was performed twice starting on 13 and 21 September. At the end of the 10-day incubation, leaves were scanned on a flat-bed scanner (Epson Perfection V500 Photo), and lesion area was measured using APS Assess 2.0 software, then analyzed by ANOVA (Proc GLM, SAS Institute).

Results

Detection of *Phytophthora* in irrigation water. An average of 20 pythiaceus CFU/liter (5 to 38 CFU/liter) and 16 pythiaceus CFU/liter (5 to 33 CFU/liter) was detected from water collected in the retention basin throughout the growing season at nursery I in 2011 and 2012, respectively. After passing through the intake and pumping system, an average of 7 pythiaceus CFU/liter (0 to 15 CFU/liter) and 7 pythiaceus CFU/liter (0 to 23 CFU/liter) was detected from water collected at the sprinkler head used to irrigate the trap plants in 2011 and 2012, respectively. For nursery II, which pumped water directly from the French Broad River, an average of 74 pythiaceus CFU/liter (30 to 133 CFU/liter) and 59 pythiaceus CFU/liter (38 to 85 CFU/liter) was detected during the growing season in 2011 and 2012, respectively. After the river water passed through the irrigation system, an average of 25 pythiaceus CFU/liter (0 to 70 CFU/liter) and 15 pythiaceus CFU/liter (0 to 33 CFU/liter) was detected from water collected at the sprinkler head used to irrigate the trap plants in 2011 and 2012, respectively (Table 1).

Identification of isolates. In total, 80 isolates were isolated from the water at both nurseries over both growing seasons. Based on the described molecular methods employed for culture identification, isolates recovered from the irrigation water at the two nurseries represented taxa in three clades, including *P. hydropathica* (clade 9), *P. irrigata* (clade 9), and *P. pini* (clade 2), and the uncharacterized taxa *Phytophthora* sp. BF4 (clade 9), *Phytophthora* sp. BFA (clade 9), and *Phytophthora* taxon PgChlamydo (clade 6) (www.phytophthoradb.org). *P. hydropathica* and *Phytophthora* taxon PgChlamydo were the predominate species isolated from water at nursery I and nursery II, respectively. Additionally, *Phytophthora* sp. BF4 and *P. hydropathica* were the only taxa recovered from both nurseries (*data not shown*). Although some of our isolates were 99 to 100% similar to deposited sequences labeled as *P. citricola* in GenBank and the *Phytophthora* database, we are referencing these isolates as *P. pini* based on the morphological and molecular data reported in a recent publication by Hong et al. (19).

Phytophthora isolates recovered from water in 2011 from both nurseries were characterized based on microscopic morphological characteristics. *P. pini* (GenBank accession KC122237) isolates ($n = 6$) produced ellipsoid to ovoid, semipapillate sporangia, 47.5 by 35.4 μm (range 38.1 to 56.3 μm). All isolates were homothallic and produced oospores that were mostly plerotic with large, capitate

paragynous antheridia. Chlamydospores were not observed in any of the isolates examined. Other morphological characteristics of the *P. pini* isolates, such as irregular hyphal swellings, demonstrate a unique characteristic observed in *P. pini* but never observed in *P. citricola* sensu stricto (19).

Isolates ($n = 9$) of *P. hydropathica* (GenBank accession JX996049) produced ovoid to obpyriform, noncaducous nonpapillate sporangia, 47.5 by 35.4 μm (range = 29.6 to 77.5 by 19.1 to 48.6 μm). The most distinctive characteristic was obovate hyphal swellings that were longitudinally produced within mycelia on V8A media. Chlamydospores were terminal and most often observed as germinating propagules.

Phytophthora taxon PgChlamydo (GenBank accession JX996047) isolates ($n = 29$) produced ellipsoid to obpyriform, noncaducous, nonpapillate sporangia, 51.3 by 34.8 μm (range = 29.1 to 82.4 by 18.6 to 62.7 μm). Isolates from this group produced an abundant amount of intercalary chlamydospores with a mean diameter of 34 μm . Oospores were not observed in CMA for this taxon when grown at RT in the dark.

