A COMPARISON OF DETECTION AND QUANTIFICATION METHODS FOR

PHYTOPHTHORA RAMORUM IN WATER AND THRESHOLD

LEVELS OF INOCULUM IN IRRIGATION WATER

REQUIRED FOR INFECTION OF

RHODODENDRON LEAVES

by

LUCY ROLLINS

A thesis submitted in partial fulfillment of
the requirements for the degree of

MASTER OF SCIENCE IN PLANT PATHOLOGY

WASHINGTON STATE UNIVERSITY
Department of Plant Pathology

DECEMBER 2014

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To the Faculty of Washington State University:

The members of the Committee appointed to examine the thesis of LUCY ROLLINS find it satisfactory and recommend that it be accepted.

____________________________________
Gary A. Chastagner, Ph.D., Chair

___________________________________
Lori M. Carris, Ph.D.

___________________________________
Dennis A. Johnson, Ph.D.
ACKNOWLEDGEMENTS

I would like to thank Gary Chastagner and Marianne Elliott for making this opportunity available to me and for their guidance along the way. Thank you to Dennis Johnson and Lori Carris for serving on my committee. A special thanks to Katie Coats for her support and teaching in the molecular lab and to all the others in Puyallup who were always so giving of their time and friendship. I would finally like to acknowledge my husband, James Rollins, for his encouragement, love and support over the past several years in helping me achieve an important personal goal.
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Abstract

by Lucy Rollins, M.S.
Washington State University
December 2014

Chair: Gary A. Chastagner

The propagules of Phytophthora ramorum, the causal agent of sudden oak death (SOD) and ramorum blight, have been recovered from streams and irrigation runoff associated with SOD outbreaks in forests and nurseries. The potential for new infections exists if untreated water from infested sources is used for irrigation of P. ramorum hosts. Five detection methods were tested simultaneously using lab and creek water artificially inoculated with P. ramorum zoospores to determine the detection threshold for each method. In creek water, detection thresholds were found to be between 1 and 10 direct plate colony-forming units per liter (DP CFU/liter) for filtration and qPCR methods, between 1 and 35 DP CFU/liter for leaves and leaf disks and between 10 and 35 DP CFU/liter for pear baits. Filtration, qPCR and leaf disk baits were able to quantify P. ramorum zoospores in creek water up to 400 DP CFU/liter; however,
precision was lost at higher inoculum concentrations. Results indicate that filtration, qPCR and leaf disks can be used for both detection and quantification of inoculum while pear and leaf baits were most effective for detection only. The inoculum concentration of *P. ramorum* zoospores needed in irrigation water for infection of nursery hosts was investigated using a novel spray device designed to simulate over-head irrigation similar to that used in nurseries and greenhouses. Inoculum threshold concentrations were found to be 51 zoospores/ml for wounded detached leaves in laboratory assays and between 1,000 and 10,000 zoospores/ml for plant hosts in a simulated nursery study. The pressurization and spraying of *P. ramorum* zoospores out of the device did not appear to affect zoospore viability or infectivity on wounded detached rhododendron leaves, implying the device may be useful in other research involving *P. ramorum* zoospore inoculum. Results of this study will help researchers, government agencies and nursery operators assess the risk of using untreated water infested with *P. ramorum* for over-head irrigation of susceptible hosts.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>iii</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>iv-v</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>viii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>ix</td>
</tr>
<tr>
<td>CHAPTER</td>
<td></td>
</tr>
<tr>
<td>1. LITERATURE REVIEW OF <em>PHYTOPHTHORA RAMORUM</em></td>
<td></td>
</tr>
<tr>
<td>Background</td>
<td>1</td>
</tr>
<tr>
<td>Symptoms and hosts</td>
<td>2</td>
</tr>
<tr>
<td>Taxonomy</td>
<td>4</td>
</tr>
<tr>
<td>Disease biology</td>
<td>6</td>
</tr>
<tr>
<td>Impact</td>
<td>8</td>
</tr>
<tr>
<td>Management</td>
<td>9</td>
</tr>
<tr>
<td>On-going risks and concerns</td>
<td>10</td>
</tr>
<tr>
<td>Detection and quantification methods</td>
<td>11</td>
</tr>
<tr>
<td>Biological threshold</td>
<td>17</td>
</tr>
<tr>
<td>Research objectives</td>
<td>18</td>
</tr>
<tr>
<td>Literature cited</td>
<td>19</td>
</tr>
<tr>
<td>2. COMPARISON OF FIVE DETECTION AND QUANTIFICATION METHODS</td>
<td></td>
</tr>
<tr>
<td>FOR <em>PHYTOPHTHORA RAMORUM</em> IN STREAM AND IRRIGATION WATER</td>
<td></td>
</tr>
<tr>
<td>Introduction</td>
<td>36</td>
</tr>
<tr>
<td>Material and Methods</td>
<td>39</td>
</tr>
</tbody>
</table>
3. INOCULUM THRESHOLD FOR Phytophthora ramorum IN Irrigation Water Required for Infection of Rhododendron Leaves

Introduction 76
Material and Methods 77
Results 86
Discussion 88
Literature Cited 96
LIST OF TABLES

1. Water quality measurements for lab and field water used for simultaneous
detection methods testing and from field samples ........................................... 65

2. Summary statistics for the correlation between direct plate colony-forming
units/liter of lab or creek water and the number of filtration CFU, zoospore
DNA, positive leaf disks and pear or leaf lesions caused by P. ramorum .......... 66

3. A comparison of P. ramorum zoospore quantification methods for trials
using creek water ............................................................................................. 67

4. Number of colonies identified as either belonging to Phytophthora and
Pythium genus from 1 liter field samples using BOB, filtration and qPCR
recovery methods ............................................................................................ 68
LIST OF FIGURES

1. The relationship between filtration and direct plate CFU/liter for the recovery of *P. ramorum* zoospores for trials using lab and creek water .......... 69

2. The relationship between the amount of recovered DNA (ng)/liter from *P. ramorum* zoospores trapped on a filter and direct plate CFU/liter for trials using lab and creek water .................................................. 70

3. The relationship between pear lesions/liter and direct plate CFU/liter for the recovery of *P. ramorum* zoospores for trials using lab and creek water .......... 71

4. The relationship between colonized leaf disks (BOB)/liter and direct plate CFU/liter for the recovery of *P. ramorum* zoospores for trials using lab and creek water................................................................. 72

5. The relationship between leaf lesions (BOB)/liter and direct plate CFU/liter for the recovery of *P. ramorum* zoospores for trials using lab and creek water 73

6. The relationship between leaf lesions/liter and direct plate CFU/liter for the recovery of *P. ramorum* zoospores for trials using lab and creek water............. 74

7. Measured-Inoculum Spray applicator (MISA) diagram and materials list .......... 103

8. In the simulated nursery study conducted at NORS-DUC, *P. ramorum* zoospores of various concentrations were applied to the foliage of rhododendron, camellia and viburnum plants using a MISA to simulate overhead irrigation ................................................................. 104

9. Precipitation, maximum and minimum daily temperatures and disease incidence during the 10 week simulated nursery study conducted at NORS-DUC ................................................................. 105
10. The survival of *P. ramorum* zoospores in a non-pressurized and pressurized zoospore suspension (35, 210, 420 and 630 kPa) as measured by the number of germinating zoospores (CFU/ml) ................................................................. 106

11. Results of an assay testing the effect of various air pressures on zoospore infectivity and the results of an assay conducted to determine the inoculum threshold for infection of wounded detached rhododendron leaves ..................... 107

12. A linear regression between the log_{10} transformed variables *P. ramorum* zoospores/ml and the number of lesions/set of ten leaves used to estimate an inoculum threshold density of 51 zoospores/ml for infection on detached *Rhododendron* x ‘Nova Zembla’ leaves ...................................................... 108

13. Direct plating and filtration methods were used to verify *P. ramorum* zoospore density in three suspensions used to inoculate potted rhododendron, viburnum and camellia plants in a simulated nursery study ................................................. 109

14. qPCR and BOB methods were used to verify *P. ramorum* zoospore density in three suspensions used to inoculate potted rhododendron, viburnum and camellia plants in a simulated nursery study......................................................... 110
Phytophthora ramorum Werres, De Cock & Man in’t Veld, the causal agent of sudden oak death (SOD) and ramorum blight, has over one hundred hosts in over forty genera including woodland trees, understory shrubs and woody ornamental nursery stock in North America and Europe (103). Symptoms of P. ramorum vary among hosts and may include bleeding bole cankers, foliar lesions and twig dieback (80, 114). While P. ramorum primarily infects above ground parts of its hosts, P. ramorum also is able to cause root infections and minor root rots on some hosts (72, 83, 92, 117).

Background

The first known incident of disease caused by P. ramorum occurred in 1993 in Germany and the Netherlands when an unidentified Phytophthora species was found in association with a new stem and leaf blight on ornamental rhododendron (Rhododendron spp.) and viburnum (Viburnum spp.) (113, 114). Beginning in 1994-95, a previously undescribed disease given the common name ‘sudden oak death’ was first noticed causing widespread mortality on tanoak (Lithocarpus densiflorus), California black oak (Quercus kelloggii) and coast live oak (Q. agrifolia) in the San Francisco Bay Area (31, 62). ITS sequencing and culture morphology revealed both diseases were caused by P. ramorum, formally described as a new Phytophthora species in 2001 (80, 114). Currently, SOD continues to cause mortality of tanoak and oak (Quercus spp.) in forests in fifteen counties along the California coast and in Curry County, Oregon, near the California border (9, 56). Ramorum blight has been found on nursery stock in Canada and twenty states throughout the U.S., especially in Washington, California and Oregon, triggering the creation of federal and state quarantine regulations that govern the movement of
host nursery stock and other products made from host materials (102). *P. ramorum* propagules have been detected in streams associated with positive forest and nursery sites, in some cases years after the pathogen was eradicated from the source (20, 87). The United States Department of Agriculture-Forest Service (USDA-FS) 2013 National *Phytophthora ramorum* Early Detection Survey of Forests identified sixteen positive waterways in the U.S., including five sites in California and Washington where *P. ramorum* was detected for the first time (69).

*P. ramorum* has been detected in at least twenty-two European countries, spread largely through the movement of infected nursery stock (27). In the United Kingdom, *P. ramorum* has moved from nurseries to public parks and gardens, woodlands and more recently heathlands where the pathogen causes ramorum blight on rhododendron, camellia (*Camellia* spp.), viburnum and wild bilberry (*Vaccinium myrtillus*) (55). Initially, tree infections of SOD were rare, found mainly on beech (*Fagus sylvatica*), sweet chestnut (*Castanea sativa*) and non-native oaks (6, 111). In 2009, *P. ramorum* was found in association with widespread mortality and dieback of mature and juvenile plantation-grown Japanese larch (*Larix kaempferi*) in southwest England (111). New SOD infections were found on larch in Wales, Northern Ireland and Scotland in 2010 and 2011(6, 27). As of October 2013, 16,000 hectares of larch trees in the U.K. have been felled in response to SOD (27, 100).

**Symptoms and hosts**

*P. ramorum* symptoms can be divided into two broad categories, bole or trunk cankers on hosts with SOD and leaf spotting with or without twig dieback on hosts with ramorum blight (39). Oak, tanoak and Japanese larch with SOD form cankers on the lower trunk, from the soil line up to approximately 20 m above the soil line (80, 111). The outer bark over the canker turns
a red-brown to blackish color and exudes a dark red sap. The bark is often intact but may crack in later stages of the disease (74). When the outer bark is removed, areas of discolored inner bark (phloem) outlined by a black margin are revealed (80). Cankers range in size from a few centimeters to over 3 m in length (74) and tree girdling occurs as the pathogen colonizes and destroys the vascular cambium layer. *P. ramorum* can colonize the sapwood (xylem) restricting water transport, resulting in the browning and wilting of the tree canopy (31, 80). While this process can occur rapidly, within 2 to 4 weeks, it can take two or more years for a tree to die after the initial infection, during which time little or no foliar symptoms may occur (79). Tanoak and Japanese larch can display both stem cankers and leaf or needle necrosis and shoot tip dieback (19, 111).