Phytophthora sp. BF4 (GenBank accession JX996050) isolates ($n = 5$) produced small, ellipsoid, noncaducous, nonpapillate sporangia that were, on average, 33.5 by 29.4 μm (range = 13.7 to 61.9 by 18.2 to 41.3 μm). Chlamydospores, hyphal swellings, and oospores were not observed in CMA for this taxon when grown at RT in the dark.

P. irrigata (GenBank accession JX996046; formerly taxon *Phytophthora* sp. 23J7) isolates ($n = 3$) produced ellipsoid, nonpapillate, noncaducous sporangia, 47.1 by 27.1 μm (range = 33.8 to 59.4 by 20.5 to 34.4 μm). Chlamydospores and hyphal swellings were not observed. In addition, oospores were not observed in CMA when grown at RT in the dark.

In situ plant bioassays. Foliar and root infections caused by *Phytophthora* taxa present in the irrigation water were not observed among the trap plants at either nursery during this study, except for two rare events where *Phytophthora* taxon PgChlamydo was isolated from the roots of an asymptomatic *Pieris* plant in

2011 at nursery II, and *P. hydropathica* was recovered from one symptomatic leaf of a *Rhododendron* plant at nursery I in 2012. To test for the presence of latent infection of the roots caused by species of *Phytophthora*, *Rhododendron* bait leaves were placed inside the bottom of the pot directly under the root ball growing within soilless media of a subset of plants at both nurseries, and processed as previously described by Vercauteren (43). *Phytophthora* spp. were not detected with baiting methods in 2011 or 2012. Root rot ratings and aboveground weights of the *Rhododendron* and *Pieris* plants were not statistically different ($P = 0.05$) at each nursery and *Phytophthora* spp. were only detected and isolated from one symptomatic *Rhododendron* leaf and one asymptomatic *Pieris* root system.

Pathogenicity assays of isolated *Phytophthora* taxa. No visual root rot symptoms were observed on *Rhododendron* or *Pieris* test plants at either trial location 8 weeks postinoculation with isolates of *P. hydropathica*, *P. pini*, *Phytophthora* sp. BF4, and *Phytophthora* taxon PgChlamydo. Aboveground weights and root rot ratings were not significantly different ($P = 0.05$) on plants harvested from the MHCRS on 11 September (Table 2). At the HFL location at 8 weeks postinoculation on 26 July, no symptoms were apparent and plants were reinoculated as described above. Six weeks following reinoculation, all plants remained symptomless, except for the positive control *Rhododendron* plants inoculated with *P. cinnamomi*. Although no root rot symptoms were observed on plants inoculated with the four *Phytophthora* isolates, aboveground fresh weights of *Rhododendron* root-inoculated with *P. pini* and *P. hydropathica* were significantly less ($P = 0.05$) than the noninoculated control plants (Table 2); however, average root rot ratings were statistically the same as the noninoculated control.

Root assays. *P. cinnamomi*, *P. hydropathica*, *P. pini*, and *Phytophthora* taxon PgChlamydo were detected from *Rhododendron* bait leaves placed in the root zone of inoculated plants or by direct isolation from roots (Table 3). *Phytophthora* sp. BF4 was not detected with *Rhododendron* bait leaves in any of the respective inoculated treatments. At the HFL location, *P. pini* was recovered from

Table 1. Concentration of pythiaceous propagules detected in irrigation water at nurseries I and II during 2011 and 2012