Most *P. ramorum* hosts get the disease ramorum blight, which is usually non-lethal. The expression of symptom type depends on the host and can include dark discoloration on stems, shoot-tip dieback, cankers on small branches, needle discoloration, foliar lesions and spots that sometimes appear to be water-soaked (19, 29, 114). Foliar lesions can occur along the petiole and mid-rib, leaf tip or in random locations along the entire leaf surface (8). Hosts include several species of ornamental and native trees and shrubs and some herbaceous plants (19, 29, 80, 114). An key native host of *P. ramorum*, California bay laurel (*Umbellularia californica*), develop foliar lesions, usually at the leaf tip, which produce abundant sporangia and chlamydospores (8) and act as inoculum reservoirs for *P. ramorum*, contributing to the spread of SOD in California forests (18, 20, 29). Tan oak (81) and *Rhododendron ponticum* (3, 21, 81) are important sporulating hosts of *P. ramorum* in Oregon and the U.K., respectively.
**Taxonomy**

*P. ramorum* classification according to MycoBank (68) is as follows: Kingdom Chromista (Stramenopila); division Oomycota; class Oomycetes; order Pythiales (Peronosporales); family Pythiaceae; genus *Phytophthora*; species *ramorum*. Oomycetes are fungal-like organisms related to brown algae, golden-brown algae and diatoms (23) that produce asexual and sexual spores, display filamentous growth and can be obligate plant parasites like fungi; but, unlike fungi, Oomycetes have cellulosic cell walls, coenocytic hyphae, a diploid life cycle and biflagellate motile zoospores (23, 44). The Pythiales include the well known plant pathogen genera *Phytophthora* and *Pythium* that are adapted to live part of their life cycle in free water found in soil and on plant foliage (23). Both genera contain species that cause rots of the lower stems and tubers, roots, fruits, crowns and foliage (23). Within *Phytophthora*, *P. ramorum* is placed in clade 8c (2) with *P. lateralis*, the cause of root and crown rot of Port-Orford-cedar (*Chamaecyparis lawsoniana*) (36, 42); *P. hibernalis*, the cause of brown rot on citrus (5); and *P. foliorum*, a pathogen that causes leaf blight of azalea (90).

Morphological characteristics can be used to identify *P. ramorum* growing on media and include coralloid, highly branched hyphae and large, abundant thin-walled chlamydospores that turn golden-brown when growing near plant tissue (114). Chlamydospores have an average diameter of 50 µm (94) and form intercalarily, terminally and sometimes laterally (74). Sporangia, borne singly or in clusters, are elongate, ellipsoid, semipapillate and caducous with a short to nonexistent pedicel and measure 25 to 97 x 14 to 34 µm (length x width) (114). Optimal temperatures for growth on V8 media occurs between 16 to 26 °C (114). *P. ramorum* is heterothallic, requiring mating types A1 and A2 for sexual reproduction (4, 114). Oospores are plerotic with an average diameter of 30 µm. Terminal oogonia are smooth and spherical and
range in size from 24 to 40 µm and antheridia are barrel-shaped and amphigynous (114). Mating type A1 is most commonly found in nurseries, gardens and woodlands in Europe and in some nurseries in the U.S. while mating type A2 is primarily found in forests and nurseries in the U.S. (39). While sexual reproduction is possible in areas where both mating types occur, the formation of oospores has only been seen within a laboratory setting and the role of oospores in the disease cycle is not well understood (4, 112).

Currently, there are four known clonal lineages of *P. ramorum* distinguished from one another through molecular marker analysis (54, 107). Their informal designations are based on the location of their initial outbreak and order of appearance (37). The North America lineage, NA1 (mating type A2) has been found in California and southwestern Oregon coastal forests, west coast nurseries and in nurseries in a number of other states associated with the shipment of infected nursery stock received from west coast nurseries (34,54); NA2 (mating type A2) has been found less frequently in nurseries in the U.S. and British Columbia (35,54). The European lineage EU1 (mating type A1) has been found in both Europe and western U.S. nurseries as well as in European parks, gardens and woodlands (38). While mating type A1 is most commonly found, isolates of EU1 of mating type A2 have been found in Belgium in 2002 and 2003 (108). A second European lineage, EU2 (mating type A1), was recently identified on four hosts including Japanese larch in Northern Ireland and western Scotland (65,107). The geographic origin of *P. ramorum* is not known; however, distinct genetic lineages, phenotypic variations in colony growth, mating type distribution, stability and aggressiveness between lineages suggest independent introduction occurred in the U.S. and in Europe (53, 99, 112).
Disease biology

The disease cycle of *P. ramorum* is polycyclic and similar to that of other aerial dispersed *Phytophthora* species (74). The disease cycle can be broadly defined as beginning with the production of inoculum (sporangia) on infected host leaves and twigs, followed by the dispersal of inoculum by rain splash and wind-blown rain to a new host. At temperatures of 25°C, direct germination of sporangia occurs with the formation of a germ tube (18, 74). At temperatures between 15 to 20°C, sporangia produce abundant zoospores which are released onto the surface of wet leaves. The zoospores penetrate and colonize host tissue to begin a new infection process. Chlamydospores are produced in plant tissue and are thought to serve as survival structures, serving as an inoculum reservoir during times not conducive to disease development (39, 74).

Zoospores possess two flagella, one anterior and one posterior that enable the zoospore to move through water (23, 44). Zoospores are chemotactically and electrotactically attracted to favorable infection sites such as a stoma or wound (78, 98). Once at a favorable site, zoospores encyst, a 5 to 10 min process that involves flagella detachment, the formation of a cellulosic cell wall and attachment to the plant surface (43, 44). Germination begins within 20 to 30 min after zoospore encystment with the formation of a germ tube that penetrates and colonizes the leaf tissue. *P. ramorum* is hemibiotrophic, acquiring its nutrition from plant tissue both through biotrophic and necrotrophic processes (99).

Although rain splash transmits sporangia locally, Davidson et al. (20) found wind-blown rain dispersed *P. ramorum* sporangia up to 15 m in a California forest and transmission distances of sporangia by air currents have been reported up to 4 km in southwest Oregon (40). Propagules of *P. ramorum* are dispersed long distances in streams, reportedly up to 4 to 6 km (20), and through the movement of infected nursery stock and contaminated soil (28). *P. ramorum* spreads
to new hosts within a nursery by way of rain puddles, standing water, rain splash, infested growing media, plant-to-plant contact and by overhead irrigation with contaminated water (74).

Originally considered primarily a foliar pathogen (39, 114), *P. ramorum* has been shown to infect the roots of both nursery (92) and forest hosts (26, 117). Parke et al. demonstrated *P. ramorum* had the ability to infect the roots of tanoak seedlings growing in a California forest (73) and the roots of lab-grown *Rhododendron* cv. ‘Nova Zembla’ and Pacific rhododendron (*Rhododendron macrophyllum*) (72). Colburn et al. (14, 15) isolated *P. ramorum* from symptomless rhododendron 4 weeks after inoculation of soil with chlamydospores. The soil phase of the *P. ramorum* disease cycle is not well understood; however, the lack of root symptoms found in some infected hosts suggests the soil phase may be connected to pathogen survival (109).

Davidson et al. (20) found *P. ramorum* sporulation to be seasonal in California forests where high levels of rainfall in winter and spring were generally followed by high levels of disease. Furthermore, host production of sporangia occurred when temperatures were between 18 and 22°C but was absent during hot, dry summers. California forest streams have shown a similar seasonal pattern reflecting the propagule density of *P. ramorum*. Inoculum levels were highest during winter and spring and lower during the summer months (20, 66, 91, 93). In contrast, Sutton et al. (87) found higher densities of inoculum in Oregon streams during the summer. They suggest Oregon’s moderate temperatures allow for year-round sporulation, thus winter inoculum levels appear to be lower relative to summer because inoculum densities are diluted by high winter stream flows.
Impact

Diseases caused by *P. ramorum* have had a significant impact on forests, public spaces and nurseries in North America and Europe. An estimated 3 million tanoak and oak trees in coastal forests of California have been killed by SOD since the mid 1990s (9). While long-term impacts of SOD on the forest landscape will become more apparent over time, high losses of oak and tanoak in redwood (*Sequoia sempervirens*) and mixed evergreen forests along coastal California have been linked to changes in forest stand structure and species composition (63, 64). Other significant ecological effects include loss of wildlife habitat and food sources, increased soil erosion, decreased soil moisture levels and increased fuel loads (57). Increased fuel loads have been linked to an increase in canopy fire severity in redwood crowns and higher redwood die-off rates (10). Regulations and restrictions aimed at preventing the spread of *P. ramorum* in recreational areas can have a negative effect on local tourism and are costly for park and forest officials to implement (57).

Many nursery and landscape operations have been financially impacted in areas where *P. ramorum* has become established (28). When *P. ramorum* is found within a nursery, federal and state regulatory actions designed to prevent the interstate movement of infected plant and plant material are triggered (102). Nursery operators must destroy and dispose of infected and at-risk plants at their own expense while holding additional plants for a 90 day observation period (28, 102). Washington nursery owners lost an estimated retail value of US $423,043 in destroyed plants alone during 2004 and 2005 (17). Additional money may need to be spent for soil fumigation, improvements to drainage systems, staff education and the implementation of other best management practices (28).
Management

Management strategies for *P. ramorum* diseases in U.S. and European forests and nurseries focus on early detection, eradication, containment and avoidance. Early detection methods aim to find outbreaks quickly and include aerial and ground surveys, stream baiting in high-risk areas and regular nursery inspections of plant material (74). Eradication, the rapid removal of diseased and at risk hosts within designated treatment areas, was initially attempted in Oregon, where the SOD outbreak is limited to forests in Curry County. Hosts within a designated quarantine area and buffer zone were cut and burned (81). Currently, Oregon has shifted its goal of complete eradication to one that strives to slow disease spread to new areas (56). SOD is managed through containment in California, where the focus is to limiting the spread of disease through the removal of infected tanoak and bay laurel (39). Additional attention is given to lowering the risk of wildfire by removing standing dead tanoak (28). California homeowners have the option of treating high-value trees with phosphite fungicide (Agrifos with Pentrabark, Agrichem, Medina, Ohio) to help prevent infection (30). In the U. K., sporulating hosts such as *R. ponticum* and Japanese larch are eliminated from infected and high-risk sites and infected larch plantation trees are cut and processed at authorized facilities to lower the risk of accidental spread (27).

Nurseries in the U.S. undergo government inspections, testing and certification procedures in an effort to avoid the spread of *P. ramorum* through infected nursery stock and related materials. In the U.K., the movement of high-risk hosts, camellia, rhododendron and viburnum, require a plant passport that certifies the plant was produced in a pathogen-free facility and is pathogen-free (88). Animal and Plant Health Inspection Service (APHIS) revised the *P. ramorum* domestic quarantine regulations for interstate movement of host nursery stock in
March 2014, relaxing the regulatory requirements for nurseries within quarantine and regulated areas of Washington, California and Oregon where \( P. \text{ramorum} \) is consistently absent. Additionally, APHIS regulations now apply to nurseries outside regulated states that ship \( P. \text{ramorum} \) host plants in the event the nursery tests positive for \( P. \text{ramorum} \). Nurseries with positive detections undergo additional sampling for \( P. \text{ramorum} \) from sources such as soil, water and growing media and must agree to eliminate \( P. \text{ramorum} \) from these sources if found to exist (106).

**On-going risks and concerns**

Despite state and federal government management efforts, \( P. \text{ramorum} \) continues to spread. In 2013, the quarantine area in Curry County, Oregon was expanded 60 miles to include new infestations found outside the existing quarantine area (56) while 10,000 additional hectares of infected larch were identified in the U.K. (100). Additionally, \( P. \text{ramorum} \) was found in eighteen nurseries located in California, Oregon, Washington and New York, all of which had prior \( P. \text{ramorum} \) infestations (82), and in sixteen waterways, including five first-time detections in Washington, California and Texas (69).

In the U.S., \( P. \text{ramorum} \) has not been detected in forests outside California and Oregon; however, establishment of \( P. \text{ramorum} \) in eastern U.S. forests is plausible. Many oak species and understory plants are confirmed hosts of \( P. \text{ramorum} \) (94, 97) and the climate could support \( P. \text{ramorum} \) growth (52). \( P. \text{ramorum} \) has been found in some eastern U.S. nurseries and associated streams, brought to the area on infected nursery stock (28, 110). While federal regulations are in place to help prevent the movement of infected nursery stock into eastern states, \( P. \text{ramorum} \) has been found on asymptomatic roots (26) and foliage (22), and has been shown to survive in
potting mix up to 12 months as chlamydospores (58). It may be possible to unknowingly ship infected plants to eastern U.S. nurseries where the infected plants could be planted in landscapes in the urban-forest interface areas.

There is concern that *P. ramorum* could spread from infested waterways to riparian, nursery or landscape plants either by a flood event or through the use of infested water for irrigation. There have been two incidences where *P. ramorum* has been recovered from vegetation growing along an infested waterway. The first occurred in December 2007 and February 2008 where *P. ramorum* was found on vegetation growing adjacent to an infested drainage ditch outside a Mississippi nursery (12). The second occurred in Washington State in 2009 where salal (*Gaultheria shallon*) growing along a drainage ditch developed ramorum blight after the salal came into contact with infested water during a flood event. The following year, infested soil was found in the same area (13). To date, streamside vegetation surveys at these and other sites have always been negative. Nursery owners often collect stream water or recycle run-off water to use for irrigation. Water from these sources has been shown to be infested with many *Phytophthora* and *Pythium* spp. including *P. ramorum* (7, 50, 76, 115). Repeated use of infested water for irrigation has been shown to cause disease in container crops over time (59). Werres et al. (115) found that water infested with *P. ramorum* caused infection on *Rhododendron* and *Viburnum* spp. in a simulated overhead irrigation study.