Time	Sample	CFU/liter ($\bar{x}$) ^z			
		Nursery I		Nursery II	
		2011	2012	2011	2012
May	Source	38 $\pm$ 3.5	33 $\pm$ 3.5	90 $\pm$ 7.1	85 $\pm$ 7.1
	Sprinkler	10 $\pm$ 0.0	23 $\pm$ 3.5	70 $\pm$ 21.2	5 $\pm$ 0.0
June	Source	3 $\pm$ 3.5	13 $\pm$ 3.5	30 $\pm$ 0.0	38 $\pm$ 3.5
	Sprinkler	0 $\pm$ 0.0	5 $\pm$ 0.0	23 $\pm$ 3.5	33 $\pm$ 14.1
July	Source	18 $\pm$ 3.5	—	55 $\pm$ 21.2	—
	Sprinkler	15 $\pm$ 7.1	—	23 $\pm$ 3.5	—
August	Source	5 $\pm$ 7.1	5 $\pm$ 0.0	60 $\pm$ 0.0	50 $\pm$ 14.1
	Sprinkler	8 $\pm$ 10.6	0 $\pm$ 0.0	0 $\pm$ 0.0	20 $\pm$ 0.0
October	Source	33 $\pm$ 10.6	13 $\pm$ 3.5	133 $\pm$ 24.8	60 $\pm$ 14.1
	Sprinkler	0 $\pm$ 0.0	0 $\pm$ 0.0	5 $\pm$ 0.0	0 $\pm$ 0.0

^z Water sources of nursery I and II represent a retention basin and a river, respectively. Average CFU/liter are represented by $\bar{x}$ and followed by $\pm$ the standard deviation of CFU/liter water; — indicates sample was not collected.

Table 2. Pathogenicity of *Phytophthora* isolates recovered from irrigation water on *Rhododendron*^y

<i>Phytophthora</i> taxa	HFL		MHCRS	
	Root rot (1–5) ^z	Weight (g)	Root rot (1–5) ^z	Weight (g)
<i>Phytophthora</i> sp. BF4	1.0 b	42.0 ab	1.0	35.0
<i>Phytophthora pini</i>	1.0 b	33.0 bc	1.0	29.0
<i>Phytophthora hydropathica</i>	1.0 b	34.0 bc	1.0	28.0
<i>Phytophthora</i> taxon PgChlamydo	1.0 b	48.0 ab	2.0	28.0
Noninoculated control	1.0 b	54.0 a	2.0 ns	33.0 ns
<i>P. cinnamomi</i> (positive control)	4.0 a	16.0 c	NT	NT

^y HFL and MHCRS represent the Horticultural Field Lab and Mountain Horticultural Crop Research Station, respectively. Values followed by different letters in each column are statistically different from one another according to Waller-Duncan k -ratio means separation, $P = 0.05$; ns = not significant and NT = not tested.

^z Root rot ratings represent the average of each treatment across eight replications on a visual disease rating scale of 1 to 5, with 5 = severe root necrosis or dead plant (see text).

roots of 100% of the inoculated plants tested. At the MHCRS, *P. pini* was recovered from only one asymptomatic *Pieris* plant. The identity of the isolates recovered from bait leaves was determined by ITS sequence analysis of a representative subset of isolates from each treatment; all recovered isolates were the same ITS genotype used to inoculate the plants.

Leaf assays. Zoospore or mycelial spray. Foliage of *Rhododendron* and *Pieris* plants inoculated with a zoospore or mycelial suspension of the isolates at the MHCRS and HFL developed lesions depending on taxa or location. Final foliar ratings were conducted at 5 and 6 weeks postinoculation at MHCRS and HFL, respectively. If produced, lesions developed within 14 days postinoculation for all isolates. For instance, at HFL, both *Rhododendron* and *Pieris* foliage developed lesions within 5 days of inoculation with *P. hydropathica*. On *Rhododendron* spp., the isolate of *P. hydropathica* used for inoculation caused the most severe dieback at HFL; however, when the same isolate of *P. hydropathica* was used to inoculate *Rhododendron* spp. at MHCRS, dieback was significant ($P = 0.05$) but only moderately severe (Table 4). *P. pini* caused the most severe symptoms on *Rhododendron* and *Pieris* plants at the MHCRS but, at the HFL, foliar infection of *Pieris* plants inoculated with this taxon was not significant when compared with the noninoculated control. *Phytophthora* sp. BF4 did not infect *Pieris* foliage at either location but some disease, based on the described rating scale, was observed on *Rhododendron* plants at HFL (Table 4). At the HFL, *Phytophthora* taxon PgChlamydo caused disease on the foliage of *Rhododendron* but not *Pieris* plants whereas neither host was infected in the experiment at MHCRS. Temperature differences, as mentioned earlier, or other environmental parameters could have caused these differences.