**Detection and quantification methods**

Managing the risk of spreading *P. ramorum* to hosts through contact with infested water involves the confirmation of the pathogen in the water source. Common detection methods for finding *P. ramorum* in streams and nursery runoff include the use of baits and filtration (86).
Each method has a detection threshold, the minimum number of propagules that the method can
detect. Knowing the detection threshold of the method will help determine how many samples to
take and what time of year is the best time to collect them (47). Knowing how much of the
pathogen is present in the water is important for disease management because the amount of
inoculum required to initiate disease varies by host. Some detection methods can also be used to
quantify the amount of pathogen propagules in the water (47). Because each method only
captures a portion of the actual population (33), the ratio of the captured population to the actual
population is an important consideration.

Baiting involves placing host material such as leaves or fruits in irrigation or stream
water to expose the bait to the pathogen, which is chemically attracted to the bait. Colonized
plant tissues are plated on selective media or molecular techniques are performed to confirm
infection of the bait by *P. ramorum*. Unripe D’Anjou pears have been used as bait for the
detection of *Phytophthora* spp., including *P. ramorum*, in streams (32, 93) and irrigation water
(61, 118). Pears are floated two to three days in infested water, either in situ or in vitro, removed
from the water, and incubated at 20°C until lesions form on the pear surface. Lesions caused by
*Phytophthora* spp. are chocolate brown, firm, often diffuse, and in a rosette pattern and can be
distinguished from those caused by *Pythium* spp. which are soft, not diffuse and sunken (93,
118). When baiting is done in vitro, water volume and temperatures can be standardized (93).
Pear baiting is relatively easy to do and could be used by nursery operators to monitor streams or
irrigation water; however, pears are more costly to use than leaf baits and break down much
faster than leaves (25, 93). Tjosvold et al. (93) used D’Anjou pears to measure *P. ramorum*
density in an infested stream and found 2.5 *P. ramorum* lesions per pear in 1 liter of stream
water. While pear baiting can be done year-round, the highest recovery of *P. ramorum* in streams was found to occur March through May, following the end of the rainy season (20, 93).

Whole plants, detached whole leaves and leaf disks have been successfully used to detect pathogenic *Phytophthora* spp. in horticultural irrigation systems (7, 33, 89) and *P. ramorum* in streams (70). Streams running through or adjacent to forests infested with *P. ramorum* and waterways associated with nurseries that have had outbreaks of ramorum blight are monitored using leaf baits as part of a national survey for early detection of *P. ramorum* (28). APHIS standard baiting protocol for water sampling (105) for detection of *P. ramorum* includes the use of bait bags (in situ) and the Bottle O’ Bait, or ‘BOB’ (in vitro) methods. APHIS water sampling protocols for baiting methods are briefly described as follows:

Bait bags are made from plastic window screen and contain pockets that hold four to eight leaves from healthy native or naturalized rhododendron, camellia or viburnum. Bait bags are placed at or near the water surface in an area where the water is relatively calm and secured with rope to prevent the bag from floating away. The bags are left in the stream either 7 or 14 days, depending upon water temperature. Bait bags work best when water temperatures are between 8 and 22°C (105). At temperatures above 22°C, leaves within the mesh bag can start to decompose. After retrieval of the bags, leaves are rinsed and incubated 7 to 14 days at 20°C to allow for the formation of lesions. Pieces of symptomatic tissue are removed and plated onto PARPH-V8 media (one-third V8 agar amended with 10 mg Delvocid (50% pimaricin), 250 mg sodium ampicillin, 10 mg Rifamycin-SV (sodium salt), 66.7 mg Terraclor (75% PCNB) and 50 mg Hymexazol). Colonies that grow on the plate can be identified morphologically and tested using APHIS-approved PCR protocols to confirm infection by *P. ramorum*. Whole leaf baits have been used successfully to detect *Phytophthora* spp. (33,77) and *P. ramorum* (67, 70, 77, 87).
in streams, irrigation systems and other watercourses. Rhododendron and camellia whole leaf baits were shown to detect more *Phytophthora* spp. than leaf disks (33); however, whole leaves generally cannot be used for quantification (48). Whole leaf baits were shown to be able to recover *P. ramorum* propagules during an entire year in areas where *P. ramorum* was well established (87); however, the highest rate of recovery in western (66, 93) and southeastern (51, 71) U.S. states has been found to be from late winter through spring.

The BOB method involves collecting stream or irrigation water in a one-liter plastic bottle, placing one whole native or naturalized rhododendron leaf and 20 leaf disks in the bottle and incubating the bottle and leaf material for three days at 20°C. The leaf disks are removed and immediately plated onto PARPH-V8 media. The leaf is removed and receives additional incubating in a moist chamber at 20°C, after which sections of symptomatic leaf tissue are removed and plated. Colonies are identified as described above. The number of water samples taken depends on the size of the nursery and the number of irrigation and drainage sources. Leaf disk have been used to detect *Phytophthora* spp. (7, 33) including *P. ramorum* (1, 70, 71). Oak et al. (71) found that whole leaf baiting and BOB gave similar results in the recovery of *P. ramorum* in eastern U.S. waterways. Whole leaf baiting and the BOB method had an 88% and an 83% detection rate, respectively (1). Unlike bait bags, the BOB method can be used in ephemeral streams, requires one trip to the sampling site rather than two, can be used when water temperatures exceed 22°C and the risk of losing bags is eliminated (71, 105). A disadvantage to using the BOB method is that fast-growing *Pythium* and *Phytophthora* spp. can colonize leaf disks before *P. ramorum*, preventing detection (24).

Filtration can be used for detection of Pythiaceous species and is capable of providing quantitative data on inoculum density (7, 48). APHIS protocols (105) for water sampling using
filtration are briefly described next. A 1-liter plastic bottle is filled by taking ten 100-ml samples of stream or irrigation water. Ten 100-ml aliquots are vacuum-filtered through ten polycarbonate membrane filters with either 3 or 5-µm pores, depending on water turbidity. The filters are plated directly onto PARPH-V8 selective media, incubated at 20°C for 2 to 3 days, after which the filters are removed, the agar is rinsed and the plates are visually inspected for *P. ramorum* colonies. Plates should be checked at regular intervals for colonies displaying morphological characteristics of *P. ramorum*. Colonies can be positively identified following the APHIS-approved PCR protocols (101). Filtration has been used to quantify *Phytophthora* spp. in nursery effluent (7, 60) and in streams (7, 51, 77). Reeser et al. (77) used filtration to quantify the total *Phytophthora* spp. recovered biweekly in two Oregon streams during April through June of 2006 and found between 0 and 185 CFU/liter. Oak et al. (70) found *P. ramorum* inoculum densities ranged between 1 and 130 CFU/liter in samples taken from streams. Filtration was found to be better than whole leaf baits for the detection of *Phytophthora* spp. in streams (48); however, turbid water samples may clog the membrane filter and colonies can be difficult to count accurately because they are in close proximity to one another on the filter (48).

When designed properly, polymerase chain reaction (PCR)-based assays offer a fast, specific and sensitive method for detection of plant pathogens in host material, soil and water sources (47). PCR assays have an advantage over other detection methods in that they are able to detect low populations of target pathogens. Molecular detection has expanded to include the use of quantitative PCR (qPCR) assays that employ traditional PCR primers as well as either Sybr green chemistry, or TaqMan chemistry that includes a sequence-specific fluorescent probe (16, 45). As the target DNA is amplified in the qPCR reaction, fluorescence increases proportionally and is recorded by the instrument. qPCR data is presented as a cycle threshold
(Cₜ) value representing the PCR cycle number at which the fluorescence signal exceeds a designated intensity. Low Cₜ values represent higher levels of pathogen DNA, because they are able to reach the threshold at an earlier PCR cycle.

Hughes et al. (49) developed P. ramorum-specific primers in the internal transcribed spacer (ITS) region of the nuclear ribosomal gene for a TaqMan real-time PCR assay. The APHIS protocols (104), based on the Hughes assay, calls for the use of primers Pram-114F and Pram-1527-190R, probe Pram-1527-134-T, one P. ramorum positive DNA control and one non-template control. A sample is considered positive for P. ramorum if the Cₜ value is between 14 and 36, negative if the Cₜ value is 0 and inconclusive if the Cₜ value is in the range of 36 to 46 and the positive and negative controls give the expected results. Samples with values lower than 14 are possible but unlikely and should be rerun. Development of a sensitive qPCR assay, such as the one to detect P. capsici in soil should investigate the relationship between the number of oospores in artificially infested soil and the quantity of pathogen DNA recovered by qPCR (75). qPCR assays are faster to perform, offer increased sensitivity and specificity and decreased risk of cross contamination when compared to conventional PCR assays (49). A caveat of PCR-based assays in general is that they do not distinguish between DNA from viable versus non-viable pathogens, and therefore the pathogen density may be over-inflated (45, 49). Samples taken from plant tissue, soil and water may also contain inhibitors such as microbes, chemicals and organic contaminants that mask pathogen DNA and lead to false negatives. Cycle cut off values, above which a Cₜ value would represent a negative sample, should be determined for each assay in order to lower the risk of obtaining false positive or false negative results (11).
**Biological threshold**

The biological threshold of a pathogen refers to the minimum number of infective propagules required to cause disease and is a key management consideration for nursery managers who use untreated stream or recycled water for irrigation (47). If irrigation water is infested but the quantity of infective propagules is below the biological threshold of the pathogen, the risk of subsequent plant infection may be lowered; however, the biological threshold of a pathogen is influenced by factors such as irrigation method (85), irrigation frequency (46), growing media (84) and the mix of other pathogens and microbes in the water (47). When biological threshold testing is performed for *Phytophthora* spp. in research, inoculum suspensions of zoospores or sporangia are typically prepared at various concentrations and applied to plant material by placing drops of suspension onto wounded or non-wounded leaves (116), dipping detached leaves (95) or whole plants (41) into the inoculum suspension, spraying leaves with an inoculum suspension to simulate overhead irrigation (115) or by applying the inoculum suspension to the soil to simulate drip irrigation (46).

Biological threshold testing has been done for eastern U.S. forest (96) and western U.S. coastal forest hosts (41, 115). Tooley et al. (96) dipped detached leaves of *Quercus prinus* (Chestnut oak), *Q. rubra* (Northern red oak), *Acer rubrum* (red maple), *Kalmia latifolia* (mountain laurel) and *Rhododendron* ‘Cunningham’s White’ into sporangia suspensions of various concentrations and recorded the number of diseased leaves that developed. Threshold estimates for 50% infected leaves ranged between 36 and 750 sporangia/ml for the hosts. Hansen et al. (41) dip-inoculated 1 and 4-month-old detached leaves of tanoak and California bay laurel into zoospore suspensions and visually assessed foliar necrosis after 7 days. Workers found the tanoak leaves developed leaf necrosis at an inoculum concentration of 860 zoospores/ml and the
1 and 4-month-old California bay laurel leaves developed necrosis at 8,600 and 86,000 zoospores/ml, respectively. Werres et al. (115) infested water reservoirs with *P. ramorum* and used the water for over-head irrigation of rhododendron and viburnum container plants over a full growing season in a simulated nursery study. Workers found the maximum amount of infection on rhododendron to be less than 19% at an inoculum concentration of 74 zoospores/ml.

**Research objectives**

The objectives of this research were to: (i) test five detection methods simultaneously using the same zoospore suspension to determine the detection threshold concentration of zoospores for each method. Methods tested were pear baiting, whole leaf baiting, Bottle O’ Bait-BOB, filtration and TaqMan Real-time (quantitative or q) PCR. Methods were tested using autoclaved deionized water, non-infested creek water and water from two nursery retention ponds; (ii) determine if the detection methods could provide quantification data on inoculum density; (iii) assess the biological threshold of *P. ramorum* in irrigation water needed for infection of detached wounded Rhododendron x ‘Nova Zembla’ leaves.
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CHAPTER 2

Comparison of five detection and quantification methods for *Phytophthora ramorum* in stream and irrigation water

ABSTRACT

Propagules of *Phytophthora ramorum*, the causal agent of sudden oak death (SOD) and ramorum blight, can be recovered from infested stream and nursery irrigation runoff using baiting and filtration methods. Five detection methods, including pear and rhododendron leaf baits, Bottle O’ Bait (BOB), filtration and qPCR performed on zoospores trapped on a filter were compared simultaneously in laboratory assays using lab or creek water spiked with known quantities of *P. ramorum* zoospores to determine the detection threshold for each method and to verify which methods could be used to quantify inoculum density. In creek water, filtration and qPCR methods had the lowest detection thresholds: between 1 and 10 direct plate colony-forming units per liter (DP CFU/liter); leaves and leaf disks had detection thresholds between 1 and 35 DP CFU/liter while pear baits had the highest detection threshold, between 10 to 35 DP CFU/liter. Filtration, qPCR and leaf disks were able to quantify *P. ramorum* zoospores ranging from 1 to 400 DP CFU/liter; however, precision was lost at higher inoculum concentrations. The ability to detect and quantify *P. ramorum* inoculum in water will assist scientists, regulatory agencies and nursery personnel in assessing the risk of using untreated infested water for irrigation.