Detached leaf assay. In the second foliar inoculation experiment which utilized agar plugs on detached leaves, only the wounded sites of *Rhododendron* leaves developed lesions regardless of the *Phytophthora* taxa tested. Ten weeks postinoculation, lesions were not observed on nonwounded sites of the *Rhododendron* leaves. However, there were significant differences in lesion areas on the wounded site of the leaves tested. *P. hydropathica* produced the

largest lesions with an average lesion area of 2.4 cm² (standard deviation [SD] = 0.97 cm²). All wounded sites showed small, necrotic areas as a wound response to puncturing the tissue with an inoculation needle. *Phytophthora* sp. BF4 was reisolated from small lesions but, overall, the average lesion size was not significantly different from the noninoculated control (Table 5).

Discussion

Phytophthora taxa were recovered from irrigation water at both nurseries at all sampling times in 2011 and 2012. However, *Phytophthora* spp. were only isolated from the foliage of 1 *Rhododendron* plant (*P. hydropathica*) and from the roots of 1 *Pieris* plant (*Phytophthora* taxon PgChlamydo) among the 384 susceptible trap plants monitored during the study. Numerous studies have identified *Phytophthora* taxa in irrigation water (1,3,6,9–11,16,22, 24,27,29,36,38–41,48) and numerous studies have demonstrated that several *Phytophthora* spp. recovered from irrigation water or other sources can cause plant disease (13,15,17,23,28,30,42, 44,47,48). The overall goal of this study was to determine whether

Table 5. Average lesion area of wounded sites on *Rhododendron* leaves in the detached leaf assay

Taxa	Lesion area (cm ²) ^y	
	Test I	Test II
<i>Phytophthora</i> sp. BF4	0.50 cd	0.82 bc
<i>Phytophthora pini</i>	1.60 b	1.95 a
<i>P. hydropathica</i>	2.60 a	2.23 a
<i>Phytophthora</i> taxon PgChlamydo	1.05 bc	2.11 a
Noninoculated control ^z	0.04 d	0.07 c
<i>P. heveae</i> (positive control)	1.65 b	1.60 ab

^y Represents the mean lesion area across all replications. Test I and Test II were analyzed separately using SAS 9.3. Values in each column followed by different letters are statistically different from one another according to the Waller-Duncan *k*-ratio means separation, $P = 0.05$.

^z The noninoculated control was a noncolonized corn meal agar plug (3 mm).

Table 3. Recovery of *Phytophthora* isolates from roots using leaf baits (Leaf) and direct root plating (Root)^y

<i>Phytophthora</i> taxa ^z	<i>Rhododendron</i>				<i>Pieris</i>			
	HFL		MHCRS		HFL		MHCRS	
	Leaf	Root	Leaf	Root	Leaf	Root	Leaf	Root
<i>Phytophthora</i> sp. BF4	–	–	–	–	–	–	–	–
<i>Phytophthora pini</i>	++++	+	–	–	++++	+	+	–
<i>P. hydropathica</i>	+	–	–	–	–	–	–	–
<i>Phytophthora</i> taxon PgChlamydo	+	–	–	–	–	–	–	–
<i>P. cinnamomi</i> (positive control)	++	++	NT	NT	NT	NT	NT	NT

^y Plants were inoculated 6 and 8 weeks prior to baiting and direct root plating at the Horticultural Field Lab (HFL) and Mountain Horticultural Crop Research Station (MHCRS), respectively. Four plants were assayed at both locations. Each + represents recovery of *Phytophthora* taxon from one plant, while – represents not recovered and NT = not tested.

^z Nine infested rice grains of each taxon were used to infest individual root zones.

Table 4. Foliar disease assessment of *Rhododendron* and *Pieris* plants inoculated with zoospore or mycelial solutions of four isolates of *Phytophthora* recovered from irrigation water^y

<i>Phytophthora</i> taxa	<i>Rhododendron</i>		<i>Pieris</i>	
	HFL	MHCRS	HFL	MHCRS
<i>Phytophthora</i> sp. BF4 (mycelium)	2.0 bc	1.0 c ($n = 7$)	1.0	1.0 b
<i>Phytophthora pini</i> (zoospores)	3.0 b	4.0 a ($n = 7$)	2.0	3.0 a
<i>P. hydropathica</i> (mycelium)	5.0 a	2.0 b ($n = 7$)	2.0	2.0 b
<i>Phytophthora</i> taxon PgChlamydo (zoospores)	3.0 b	1.0 c	2.0	1.0 b
Noninoculated control ^z	1.0 c	1.0 c ($n = 5$)	2.0 ns	1.0 b

^y Experiments were conducted at the Horticultural Field Lab (HFL) and Mountain Horticultural Crop Research Station (MHCRS) in fall 2012. Foliar disease ratings represent the average of each treatment across eight replications (unless *n* is specified differently) on a visual disease rating scale of 1 to 5, with 5 = dead plant (see text); six *Rhododendron* individuals at MHCRS were thrown out because plants were infected prior to inoculation. Values in each column followed by different letters are statistically different from one another according to the Waller-Duncan *k*-ratio means separations, $P = 0.05$; ns = not significant.

^z The noninoculated control was a diluted nonsterile soil extract at a proportion of 1:4 with a total volume of 200 ml.

disease would develop on ornamental plants irrigated with water naturally infested with *Phytophthora* spp. Similar studies have been conducted with *P. ramorum* and yielded few to no infections on susceptible plants (42, 5). As mentioned previously, Tjosvold et al. (42) found a low incidence of disease over four growing seasons when hybrid *Rhododendron* plants were irrigated with stream water containing *P. ramorum*. In the present study, two nurseries were selected with histories of *Phytophthora* taxa present in irrigation water. The irrigation system at nursery I represented a retention basin that recycled almost total runoff water from the container growing area. *P. hydropathica*, a foliar pathogen of *Rhododendron* (21), had been previously recovered from the retention basin at Nursery I. The irrigation system at nursery II represented a natural irrigation source, the French Broad River, which drains from a forested watershed. *P. pini* had been previously recovered from this river at this location and this species has been documented as a stem, leaf, and root pathogen of *Rhododendron* (5,9,19), as well as many other shrubs and trees.

Isolates recovered from water represented six unique taxa. Four of the six taxa detected were tested for their ability to cause disease on leaves and roots of *Rhododendron* and *Pieris* plants. The four taxa chosen were the most commonly recovered species from irrigation water, *P. hydropathica* and *Phytophthora* taxon PgChlamydo from nursery I and II, respectively; as well as the taxa recovered from both nurseries, *Phytophthora* sp. BF4; and, finally, an isolate representing an aggressive plant pathogen, *P. pini* (5). *P. hydropathica*, *P. pini*, and *Phytophthora* taxon PgChlamydo caused foliar disease with both inoculation assay methods tested but no isolates caused significant root rot when compared with the positive control of *P. cinnamomi*. Benson and Jones (5) demonstrated that isolates of *P. citricola*, now referred to as *P. pini* by Hong et al. (19), caused *Phytophthora* root rot on *Rhododendron* plants (5). However, we found no evidence to suggest that the isolate of *P. pini* recovered in this study caused root rot on *Rhododendron* or *Pieris* spp. Host specificity may be operating with our isolates, because Olson (33) showed differences in host range and aggressiveness with isolates of *P. drechsleri* on floriculture crops. The host of origin of our isolates is unknown because we recovered them from irrigation water.