INTRODUCTION

Nurseries collect and reuse effluent as a way to conserve water and to prevent the discharge of pollutants into the surrounding environment. Effluent can be supplemented with
surface water taken from nearby rivers and streams and then used for irrigation. Both sources have been shown to contain plant pathogenic species of the genera *Phytophthora* and *Pythium* (1, 29, 30). Pythiaceous species are well adapted to survive in water, which is required for dispersal as well as for the formation of sporangia and motile zoospores, the primary propagules for the initiation of new infections. Nursery managers need reliable ways to detect and quantify pathogenic Pythiaceous species in irrigation water before its use on plant stock to mitigate the risk of spreading disease through the use of contaminated water.

*Phytophthora ramorum* is the causal agent of sudden oak death (SOD) on tanoak (*Lithocarpus densiflorus*), oak (*Quercus* spp.), and Japanese larch (*Larix kaempferi*) and ramorum blight on native and nursery hosts such as rhododendron and viburnum (40, 50, 51). While *P. ramorum* is described as primarily a foliar pathogen that is dispersed to new hosts via airborne sporangia, *P. ramorum* propagules have been shown to spread long distances in streams that run through areas with high incidences of SOD (8). Early detection of *P. ramorum* in forests is a vital management tool. Detection methods include aerial and ground surveys and stream monitoring to detect *P. ramorum* propagules in water. A positive detection in a stream signals an infestation somewhere upstream and can trigger intensive ground surveys to locate infected hosts (44). Effluent in retention ponds, streams and drainage ditches are monitored for *P. ramorum* in nurseries where the pathogen has been detected on container-grown plants. In addition to the risk of using *P. ramorum* infested water for irrigation of nursery hosts, there is concern the pathogen will move off-site in runoff water and become established in adjoining forests and natural areas (25).

As with irrigation water, stream monitoring requires effective methods for the detection and quantification of *P. ramorum*. Baiting and filtration are commonly used for the detection of
*Phytophthora* species in both systems (17). Baits used for the detection of *P. ramorum* include D’Anjou pears (8, 45, 46), whole leaves (3, 8, 32, 44) and leaf disks (1, 12, 45) of *Rhododendron* spp. as well as leaves from other *P. ramorum* hosts (39). Stream monitoring conducted as part of the United States Department of Agriculture-Forest Service (USDA FS) 2013 National *Phytophthora ramorum* Early Detection Survey of Forests used leaf baits to detected *P. ramorum* in sixteen waterways, including five first-time detections in Washington, California and Texas (34). Monitoring was conducted by means of an in situ baiting method using whole rhododendron leaves placed in plastic mesh leaf bags which were left in the stream for 1 to 2 weeks or by a relatively new in vitro method, Bottle O’ Bait (BOB), where water samples were collected from streams in plastic bottles, baited with rhododendron leaves and leaf disks and taken to a lab for incubation and identification (35).

Filtration has been used for detection of *Phytophthora* spp. in nursery settings (1, 18, 19, 29) and in forest streams (4, 21, 23, 24, 39) and has been used to quantify *P. ramorum* in rainwater (7) and streams (22). Quantifying propagule density enables the calculation of disease threshold levels in irrigation water. The sensitivity of the filtration assay is important to correctly assess the disease potential of *P. ramorum* in irrigation and stream water.

Traditional detection methods are time-consuming to perform and may not be sensitive enough to detect low densities of the propagules in stream and nursery water. Polymerase chain reaction (PCR) offers a fast, specific and sensitive method for the detection of pathogenic fungi and oomycetes in plant tissue, soil and water (42, 53). Propagules in water samples are collected through filtration, after which the filters are treated with chemicals to cause cell lysis and the release of nucleic acids. Commercial DNA extraction kits can be used for DNA purification (20). While quantitative real-time polymerase chain reaction (qPCR) assays have been used for the
detection and quantification of P. ramorum in plant tissue (14, 15) and soil (5, 43), a qPCR protocol has not been developed for the detection and quantification of P. ramorum in water (41).

Objectives for this research were to: (i) test five detection methods simultaneously using lab and creek water spiked with known quantities of P. ramorum zoospores to determine the detection threshold concentration of zoospores for each method; (ii) determine if the detection methods could be used to quantify propagule density; (iii) develop a qPCR assay to quantify P. ramorum zoospore density in spiked lab and creek water samples; (iv) test the methods at field locations, including sites where P. ramorum positive detections have been made.

MATERIALS AND METHODS

P. ramorum inoculum preparation. Cultures of a NA1 lineage isolate (03-74-N10A-A from Rhododendron x ‘Unique’, A2 mating type, collected from a production nursery in Oregon) were grown on one-third V8 agar (66 ml of clarified V8 concentrate (100 ml V8 juice, 1.43 g CaCO3 centrifuged 15 minutes at 5000 rpm), 15 g Difco Bacto agar, 950 ml H2O) for 4 weeks in the dark at 20°C. Zoospore release was stimulated by flooding plates with 7 ml of autoclaved deionized water, refrigerating at 5°C for 2 h, and then bringing plates to room temperature for 1 h. Released zoospores were harvested from plates by pouring the deionized water and its contents through eight layers of cheesecloth to remove mycelium and sporangia. Zoospore concentration (zoospores/ml) of the initial stock was determined with a hemacytometer. The zoospores were diluted to the required concentrations by carefully pipetting a specific amount of zoospore suspension into 1 liter of water. The zoospore suspension was gently stirred to help homogenize the suspension without causing the zoospores to encyst before pipetting. A negative
control (0 zoospores) was made by flooding a non-colonized one-third V8 agar plate and pipetting 1 ml into 1 liter of water. Zoospore densities were verified using a hemacytometer (concentrations greater than 1,000 zoospores/ml) and by direct plating (DP) of zoospores onto PARPH-V8 medium (one-third V8 agar amended with 10 mg Delvocid (50% pimaricin), 250 mg sodium ampicillin, 10 mg Rifamycin-SV (sodium salt), 66.7 mg Terraclor (75% PCNB) and 50 mg Hymexazol) (11) in 100-mm diameter disposable Petri plates. Colony-forming units (CFU) were counted using a dissecting microscope at 24, 48 and 72 h. Three plates were made for each treatment and the average CFU/plate was determined for each trial. Zoospore concentration (zoospores/liter of water) was represented as direct plate colony-forming units/liter of water (DP CFU/liter).

**Experimental setup and water preparation.** Detection methods tested were: (i) baiting with a D’Anjou pear; (ii) baiting with a single wounded rhododendron leaf; (iii) the BOB method; (iv) filtration and (v) qPCR. Nine zoospore concentrations (treatments) were used: 100,000, 10,000, 1,000, 500, 250, 100, 50, 25 and 0 zoospores/liter of water to determine the detection threshold of each method. Lab and creek were used in separate experiments. Deionized lab water was put into clean 1-liter Nalgene bottles (Thermo Fisher Scientific Inc., Waltham, MA) and autoclaved on a liquid cycle for 20 min. Water was cooled to room temperature before adding zoospores and baits. There were three replicates for each zoospore concentration for each of detection method tested. The experiment was repeated four times.

Water was collected in buckets from Clarks Creek, a small spring-fed tributary to the Puyallup River, at the Washington State University Puyallup Research and Extension Center in Puyallup, Washington to evaluate how the detection methods performed under field conditions.
Water was taken near the surface to avoid gathering any sediment in the sample in an area of the creek where the water flow-rate was slow. The buckets of water were mixed together in an 18-gallon plastic storage box (Newell Rubbermaid Inc., Atlanta, GA) to homogenize the sample for each replication. There were three replicates for each zoospore concentration for each of the detection methods tested. The experiment was repeated twice. Lab and creek water quality measurements were taken (Table 1) with a portable meter (Hach Co., Loveland, CO) and associated probes for water temperature, pH, electrical conductivity (EC), dissolved oxygen (DO) and oxidation-reduction potential (ORP).

**Pear baiting.** Green (unripe) D’Anjou pears were acquired from a local store as needed and used within 24 h of purchase. Organic pears were used when available. Pears were washed with liquid (non-antibacterial) dish soap, rinsed, and allowed to air dry on paper towels. One pear was placed into a container (Glad 1.89 liter ‘Deep Dish’ style) that was filled with 1 liter of lab (or creek) water that had been spiked with *P. ramorum* zoospores at the desired concentration. Each concentration was replicated 3 times. Plastic bird netting was used to hold the pears in place so that only half of the pear would be submerged in the water at any given time. Pears and water were held in an APHIS-approved biocontainment unit at WSU Puyallup in 12 h of light (7.00 to 8.00 µmolS⁻¹ m⁻²) and 12 h of darkness, at a temperature of 20°C. Pears were turned to expose the entire surface to the pathogen after 24 h. Pears were removed from the water after a second 24 h, placed on paper towels for 2 to 4 days incubation in the biocontainment unit under the same conditions listed above. Lesions caused by *P. ramorum* were visually identified as described by Tjosvold et al. (firm, diffuse and in a rosette pattern) (46) and counted for each pear. Tissues from five discrete lesion margins were removed and plated onto 100-mm Petri
dishes containing PARPH-V8 medium. If pears had less than five lesions, tissues were removed from randomly chosen asymptomatic areas of the pear and plated onto PARPH-V8 so that there were always five tissue samples plated per fruit. Identification of *P. ramorum* was made based on morphological features of the colonies growing on PARPH-V8.

**Whole leaf baiting and BOB.** Bait leaves were harvested from field grown *Rhododendron x* ‘Nova Zembla’ at WSU Puyallup. Leaves that were fully expanded and hardened off were selected. Leaves were surface-sterilized with 0.5% hypochlorite solution for 30 s, rinsed twice with distilled water and blotted dry. A sterile 6-mm cork borer was used to make leaf disks. Whole leaves were wounded by folding the leaf into thirds and breaking across the midrib in two places. One wounded leaf was placed into a plastic bottle containing 1 liter of lab (or creek) water spiked with *P. ramorum* zoospores at the desired concentration. Each concentration was replicated 3 times. Bottles were sealed, placed on their sides and incubated in the biocontainment unit at 20°C for 3 days. Leaves were removed from the bottle and placed between layers of moist paper towels in sealed plastic containers and incubated for up to 7 days to allow for lesion development. Leaves were removed from the moist chamber and blotted dry. Tissue from the leaf tip, petiole and four discrete lesion margins was excised and transferred to PARPH-V8 medium. If leaves that had less than four lesions, tissue was removed from asymptomatic leaf areas that had been wounded so that there were always six tissue samples plated per leaf. Tissue material was incubated in the dark at 20°C for 7 days. Identification of *P. ramorum* was made based on morphological features of the colonies growing on PARPH-V8.

For the BOB method, one wounded leaf and ten leaf disks were placed into a plastic bottle containing 1 liter of lab (or creek) water spiked with *P. ramorum* zoospores at the desired
concentration. There were 3 replications for each concentration. Bottles were incubated on their sides for 3 days in the biocontainment unit. The wounded leaves were processed as described above; the leaf disks were removed, blotted dry, and transferred to PARPH-V8 medium immediately after removal from the bottle. Identification of *P. ramorum* colonies growing on the PARPH-V8 media was made as described above.

**Filtration.** One liter of lab (or creek) water was placed in a plastic bottle and spiked with *P. ramorum* zoospores to obtain the desired concentration. There were 3 replications for each concentration. Bottle contents were vacuum-filtered using a Nalgene filter funnel and 1,000-ml flask (Thermo Fisher Scientific Inc., Waltham, MA) through a Whatman 3-µm pore size, 47 mm in diameter polycarbonate membrane filter (GE Healthcare Life Sciences, Pittsburgh, PA) using a rotary vane vacuum pump (Gast Manufacturing, Inc., Benton Harbor, MI) at approximately 4 to 7 kPa for lab and creek water. Occasionally, a higher vacuum pressure was required to filter the creek samples. The vacuum pressure did not exceed 60 kPa in those cases. Vacuum pressure was released as soon as all the water had passed through the filter to help prevent damage to the zoospores. Filters were inverted onto thinly-poured PARPH-V8 medium in 60-mm diameter disposable Petri plates. Plates were incubated at 20°C in the dark for 1 to 2 days. The filters were removed and the surface of the agar was rinsed with de-ionized water to remove debris. Emerging colonies of *P. ramorum* were counted up to 72 h. Creek water was filtered in 250-ml aliquots to prevent the filter from becoming clogged. The entire bottle contents were filtered for concentrations of 0, 25, 50 and 100 zoospores/liter. A single 250-ml aliquot was filtered per bottle for concentrations of 250, 500 and 1,000 zoospores/liter so as to obtain countable plates of approximately 30 to 50 CFU per plate. A single 25-ml and one 5-ml aliquot was filtered per
bottle for concentrations of 10,000 and 100,000 zoospores/liter, respectively.

**qPCR assays for quantifying zoospores in water.** One liter of lab (or creek) water was prepared to the desired zoospore concentration and filtered as previously described. There were 3 replications for each concentration. Filters containing the trapped zoospores were placed into individual 2-ml screw-cap tubes and stored at -80°C until DNA extraction. Preliminary testing identified cell lysis with sodium dodecyl sulphate (SDS) and proteinase K combined with DNA purification using silica-membrane columns from a NucleoSpin Plant kit® as yielding significantly higher amounts of DNA product when compared to methods using Cetyl trimethylammonium bromide (CTAB) combined with phenol chloroform, CTAB combined with silica-membrane columns and SDS combined with phenol chloroform. Therefore, zoosporic DNA was extracted using a NucleoSpin Plant II kit (Macherey-Nagel Inc., Bethlehem, PA) following the protocol for genomic DNA from plants with the following modification: cell lysis of zoospores was done following the method of Lefevre et al. (28). Filters were soaked overnight at 37°C in 12 µl of 1mg ml⁻¹ proteinase K and 500 µl of a buffer containing 1% SDS (sodium dodecyl sulfate) and TE (Tris-EDTA, 1mM EDTA and 10 mM Tris-HCl). The entire sample DNA was bound to a single column (step 5 of the protocol) by multiple repetitions of the loading step in samples where multiple filters were used. A TaqMan Real-Time (quantitative or q) PCR assay, based on the APHIS protocol (48) was performed on a 7500 Sequence Detection System (Applied Biosystems) instrument. Each 25-µl qPCR reaction included 2 µl of DNA and 23 µl of master mix (12.5µl 2X TaqMan Universal PCR Master Mix (no AmpErase UNG), 0.0125µl Pram-114F (100µM), 0.05µl Pram-1527-190R (100µM), 0.025µl probe Pram-1527-134-T (100µM), 0.025µl IPC Sketa F (100µM), 0.025µl IPC Sketa R (100µM), 0.025µl IPC Sketa
probe (100µM), 0.125µl BSA (10mg/ml), 2.375µl Trehalose (50%), 1 µl salmon sperm DNA, 6.8375 nuclease-free water). Cycling conditions were set at: 50°C for 2 min, 95°C for 10 min and 46 cycles of 95°C for 15 s, 58°C for 15 s and 60°C for 30 s. Salmon sperm DNA (10) was added to the reaction mix as an internal amplification control and detected by Sketa primers and probe (2). The *P. ramorum* probe was labeled with a 5’ fluorescent FAM dye and non-fluorescent quencher and the Sketa probe was labeled with a 5’ fluorescent VIC dye and TAMRA quencher. Bovine serum albumin (BSA) was added to reduce the effect of inhibitors on the yield of qPCR product. The optimal cutoff point (*C*<sub>t</sub>, cycle number) was determined using the Youden index in the context of the receiver operating characteristic (ROC) curve analysis as described by Nutz et al. (33). Samples with a FAM *C*<sub>t</sub> value of 38 and above were considered to be negative for tests using lab and creek water. Standard curves were generated based on a dilution series of *P. ramorum* DNA from a NA1 lineage isolate (Pr-1418886 from *Camellia x Coral Delight*, A2 mating type, collected from a nursery in Fairfax, Marin Co., CA) containing 7 x 10<sup>-1</sup> to 7 x 10<sup>-6</sup> ng to of DNA. Each DNA sample including standards was assayed in triplicate. A water control was included as a negative control.

The estimated number of *P. ramorum* zoospores was determined by the equation:

\[ Y = \frac{X}{0.0000665 \text{ ng DNA}} \]

where \( Y \) = the estimated number of zoospores, \( X \) = the amount of recovered DNA (ng) and 0.0000665 = the DNA content of a single *P. ramorum* zoospore (ng). The DNA content of one zoospore was determined by using the conversion factor of Dolezel et al. (9) where 1 pg of DNA represents 0.978 x 10<sup>9</sup> base pairs (bp) and the *P. ramorum* genome size of 65 Mb (0.065 x 10<sup>9</sup> bp) estimated by Tyler et al. (47). Assuming there is one copy of the genome per zoospore, the
DNA content of a single *P. ramorum* zoospore was determined by the equation:

\[
0.065 \times 10^9 \text{ bp} \div 0.978 \times 10^9 \text{ bp} = 0.0665 \text{ pg DNA (0.0000665 ng DNA)}
\]

**Field sampling.** Detection methods were tested at two Washington wholesale nurseries (henceforth referred to as Nursery 1 and Nursery 2) located in King and Thurston counties, and at the Dungeness River and Gierin Creek, both located in Clallam County, WA. Filtration and the BOB method were used to detect *Phytophthora* spp. in a retention pond and swale that connects the pond to a drainage ditch bordering the property at Nursery 1. *P. ramorum* had not been previously recovered from either the pond or swale. Eight 1-liter samples were collected from various locations around the perimeter of the pond and swale in March 2013 using a 1-liter Nalgene bottle attached to a pole approximately 10 ft from the water edge and at roughly 12 in below the water surface. Filtration and BOB methods were conducted as described in sections above; however, 1 liter water samples were filtered in 100-ml aliquots for filtration to prevent the filters from becoming clogged. Colonies were identified using morphological characteristics to either *Pythium* or *Phytophthora* genus.

Filtration, BOB and qPCR were used to detect the presence of *P. ramorum* at the river, creek and drainage pond at Nursery 2 in July 2013. *P. ramorum* had previously been recovered from the river and drainage pond. Three 1-liter samples were collected at single locations at the river and creek while 1-liter samples were collected from four locations around the drainage pond perimeter. Samples were collected as described for Nursery 1. At the river and creek, the three 1-liter aliquots were pooled, mixed and poured into 1-liter bottles to homogenize the sample. Temperature, pH, EC, DO and ORP were recorded as previously described.

Filtration, BOB and qPCR methods were conducted as described in sections above;
however, 1 liter water samples were filtered in 100-ml aliquots for filtration and in 250-ml aliquots for qPCR to prevent the filters from becoming clogged. DNA from multiple filters was combined onto a single column by multiple repetitions of the loading step. A positive control consisting of an additional 1 liter water sample that was ‘spiked’ with a known quantity of *P. ramorum* zoospores was included for qPCR. Isolates from the river, creek and Nursery 2 were identified morphologically and DNA sequencing of the internal transcribed spacer (ITS) region of the nuclear ribosomal DNA, using the ITS4 and ITS6 primers (6, 52) was performed for identification of select colonies from the drainage pond at Nursery 2.

**Statistical analysis.** The relationship between DP CFU/liter and the number of lesions on pear, leaves, infected leaf disks, filtration CFU and DNA quantity was assessed with simple linear regression (PROC REG; SAS 9.2, SAS Institute Inc., Cary, NC) using mean values of 3 replicates of each treatment (zoospore concentration) for each trial. Before analysis, pear, leaf and leaf disk data sets were transformed using a logarithmic (base e) transformation with a “1” added to each mean value to retain the ability to analyze means with values below 1. Initial analysis of scatter plot data showed filtration and DNA means had a strong linear relationship with DP CFU and were not transformed.

**RESULTS**

**Water quality measurements.** Water pH, EC, DO and ORP readings were similar for the two nursery ponds and for Clarks Creek (Table 1). The water temperature difference between the nursery ponds was likely due to seasonal variation. The Dungeness River and Gierin Creek had a lower pH and EC than the nursery ponds although water temperatures were similar to that of Nursery 1. Deionized lab water had a lower pH and EC and higher ORP and temperature than
Clarks Creek. DO was slightly higher in water from Clarks Creek than in the lab water.

**Detection methods.** The relationship between DP CFU/liter and filtration CFU/liter (Table 2) was strongly correlated for all trials. There was a similar correlation trend between trials using lab water (Fig. 1A) and creek water (Fig. 1B). The detection threshold ranged from 1 to 10 DP CFU/liter in lab and creek trials. The filtration method was able to estimate zoospore density over 400 DP CFU/liter in lab and creek water (Table 3); however, variability between replications in lab trials 1 through 3 was greater for filtration CFU/liter at higher zoospore concentrations. None of the non-infested filtration replications in any trials had false positives and filtration colony-forming units were detected at all zoospore concentrations in all replications.

The relationship between DP CFU/liter and recovered nanograms of DNA/liter (Table 2) was strongly correlated in all trials with the exception of lab trial 3 \( (P = 0.5051, R^2 = 0.1179) \). The zoospore detection threshold ranged between 1 and 10 DP CFU/liter for both lab (Fig. 2A) and creek water (Fig. 2B). The qPCR method was able to quantify *P. ramorum* DNA in creek water over 400 DP CFU/liter (Table 3), although the variability between replications in both lab and creek trials was greater at higher DP CFU/liter concentrations. qPCR failed to detect spiked *P. ramorum* zoospore DNA in three of the six replications at the lowest concentrations, 7 and 8 DP CFU/liter in the creek trials.

Data for the pear trials (Fig. 3) showed an overall trend of increasing pear lesions/liter as the number of DP CFU/liter increased; however, the trend was only significant in creek trials (Table 2). The detection threshold ranged between 10 and 100 DP CFU/liter in lab trials (Fig. 3A) and between 10 and 35 DP CFU/liter in creek trials (Fig. 3B). Lab trial 2 was an exception;
pear lesions formed in DP CFU/liter concentrations as low as 5 DP CFU/liter. There was a high rate of variability between replications at all concentrations. *P. ramorum* was recovered once from asymptomatic pear tissue in the creek trials. Pears failed to develop lesions in three replicates at high zoospore concentrations, above 1,000 DP CFU/liter. Individual lesions on pears could not be accurately counted above concentrations of 400 DP CFU/liter.

The detection threshold for *P. ramorum* using rhododendron leaf disks (Fig. 4A and B) was between 1 and 50 DP CFU/liter for lab trials and between 1 and 35 DP CFU/liter for creek trials. There was a strong correlation between DP CFU/liter and colonized leaf disks/liter in all trials (Table 2). Infected disks appeared to be asymptomatic and were quantitative in creek trials between the range of 1 and 400 DP CFU/liter. Approximate disk infection rates were as follows: between the range of 1 to 10 DP CFU/liter, < 1%; between 11 to 100 DP CFU/liter, 10%; between 101 to 200 DP CFU/liter, 20%; between 201 to 400 DP CFU/liter, 50%.

There was a strong correlation between DP CFU/liter and the number of *P. ramorum* lesions/liter in one creek and two lab BOB leaf trials (Table 2); however, in lab trial 1, BOB leaves failed to detect *P. ramorum* in all three replications at concentrations of 206 DP CFU/liter and below. There was a trend towards an increase in the number of lesions/liter as DP CFU/liter increased in some leaf only trials (Table 2), but none of the trials had relationships that were significant. BOB leaf (Fig. 5A and B) lab and creek trials had detection thresholds in the ranges of 1 to 100 and 1 to 35 DP CFU/liter, respectively. Leaf only (Fig. 6A and B) lab and creek trials had a detection threshold in the ranges of 1 to 20 and 1 to 10 DP CFU/liter, respectively. Symptomatic leaf petioles and leaf tips were infected with *P. ramorum* between 43 and 65% of the time. One symptomatic piece of leaf tissue was negative for *P. ramorum* and three asymptomatic pieces of leaf tissue were positive in the trials using lab water. Lesions caused by
*P. ramorum* could not be visually distinguished from those caused by other *Phytophthora* and *Pythium* spp. in the trials using creek water. Only six pieces of symptomatic leaf tissue were sampled per leaf in the creek trials; therefore, the highest potential number of *P. ramorum* lesions reported (Fig. 5B and 6B) was six.

**Field sampling.** *P. ramorum* was not found at the river, creek or Nursery 2 pond using the detection methods. More colonies were identified as *Phytophthora* from lesions that developed on non-wounded leaves and more colonies were identified as *Pythium* when using filtration (Table 4). More colonies were identified as *Pythium* when collected from leaf disks at Nursery 1 while results were mixed at the other sites. *P. ramorum* was not detected in the ‘unspiked’ river and creek samples using qPCR methods but it was detected in the ‘spiked’ samples. Spiked samples from Nursery 2 required an additional dilution step (1:10) before *P. ramorum* DNA could be detected. While *P. ramorum* DNA was detected in diluted ‘unspiked’ samples from Nursery 2, the C\textsubscript{i} values were above the C\textsubscript{i} cutoff value of 38 as determined by ROC analysis (33).