Both inoculation methods we employed for foliar pathogenicity assays showed that isolates of *P. hydropathica* and *P. pini* were the most aggressive. The zoospore or mycelial spray inoculation method simulated application of *Phytophthora* propagules during sprinkler irrigation at a nursery. With this inoculation method, the isolate of *P. pini* used in this test was consistently the most aggressive across the two trials but isolates of *P. hydropathica* and *Phytophthora* taxon PgChlamydo also caused a significant level of foliar disease when compared with the negative control. Temperature differences, age of the plants, and other factors such as irrigation water quality at the two test locations may have impacted overall differences in disease severity between locations with our isolates of *P. hydropathica* and *P. pini*. The isolate of *Phytophthora* taxon PgChlamydo caused significant disease severity at the HFL trial location with the zoospore or mycelial spray inoculation method. In Minnesota nurseries, Schwingle (37) isolated *Phytophthora* taxon PgChlamydo from leaf lesions on *Rhododendron* and *Taxus* spp. In California nurseries, Blomquist et al. (8) recently demonstrated pathogenicity of *Phytophthora* taxon PgChlamydo to foliage of *Buxus sempervirens*, *Camellia sasanqua*, *Laurus nobilis*, and *Arbutus unedo*.

In the detached leaf assay, all isolates tested produced lesions on the wounded sites on the underside of *Rhododendron* leaves but no isolate produced lesions on the nonwounded sites of the detached *Rhododendron* leaves. Wound sites were made directly before inoculation. The isolate of *Phytophthora* sp. BF4 tested was recovered from the lesion but the lesion size was not significantly different from the noninfested CMA negative control. Blomquist et al. (7) and Denman (14) previously suggested that nonwounded sites on detached *Rhododendron* leaves yielded inconsistent results. Similarly, we found that wounding mature *Rhododendron* leaves

resulted in consistent infection in comparison with nonwounded leaves. Overall, the pathogenicity assays showed that the commonly recovered *Phytophthora* taxa at the two nurseries were capable of infecting foliage. *Phytophthora* sp. BF4 was recovered from wounded sites of detached *Rhododendron* leaves inoculated with this isolate but the lesion size was statistically insignificant compared with the negative control.

Although three of the four *Phytophthora* taxa tested caused foliar infections in the pathogenicity assays, trap plants irrigated with water naturally-infested with *Phytophthora* spp. at the two nurseries yielded a low frequency of only two infections throughout the entire study, representing <1% of the 384 trap plants. One hypothesis explaining the lack of *Phytophthora* infection during the in situ assays involves the zoospore concentration threshold necessary for infection. Mitchell et al. showed a zoospore concentration to infection relationship with *P. cryptogea*, where 20 and 50% of watercress plants were infected when exposed to 50 and 276 zoospores, respectively (32). Similarly, Benson and Jones (5) demonstrated that, when *P. heveae* inoculum was prepared at concentrations of 9 to 32 zoospores/ml (low), 337 to 364 zoospores/ml (moderate), and 1,875 to 5,491 zoospores/ml (high), 0, 40, and 100% of the *Rhododendron* test plants, respectively, developed *Phytophthora* dieback. In this study, the average number of zoospores exiting the sprinkler and falling into each container daily was calculated at 2 (SD = 1.4) and 5 (SD = 5.6) zoospores at nursery I and II, respectively, based on the surface area of the container and the average daily irrigation volume at each nursery. Even assuming a total of 18 zoospores applied per container per day based on the highest estimated amount of zoospores deposited on one assay date at one nursery from the sprinkler, this zoospore concentration is likely too low, even during favorable conditions, to cause infection based on the examples with *P. cryptogea* on watercress (32) and *P. heveae* and *P. ramorum* on *Rhododendron* (5,42). *P. hydropathica*, *P. pini*, and *Phytophthora* taxon PgChlamydo all caused foliar infection of *Rhododendron* and *Pieris* spp. when inoculated at 2,500 zoospores/ml but not at the natural concentrations observed at the two nurseries. Overall, *Phytophthora* sp. BF4 caused negligible disease on *Rhododendron* and *Pieris* spp. in all of the pathogenicity assays and does not appear to be a pathogen of these commonly produced hosts. This suggests that species identification of *Phytophthora* could be an important factor in determining the risks associated with irrigating woody ornamental plants with water naturally infested with *Phytophthora* spp.