**DISCUSSION**

This study provided a comparison of the performance of five *P. ramorum* detection methods run simultaneously under controlled laboratory settings using zoospores suspensions made with either autoclaved deionized lab or non-infested creek water that had been spiked with known quantities of *P. ramorum* zoospores. All bait types were effective at detecting a range of zoospores in water. The data suggest that leaf material was more sensitive at detecting low concentrations of *P. ramorum* zoospores than pears; all baits had a wider detection threshold range than filtration and qPCR. Counting the number of *P. ramorum* positive leaf disks could be
used to quantify zoospore density in lab and creek trials while the number of *P. ramorum* pear lesions could be used for limited quantification in the creek trials. The relationship between the number of positive *P. ramorum* leaf lesions on BOB leaves and zoospore concentration was significant in some trials, but in general, the data suggest that wounded leaf baits were better at detecting *P. ramorum* than quantifying inoculum density.

In situ baiting using detached rhododendron leaves in bait bags has been used since 2006 to detect *P. ramorum* as part of the National *Phytophthora ramorum* Early Detection Survey of Forests (3). The standard national survey protocol (49) was expanded to include the use of BOB in 2013 (34). The BOB method was found to be more effective at detecting *P. ramorum* when used in the spring, during which time the overall recovery rate for *P. ramorum* is higher, while bait bags were more effective in the fall when recovery rates of *P. ramorum* are generally lower in a comparison of methods conducted in 2011 and 2012 (34). Data from the creek trials suggest that BOB wounded leaves and disks have a wider detection threshold, between 1 to 35 DP CFU/liter, when compared to the leaves only baits detection threshold, between 1 to 10 DP CFU/liter, but overall, both methods were similar in their ability to detect *P. ramorum*.

A positive correlation between the number of infected leaf disks and *P. ramorum* inoculum concentration in run-off water has been shown in prior research (45). Leaf disks were able to quantify zoospore density up to 400 DP CFU/liter, where the *P. ramorum* disk infection rate was found to be approximately 50%. Creek trial data show leaf disks to be nearly 100% colonized at concentrations above 1,440 DP CFU/liter (Table 3). More research needs to be conducted to determine the quantification cut off value, when the rate of infection reaches between 80 to 100% of the disks.
Pear baits had the highest detection threshold of all the methods tested, above 10 DP CFU/liter (Fig. 3), and might not be able to detect low concentrations of zoospores in streams or nursery effluents. There were occurrences when one or two of the pear replicates failed to develop lesions at concentrations above the detection threshold, implying that pear baits might not be as reliable as other methods at detecting *P. ramorum*. There was not a noticeable difference in the number of infections between organic and non-organic pears (data not shown). Unlike leaf disks or pieces of symptomatic leaf tissue where several isolates grew from each piece of tissue that was plated onto PARPH-V8 medium, each piece of pear tissue generally resulted in a single distinct colony, which facilitated visual identification of *P. ramorum* isolates in creek water. Tjosvold et al. (46) used three D’Anjou pears to bait 8-liter aliquots of stream water and found a concentration of approximately two infective *P. ramorum* propagules per liter of stream water. Using more than one pear in a larger volume of water may increase the reliability of this method at detecting *P. ramorum* zoospores in water.

Filtration CFUs were strongly correlated to zoospore density in both lab and creek water that was spiked with *P. ramorum* zoospores. Data show that filtration CFUs were approximately 8 and 16% of the *P. ramorum* zoospores counted by hemacytometer in creek (Table 3) and lab water (data not shown), respectively. DP CFUs were approximately 20 and 23% of the creek (Table 3) and lab water (data not shown) hemacytometer counts. Fewer filtration CFUs when compared to those formed by direct-plating of zoospores onto PARPH-V8 may be the result of the loss of zoospores through the filter pores (18), damage to the zoospore caused by the vacuum pressure or may be caused by the difficulty counting individual CFUs that are in close proximity to each other.
Kong et al. (26) found a germination rate of less than 20% when *P. ramorum* zoospores were plated directly onto PARP-V8 medium while Hao et al. (13) reported 13% of motile *P. nicotianae* zoospores germinated when placed in PARP-V8 broth. Germination rates for *P. ramorum* zoospores captured on a filter have been reported to be between 10 and 15% for motile zoospores (J. Hwang and S. Oak, personal communication) while the filtration recovery rate of *P. cryptogea* zoospores was found to be in the range of 10 and 20% (29). Filtration of naturally infested water only recovers a portion of *P. ramorum* zoospores present due to the amount of water sampled relative to the total water volume. *P. ramorum* was detected using filtration at densities of 2, 36 and 130 CFU/liter in California streams (22, 35). Assuming that the filtration process captured the low end of the recovery rate (29) or 10% of *P. ramorum* zoospores, actual zoospore densities would be closer to 20, 360 and 1,300 zoospores/liter.

There were strong correlations between DP CFU/liter and recovered DNA/liter in all trials with the exception of recovered DNA in lab trial 3 (Table 2, Fig. 2A). Low amounts of recovered DNA at the concentration of 127 DP CFU/liter could be due to pipetting error while spiking the deionized water during the initial setup.

Most natural water sources contain phenolic compounds that can cause inhibition in qPCR reactions (37). Inhibitors lower the amount of qPCR product amplified, causing a reduction in the sensitivity of detection of target DNA. Humic acid, found in soil, and tannic acid, found in leaf litter, are components of natural water and inhibit qPCR reactions by binding to target DNA, thereby limiting the amount of available DNA template that can be amplified (37). Tannic acid also interacts with the *Taq* polymerase, making it inoperable during the qPCR reaction (37). Bovine serum albumin (BSA), which binds to endogenous phenolic compounds
and helps prevent them from interfering with qPCR (27) was added to the final qPCR reaction mix to bind inhibitors in creek and field water samples.

Salmon testes DNA (Sketa) (10) was added as an internal control and to evaluate the impact of inhibition in the final reaction mixture for all qPCR samples. A sample was considered inhibited if the Sketa threshold value ($C_t$) was increased by 3.0 cycles from an uninhibited reference sample analyzed on the same plate (10). Trials using lab and creek water had Sketa $C_t$ values within 1 and 3 cycles, respectively, of the reference samples. Sketa $C_t$ values were within 3 cycles of the reference samples for river and stream field samples. Nursery 2 samples required an additional dilution of 10x before the effects of inhibitors were overcome. Recovered quantities of zoospore DNA was relatively less in creek trials 1 and 2 than for the lab water trials (Fig. 2A and B), suggesting that the qPCR reactions were partially inhibited by compounds in the water, in spite of the addition of BSA.

One potential drawback of using qPCR for the quantification of $P$. ramorum zoospores is that the qPCR process amplifies all detectable DNA regardless if it comes from viable or nonviable zoospores. If non-viable zoospores are included in a qPCR water sample, it is possible to have a false positive result or an over-estimation of propagule density (38). One way to avoid false positive results is to combine culture-based methods that identify viable zoospores along with quantification using qPCR.

$P$. ramorum was not recovered from any of the field locations that had previously tested positive for the pathogen. Increasing the number of sampling dates to include late winter and spring, peak recovery times of $P$. ramorum (31, 36) may be necessary to detect the pathogen at these sites. Detection of $P$. ramorum by filtration may have been hindered by faster growing $Pythium$ and $Phytophthora$ species that interfere with visual identification of $P$. ramorum on
PARPH-V8 media (39). *P. ramorum* recovery may be enhanced by filtering smaller aliquots of water to spread out the distribution of propagules among more filters (39).

Isolates of *Phytophthora citrophthora*, commonly recovered from nursery effluent (1, 29) and *Phytophthora hydropathica* were both identified at Nursery 2. *P. hydropathica*, a pathogen that causes leaf necrosis and shoot blight on *Rhododendron catawbiense*, has been recovered from irrigation reservoirs in Virginia and neighboring states during warm summer weather (16). *P. hydropathica* was molecularly identified on two pieces of symptomatic leaf tissue and visually identified (chlamydospores with dense protoplasm and obovate hyphal swellings) (16) on approximately 44% of the isolates recovered from Nursery 2. Other species of *Phytophthora* in the pond, including *P. hydropathica*, may have been able to colonize bait material before *P. ramorum* (44). Recovery of *P. hydropathica* is highest during hot summers and rapidly declines in cooler water temperatures (16). *P. ramorum* detection may be improved if baiting is done when pond water temperatures are lower.

In conclusion, baiting with rhododendron leaves, disks and pears along with filtration and qPCR methods detected *P. ramorum* zoospores with only minor differences in sensitivity between the types of water used. The data suggest that filtration and qPCR were equally effective in detecting a range of concentrations of *P. ramorum* zoospores and could provide information on zoospore density over 400 DP CFU/liter. Leaf disks were able to estimate zoospore density in lab and creek water up to 400 DP CFU/liter while pear and wounded whole leaf baits appeared to be better at detection than quantification of zoospore density.
ACKNOWLEDGEMENTS

This research was possible with funding from the United States Department of Agriculture Animal and Plant Health Inspection Service. Cooperative Agreement 12-8130-0178-CA. Information on *P. ramorum* zoospore density in lab and stream water was provided by Jaesoon Hwang, Clemson University and Steve Oak, USDA Forest Service, Southern Region (retired). Assistance from the staff of the WSU Puyallup Ornamental Plant Pathology program at the Puyallup Research and Extension Center is acknowledged.
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24. Hwang, J., Oak, S. W., and Jeffers, S. N. 2008. Variation in population density and diversity of *Phytophthora* species in streams within a forest watershed. (Abstr.) Phytopathology 98(suppl.):S70.


48. USDA-APHIS. 2013. Quantitative multiplex real-time PCR (qPCR) for detection of *Phytophthora ramorum* (ITS target) using a TaqMan system on the Cepheid SmartCycler® and the ABI 7900/7000. WI-B-T-1-6 revision 7. USDA Animal and Plant Health Inspection Service, Plant Protection and Quarantine.

Phytophthora ramorum Nursery Survey, appendix 7.


Table 1. Water quality measurements for lab and field water used for simultaneous detection methods testing and from field samples.

<table>
<thead>
<tr>
<th>Source</th>
<th>Date</th>
<th>pH</th>
<th>Electrical conductivity (S/m)</th>
<th>Dissolved oxygen (mg/l)</th>
<th>Oxidation-reduction potential (mV)</th>
<th>Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lab</td>
<td>3-Sep-13</td>
<td>6.36</td>
<td>0.0058</td>
<td>7.43</td>
<td>347.2</td>
<td>23.0</td>
</tr>
<tr>
<td>Lab</td>
<td>8-Jun-14</td>
<td>5.52</td>
<td>0.0078</td>
<td>8.50</td>
<td>339.7</td>
<td>21.4</td>
</tr>
<tr>
<td>Clarks Creek</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trial 1</td>
<td>6-Jan-14</td>
<td>7.43</td>
<td>0.0226</td>
<td>8.92</td>
<td>215.9</td>
<td>12.6</td>
</tr>
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<td>Clarks Creek</td>
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<td>Trial 2</td>
<td>21-Jan-14</td>
<td>7.59</td>
<td>0.0225</td>
<td>9.59</td>
<td>264.4</td>
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</tr>
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<td>Nursery1 pond</td>
<td>3-Mar-13</td>
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<td>0.0226</td>
<td>ND</td>
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<td>Nursery2 pond</td>
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<td>7.55</td>
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<td>Dungeness River</td>
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<td>Gierin Creek</td>
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<td>0.0131</td>
<td>8.98</td>
<td>ND</td>
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*a Simultaneous detection methods testing was performed using cooled, autoclaved deionized lab water and water taken from Clarks Creek located at WSU Puyallup. Field sampling was conducted at two nursery sites, the Dungeness River and Gierin Creek. Water quality data shown are the average of three measurements taken per source.

b ND = no data. Water quality measurements were not taken.
Table 2. Summary statistics for the correlation between direct plate colony-forming units/liter of lab or creek water and the number of filtration CFU, zoospore DNA, positive leaf disks and pear or leaf lesions caused by *P. ramorum*.

<table>
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<tr>
<th>Detection method</th>
<th>Trial</th>
<th>DF$^a$</th>
<th>$P$-value</th>
<th>$R^2$</th>
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<td>0.0003</td>
<td>0.9710</td>
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<td>Lab 2</td>
<td>4</td>
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<td></td>
<td>Lab 3</td>
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<td>Lab 4</td>
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<td>&lt;0.0001</td>
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<td>Creek 1</td>
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<td>&lt;0.0001</td>
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<td></td>
<td>Creek 2</td>
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<td>Lab 1</td>
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<td>Lab 3</td>
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<td>0.0009</td>
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<td>Pear</td>
<td>Lab 1</td>
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<td>BOB leaf</td>
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<td>0.6150</td>
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<td>4</td>
<td>0.0752</td>
<td>0.5882</td>
</tr>
</tbody>
</table>

$^a$Degrees of freedom

$^b$ND = no data; *P. ramorum* was not recovered from any of the BOB rhododendron leaves below the concentration of 452 DP CFU/liter of lab water.
Table 3. A comparison of *P. ramorum* zoospore quantification methods for trials using creek water.

<table>
<thead>
<tr>
<th>Target zoospores a</th>
<th>Hema-cytometer b</th>
<th>DP CFU c</th>
<th>Filtration CFU d</th>
<th>BOB leaf disks e</th>
<th>C t f</th>
<th>DNA(ng) g</th>
<th>Estimated zoospores h</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Trial 1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.00</td>
<td>undetectable</td>
<td>undetectable</td>
</tr>
<tr>
<td>25</td>
<td>ND i</td>
<td>7</td>
<td>8</td>
<td>0.33</td>
<td>37.09</td>
<td>6.00E-04</td>
<td>9</td>
</tr>
<tr>
<td>50</td>
<td>ND i</td>
<td>19</td>
<td>14</td>
<td>0.33</td>
<td>36.37</td>
<td>9.95E-04</td>
<td>15</td>
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<tr>
<td>100</td>
<td>ND i</td>
<td>35</td>
<td>21</td>
<td>1.67</td>
<td>35.06</td>
<td>2.21E-03</td>
<td>33</td>
</tr>
<tr>
<td>250</td>
<td>ND i</td>
<td>71</td>
<td>36</td>
<td>2.00</td>
<td>34.59</td>
<td>3.22E-03</td>
<td>48</td>
</tr>
<tr>
<td>500</td>
<td>ND i</td>
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<td>1.33</td>
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<tr>
<td>1,000</td>
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<td>381</td>
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<td>4.33</td>
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<td><strong>Trial 2</strong></td>
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<td>undetectable</td>
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<tr>
<td>50</td>
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<td>35.63</td>
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<tr>
<td>500</td>
<td>ND i</td>
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<td>10.00</td>
<td>22.50</td>
<td>1.47E+00</td>
<td>22,105</td>
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</table>

a The target number of *P. ramorum* zoospores/liter for each treatment.
b The mean number of *P. ramorum* zoospores/liter from three hemacytometer counts.
c The mean number of direct plate colony-forming units/liter that formed on PARPH-V8 medium, n=3.
d The mean number of filtration colony-forming units/liter that formed on PARPH-V8 medium, n=3.
e The mean number of *P. ramorum* infected leaf disks (out of ten), n=3.
f Threshold cycle (Ct) number when fluorescence intensity of the sample exceeded background fluorescence intensity. The Ct value is the mean of three sample replicates.
g The mean of *P. ramorum* DNA from three 1-liter creek water samples.
h The estimated number of *P. ramorum* zoospores determined by the equation \( Y = \frac{X}{0.0000665} \) ng, in which \( Y \) = the number of zoospores, \( X \) = recovered DNA (ng) and 0.0000665 ng = the DNA content of one zoospore.
i ND = no data, hemacytometer counts were not taken for zoospore densities below 1,000 zoospores/liter.
Table 4. Number of colonies identified as either belonging to *Phytophthora* and *Pythium* from 1 liter field samples using BOB, filtration and qPCR recovery methods.

<table>
<thead>
<tr>
<th>Source</th>
<th>Phytophthora</th>
<th>Pythium</th>
<th>Phytophthora</th>
<th>Pythium</th>
<th>Phytophthora</th>
<th>Pythium</th>
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<tr>
<td>Nursery 1</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>pond</td>
<td>5</td>
<td>31</td>
<td>20</td>
<td>13</td>
<td>20</td>
<td>36</td>
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<tr>
<td>Nursery 2</td>
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<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>pond</td>
<td>7</td>
<td>8</td>
<td>12</td>
<td>3</td>
<td>8</td>
<td>18</td>
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<tr>
<td>Dungeness River</td>
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<td>Gierin Creek</td>
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<tr>
<td>Creek</td>
<td>5</td>
<td>3</td>
<td>4</td>
<td>3</td>
<td>6</td>
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<td>44</td>
<td>43</td>
<td>20</td>
<td>44</td>
<td>84</td>
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</table>
Fig. 1. The relationship between filtration and direct plate CFU/liter for the recovery of *P. ramorum* zoospores for A, trials using lab water and B, trials using creek water. Error bars represent standard error, n=3.
Fig. 2. The relationship between the amount of recovered DNA (ng)/liter from \textit{P. ramorum} zoospores trapped on a filter and direct plate CFU/liter for \textbf{A}, trials using lab water and \textbf{B}, trials using creek water. Error bars represent standard error, n=3.
Fig. 3. The relationship between pear lesions/liter and direct plate CFU/liter for the recovery of *P. ramorum* zoospores for **A**, trials using lab water and **B**, trials using creek water. Error bars represent standard error, n=3.
Fig. 4. The relationship between colonized leaf disks (BOB)/liter and direct plate CFU/liter for the recovery of *P. ramorum* zoospores for **A**, trials using lab water and **B**, trials using creek water. Error bars represent standard error, n=3.
Fig. 5. The relationship between leaf lesions (BOB)/liter and direct plate CFU/liter for the recovery of *P. ramorum* zoospores for **A**, trials using lab water and **B**, trials using creek water. Error bars represent standard error, n=3.
Fig. 6. The relationship between leaf lesions/liter and direct plate CFU/liter for the recovery of *P. ramorum* zoospores for **A**, trials using lab water and **B**, trials using creek water. Error bars represent standard error, n=3.
CHAPTER 3

Inoculum threshold for *Phytophthora ramorum* in irrigation water required for infection of rhododendron leaves

**ABSTRACT**

The concentration of *Phytophthora ramorum* zoospores needed in irrigation water for infection of nursery hosts is not well understood. To determine the inoculum threshold, a novel measured-inoculum spray applicator (MISA) was constructed to apply pressurized zoospore suspensions of *P. ramorum* to wounded detached rhododendron leaves under controlled laboratory conditions and to camellia, viburnum and rhododendron plants in a simulated nursery study. The pressurization and spraying of *P. ramorum* zoospores using the MISA did not significantly affect zoospore viability or infectivity on wounded detached rhododendron leaves. The inoculum threshold concentration for infection on wounded detached leaves was found to be 51 zoospores/ml in the laboratory assays and between 1,000 and 10,000 zoospores/ml for plant hosts in the simulated nursery study. Direct plating, filtration and qPCR methods were used to quantify *P. ramorum* zoospores between 1 and 10,000 zoospores/ml while the Bottle O’ Bait (BOB) method was used to quantify zoospores densities between 1 and 100 zoospores/ml.

Understanding the inoculum threshold necessary for infection of nursery hosts will assist nursery managers and regulatory agencies in assessing the risk of using *P. ramorum* infested water for irrigation.
INTRODUCTION

Commercial plant nurseries commonly capture and reuse irrigation water to comply with government regulations designed to prevent the escape of nutrients and pesticides into the environment and to conserve limited supplies of clean water (25). Surface water can be taken from rivers, streams and artificial waterways and used for irrigation of commercial crops and private landscapes. Both surface (22, 29, 45) and recycled irrigation water (21) have been found to contain several Phytophthora and Pythium species, some of which are pathogenic to nursery and landscape plants. One such species, Phytophthora ramorum, is the causal agent of sudden oak death (SOD) on tanoak (Lithocarpus densiflorus), oak (Quercus spp.), and Japanese larch (Larix kaempferi) as well as ramorum blight on hundreds of native and nursery hosts such as rhododendron and viburnum (30, 41, 42). Propagules of P. ramorum have been found in streams associated with areas of high incidence of SOD (6, 36) up to 5 years after P. ramorum was eradicated from the landscape (34). P. ramorum has been detected in streamside vegetation growing alongside an infested ditch exiting a positive nursery after a flooding event (4, 5). Nurseries that irrigate with untreated recycled or surface water infested with P. ramorum or other waterborne plant pathogens are at an elevated risk for the development of disease within their nursery stock.

The inoculum threshold of a pathogen refers to the amount of inoculum needed to cause plant disease on host plants (11). Some factors that influence the inoculum threshold of pathogens in irrigation systems include exposure frequency (10), irrigation method (33), host susceptibility and environmental factors such as water pH (18). The inoculum threshold of P. ramorum in irrigation systems is not well understood. Werres et al. (43) and Tjosvold et al. (36) found low incidence of disease of Rhododendron × ‘Cunningham’s White’ that received
overhead irrigation of water artificially and naturally infested with *P. ramorum*. Estimated propagule densities varied widely between the studies at 74 zoospores/ml and 0.001 zoospores/ml respectively. The purpose of this study was to examine the effect of application pressure on *P. ramorum* zoospore viability and to determine the inoculum concentration of *P. ramorum* that is necessary in irrigation water to cause infection of detached wounded rhododendron leaves in a laboratory setting and on rhododendron, camellia and viburnum plants in a simulated nursery environment.

**MATERIALS AND METHODS**

**Inoculum application.** A measured-inoculum spray applicator (MISA) was constructed using a 25.4 mm (1 in) PVC expansion coupling that is normally used to repair broken PVC water pipes (Fig. 1). An air coupling was affixed to the top of the expansion coupling that allowed the applicator to be attached to an air compressor. A threaded PVC cap with an adjustable mister was affixed to the bottom of the expansion coupling. The chamber created by the expansion coupling was filled with suspensions of inoculum by removing the hand-tightened cap on the expansion coupling. The MISA was pressurized with a portable air compressor (All Power America LLC., Chino, CA) fitted with an air pressure regulator value (Wilkerson Corp., Richland, Michigan). The MISA was held in place 40.6 cm above the surface to be sprayed by inserting the MISA into a drilled hole at the top of a wooden stand that was 61 cm tall, 46 cm wide and 15 cm deep to ensure uniformity of spray application.

**P. ramorum inoculum preparation.** Cultures of an NA1 lineage isolate (03-74-N10A-A from *Rhododendron x ‘Unique’, A2 mating type, collected from an Oregon production nursery) were grown on one-third V8 medium (66 ml of clarified V8 concentrate (100 ml V8 juice, 1.43 g
CaCO₃ centrifuged 15 min at 5000 rpm), 15 g Difco Bacto agar, 950 ml H₂O) for 4 weeks in the dark at 20°C. Plates were flooded with 7 ml of autoclaved deionized water, refrigerating at 5°C for 2 h, and then bringing plates to room temperature for 1 h to stimulate zoospore release. Deionized water and its contents were strained through eight layers of cheesecloth to remove mycelium and sporangia, the zoospore concentration (zoospores/ml) of the initial stock was determined with a hemacytometer, and adjusted to the desired concentration using autoclaved deionized water.

**Detached leaves.** Fully expanded, hardened off leaves were harvested from *Rhododendron x* ‘Nova Zembla’ grown in fields at the Washington State University Puyallup Research and Extension Center in Puyallup, Washington. Leaves were from bushes that are grown to supply plant material for research projects and were not part of any research plots. Leaves were surface-sterilized with 0.5% hypochlorite solution for 30 s, rinsed twice with deionized water and blotted dry. Leaves were wounded by making a 3 to 4 cm cut across the midrib on the lower half of the leaf using a sterile scalpel.

**Laboratory assays.** Two experiments were run inside an APHIS-approved biocontainment unit at the Washington State University Puyallup Research and Extension Center in Puyallup, Washington using the MISA setup. The first assay examined the affect different pressures had on zoospore viability and infectivity. Three pressures (treatments) were tested: 210, 420 and 630 kPa (30, 60 and 90 psi). A pressure of 35 kPa (5 psi) served as a control and was applied using a hand-operated spray bottle as described by Banko et al. (2). A single zoospore suspension was made for each trial as described above. The suspension was gently stirred while 2 ml aliquots were removed using a pipette to distribute the zoospores uniformly throughout the suspension.
The aliquots were added to 98 ml of autoclaved deionized water in a 150-ml flask for a final concentration of 773 zoospores/ml (trial 1) and 1,415 zoospores/ml (trial 2). To determine how application pressure affects zoospores viability, three 200 µl aliquots of zoospore suspension were removed from the flask before pressurizing and plated onto three hardened PARPH-V8 medium (15) (one-third V8 medium amended with 10 mg Delvocid (50% pimaricin), 250 mg sodium ampicillin, 10 mg Rifamycin-SV (sodium salt), 66.7 mg Terraclor (75% PCNB) and 50 mg Hymexazol) that had been thinly poured into 100-mm-diameter disposable Petri plates. The zoospore suspension was poured into the MISA, the MISA was sealed and the contents were pressurized at the desired pressure for 60 s. Ten wounded leaves were placed on paper towels on plastic trays under the MISA. The zoospore suspension was sprayed onto the abaxial surface of the leaves and into an open 100-mm diameter disposable Petri plate. The contents of the Petri dish halves were consolidated and three 200 µl aliquots were removed by pipette and plated onto PARPH-V8 medium. Both sets of plates were incubated in the dark at 20°C overnight and emerging colonies were counted at 24, 48 and 72 h. The leaves were placed on moistened paper towels inside a plastic container. The container was sealed and the leaves were incubated in the dark at 20°C for 14 days. The number of lesions was recorded for each set of ten leaves. Pieces of lesion tissue were removed and plated onto PARPH-V8 medium to confirm infection by P. ramorum. There were five replicates for each pressure and the test was repeated once. After each application of suspension, 100 ml of deionized water was sprayed through the MISA to remove any residual zoospores.