Werres (45) observed that *Rhododendron* plants irrigated with water containing *P. ramorum* at an estimated 74 zoospores/ml were able to infect <19% of *Rhododendron* and *Viburnum* test plants. In our study, the concentration of 74 zoospores/ml is three magnitudes greater than the highest concentrations detected at the sprinklers at any sampling date or location. Our data suggests that the practice of using *Phytophthora*-infested irrigation water as a primary water source may not be a direct risk to susceptible *Rhododendron* and *Pieris* spp. unless high levels of inoculum exist and conducive environmental conditions favorable for disease are present. A study conducted by Tjosvold et al. (42) demonstrated very limited disease incidence only when *Rhododendron* plants were irrigated with stream water that had relatively high levels of inoculum and when environmental conditions were conducive for infection in the spring. Consideration should also be given to the *Phytophthora* spp. present in irrigation water and the capability of their zoospores to induce lesions, which can vary among *Phytophthora* spp. and host plants.

Many species of *Phytophthora* that cause dieback, including *P. pini* and *P. ramorum*, have been shown to overwinter and persist as latent root infections until favorable conditions for aboveground infection occur (5,35). Although none of the *Phytophthora* taxa tested caused root infection on *Rhododendron* and *Pieris* spp., three of four taxa were detected in the root zone 6 weeks postinoculation by the *Rhododendron* leaf bait method. The *P. pini* isolate was recovered from 100% of the root balls of asymptomatic plants of *Rhododendron* and *Pieris* baited with *Rhododendron*

leaves at the HFL and from 13% of the *Pieris* and *Rhododendron* root balls at the MHCRS location. *P. hydropathica* and *Phytophthora* taxon PgChlamydo were recovered with *Rhododendron* bait leaves from 13% of plants inoculated with the respective isolate 6 weeks postinoculation at the HFL location. Although these three taxa did not cause root infection, survival in the root zones of asymptomatic plants could be a source of inoculum for foliar infection, and a means of moving *Phytophthora* spp. through the nursery trade. Parke and Lewis (35) demonstrated that asymptomatic root infection in hybrid *Rhododendron* plants by *P. ramorum* present in the potting mix could result in foliar infection as the pathogen colonized asymptomatic vascular tissues in the root system to reach leaves in the foliage.

Overall, low risk was associated with irrigating susceptible *Rhododendron* and *Pieris* plants with water naturally infested with different *Phytophthora* spp. at two woody ornamental nurseries. Even though multiple taxa of *Phytophthora* were consistently recovered from irrigation water throughout the duration of the test during both years at both nurseries, the number of propagules persisting in the irrigation system from the retention basin or river water was most likely too low to cause infection of the trap plants. These data suggest that, under shaded and irrigated conditions, one of the major factors that determine the level of risk associated with using *Phytophthora*-infested irrigation water is the concentration of *Phytophthora* propagules present. This preliminary finding should warrant future studies to evaluate infection threshold concentrations of different species of *Phytophthora* frequently found in irrigation water to determine relative risks to susceptible woody plants.

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