In a second assay, the amount of inoculum required for infection on detached rhododendron leaves was determined using simulated overhead irrigation. The study was conducted in the WSU Puyallup biocontainment unit. One pressure, 210 kPa (30 psi), and four
concentrations of zoospores (treatments) were tested: 3,000, 1,500, 300 and 150 zoospores/ml. A negative control (0 zoospores) was included. There were five replicates for each concentration and the test was repeated once. A single zoospore suspension was made containing approximately 150,000 zoospores/ ml. Varying amounts of suspension were pipetted into autoclaved deionized water inside 150-ml flasks to obtain 100 ml of zoospore suspension at the desired concentrations. Three 200µl aliquots were removed and plated onto three plates of hardened PARPH-V8 medium. The contents of the flask were poured into the MISA and pressurized for 60 s. The contents were sprayed onto the abaxial surface of ten wounded rhododendron leaves and into an open Petri plate as described above. The contents of the Petri plate halves were consolidated and three 200 µl aliquots were removed and plated onto three plates of hardened PARPH-V8 medium. The plates were incubated in the dark at 20°C and emerging colonies were counted at 24, 48 and 72 h. Leaves were incubated and processed as described above. Any residual zoospores were removed by spraying 100 ml of deionized water through the MISA after each application of suspension.

**Simulated nursery study.** Inoculum was applied to plants using the MISA previously described. The inoculum concentration before and after passage of water through the MISA was determined by direct plating of zoospores onto media, filtration, *Bottle O’ Bait* (BOB) and quantitative polymerase chain reaction (qPCR). Filtration of water through polycarbonate membrane filters and the incubation of filters on selective media has been used to detect and quantify *P. ramorum* in nursery water (14) and streams (12). BOB is a relatively new method in which a water sample is collected in a plastic bottle and baited with rhododendron leaves and disks and taken to a lab for incubation and identification (27). qPCR was performed on DNA extracted from *P. ramorum*
zoospores trapped on a filter. The work was conducted at the National Ornamental Research Site at Dominican University of California (NORS-DUC) and at WSU Puyallup from May through July 2013.

**Experimental design.** The experimental design was a split-plot with ten blocks. There were four main plots (inoculum level) within each block. Each main plot consisted of three subplots (host plants). One-gallon liners of *Rhododendron* x ‘Cunningham’s White’, *Viburnum tinus* x ‘Spring Bouquet’ and *Camellia* x ‘Kramer’s Supreme’ were obtained from a local nursery and kept in quarantine until the start of the experiment. No *P. ramorum* was found on any of the plants prior to placement in the test facility. There were three additional blocks that did not contain plants. These “blanks” were used to determine inoculum concentration before and after the zoospore suspension passed through the pressurized MISA. Each main plot was surrounded by a “stovepipe”, a thin sheet of ridged plastic shaped into a cylinder that acted as an irrigation chamber for the three plants (Fig. 2). The stovepipe was removed after the plants were inoculated. There was a separate stovepipe for each inoculum concentration. Four inoculum concentrations were applied using a separate MISA for each concentration: high (1,000 to 10,000 zoospores/ml); medium (100 to 1,000 zoospores/ml); low (1 to 100 zoospores/ml); and none (0 zoospores). Inoculum was applied twice to the plants, with a 48 h interval between applications using the stovepipe inoculum chambers each time. Inoculum quantification data from blank sets in the simulated nursery study were combined for the two inoculum applications.

**Inoculum preparation and application.** Two hundred cultures of a NA1 lineage isolate (Pr-1418886 from *Camellia* x ‘Coral Delight’, A2 mating type, collected from a nursery in Fairfax, Marin Co., CA) were grown on one-third V8 medium for 2 weeks. For sporangia formation, 80
culture plates were flooded with 7 ml of a 15% non-sterile soil extract solution (13) (7.5 g of field soil, 500 ml distilled water, mixed 4 h and allowed to settle 4 h, then filtered through two layers of Whatman No. 1 filter paper (GE Healthcare Life Sciences, Pittsburg, PA) and stored at 4°C for 24 h before the start of the test. Plates were kept at 20°C under natural lighting conditions. Zoospore release was performed by refrigerating the plates at 5°C for 2 h, and then bringing plates to room temperature for 1 h. Zoospore concentration was determined with a hemacytometer to be 2.9 x 10^5 zoospores/ml (inoculum application 1) and 3.2 x 10^5 zoospores/ml (inoculum application 2). For each inoculum application, the stock solution was diluted with deionized water to make two 1-liter bottles of zoospore inoculum for each inoculum concentration. A 100 ml aliquot was poured into the MISA, pressurized to 210 kPa for 30 s and sprayed onto the three plants within each stovepipe. After removal of the stovepipes, plants were kept moist by periodic misting for 10 min every 2 h from 8 a.m. to 6 p.m. for a 1-week period and then irrigated as needed with pathogen-free water via drip irrigation throughout the remainder of the test. Plants were monitored for signs of infection five times at two-week intervals. Pieces of symptomatic leaf tissue were cultured on PARPH-V8 medium and examined for colonization by *P. ramorum*. Rainfall, maximum and minimum daily temperatures for the 10 week period were obtained from the National Oceanic and Atmospheric Administration (NOAA) website (24) which were collected at the San Rafael Civic Center, CA US, approximately 3 km from the NORS-DUC site (Fig. 3).

Soil infestation by *P. ramorum* was determined at the end of 10 weeks by pouring 250 ml of deionized water through each pot and collecting 150 ml of soil leachate in 1-gallon Ziploc bags (SC Johnson, Racine, WI). The leachate was baited with one unwounded *Rhododendron x Nova Zembla*’ leaf and ten leaf disks. Baits were removed from the leachate after 48 h, rinsed
and mailed to WSU Puyallup. Disks were immediately plated onto PARPH-V8 medium upon arrival while leaves were incubated for 4 days in a moist chamber at 20°C. Symptomatic tissue was removed and plated onto PARPH-V8 medium and examined for colonization by *P. ramorum*.

**Verification of zoospore density:** To detect any differences in zoospore density before and after passing through the pressurized MISA, a 100 ml aliquot (non-pressurized) was removed from the zoospore suspension bottles and poured into a clean 250-ml Nalgene bottle (Thermo Fisher Scientific Inc., Waltham, MA). Next, a second 100 ml aliquot was poured into the MISA, pressurized and sprayed into a clean plastic bucket and the bucket contents were poured into a 250-ml bottle. One bottle of non-pressurized and one bottle of pressurized zoospore suspensions made up a “blank” set. Three sets were taken in total, one set after inoculation of the plants in block 3, 6 and 10.

Four methods were used to verify inoculum concentration of the blank sets. Direct plating was performed by pipetting 1 ml of zoospore solution from each Nalgene bottle onto 100-mm Petri dishes containing thinly-poured CARP medium [8.5 g corn meal agar, 10 mg Delvocid (50% pimaricin), 500 ml distilled water, 100 mg sodium ampicillin and 1 ml rifampicin solution (50 mg rifampicin dissolved in 10 ml Everclear @ 95% ABV)]. Plates were incubated at 20°C and emerging colonies were counted after 24 and 48 h. Serial dilutions were made with deionized water when necessary to get countable plates of approximately thirty colony-forming units (CFU) per plate. Filtration was performed by passing a 10 ml aliquot of zoospore suspension through a Whatman 3-µm pore size, 47-mm-diameter polycarbonate membrane filter (GE Healthcare Life Sciences, Pittsburgh, PA) using a Nalgene hand-operated vacuum pump.
Serial dilutions were made as described above. Filters were inverted and placed onto CARP media, incubated at 20°C overnight and CFUs were counted after 24 and 48 h.

The BOB quantification method was performed by transferring 10 ml of zoospore solution to a Falcon 50-ml tube (Corning Inc., Tewksbury, MA) in which ten *Rhododendron x Nova Zembla* leaf disks (~4mm) were added. Leaf disks were incubated at room temperature on the lab bench for 3 days, removed, patted dry with a paper towel and then plated onto PARPH-V8 medium. The numbers of infected leaf disks were counted for up to 5 days.

qPCR was performed by filtering 10 ml aliquots of zoospore solution as described above. Filters containing the trapped zoospores were placed into individual 2-ml screw-cap tubes and stored at -80°C until transport to Puyallup, Washington. Micro-tubes were packed in dry ice for transport. Zoosporic DNA was extracted using a NucleoSpin Plant II kit (Macherey-Nagel Inc., Bethlehem, PA) following the protocol for genomic DNA from plants with the following modification: cell lysis of zoospores was done following the method of Lefevre et al. (19). Filters were soaked overnight at 37°C in 12 µl of 1mg ml⁻¹ proteinase K and 500 µl of a buffer containing 1% SDS (sodium dodecyl sulfate) and TE (Tris-EDTA, 1mM EDTA and 10 mM Tris-HCl). A TaqMan Real-Time (quantitative or q) PCR assay, based on the APHIS protocol (39) was performed on a 7500 Sequence Detection System (Applied Biosystems) instrument. Each 25-µl qPCR reaction included 2 µl of DNA and 23 µl of master mix (12.5µl 2X TaqMan Universal PCR Master Mix (no AmpErase UNG), 0.0125µl Pram-114F (100µM), 0.05µl Pram-1527-190R (100µM), 0.025µl probe Pram-1527-134-T (100µM), 2.375µl Trehalose (50%), 8.0375 nuclease-free water). Cycling conditions were set at: 50°C for 2 min, 95°C for 10 min and 46 cycles of 95°C for 15 s, 58°C for 15 s and 60°C for 30 s. The *P. ramorum* probe was
labeled with a 5’ fluorescent FAM dye and non-fluorescent quencher. The optimal cutoff point (Ct cycle number) was determined using the Youden index in the context of the receiver operating characteristic (ROC) curve analysis as described by Nutz et al. (26). Samples with a FAM Ct value of 31 and above were considered to be negative. Standard curves were generated based on a dilution series of \textit{P. ramorum} DNA from isolate Pr-1418886 containing \(7 \times 10^{-1}\) to \(7 \times 10^{-6}\) ng of DNA. Each DNA sample including standards was assayed in triplicate. A water control was included as a negative control.

The estimated number of \textit{P. ramorum} zoospores was determined by the equation:

\[
Y = \frac{X}{0.0000665 \text{ ng DNA}}
\]

where \(Y\) = the estimated number of zoospores, \(X\) = the amount of recovered DNA (ng) and 0.0000665 = the DNA content of a single \textit{P. ramorum} zoospore (ng). The DNA content of one zoospore was determined by using the conversion factor of Dolezel et al. (7) where 1 pg of DNA represents \(0.978 \times 10^9\) base pairs (bp) and the \textit{P. ramorum} genome size of 65 Mb (\(0.065 \times 10^9\) bp) estimated by Tyler et al. (38). Assuming there is one copy of the genome per zoospore, the DNA content of a single \textit{P. ramorum} zoospore was determined by the equation:

\[
0.065 \times 10^9 \text{ bp} \div 0.978 \times 10^9 \text{ bp} = 0.0665 \text{ pg DNA (0.0000665 ng DNA)}
\]

**Statistical analysis.** Differences between the number of direct plate and filtration CFUs, infected leaf disks and the amount of recovered zoospore DNA from non-pressurized and pressurized zoospore suspensions were tested with a Wilcoxon signed-rank test (SAS 9.2, SAS Institute INC., Cary, NC). Rhododendron leaf lesion data (number of lesions/set of ten leaves) were subjected to analysis of variance (ANOVA) using the PROC MIXED procedure (SAS 9.2), where means were estimated as least-squares means (lsmeans) and \(n = 5\). The relationship
between zoospore concentration (zoospores/ml) and the number of lesions/set of ten leaves was assessed with simple linear regression (PROC REG; SAS 9.2, SAS Institute Inc., Cary, NC) using mean values of 5 replicates of each treatment (zoospore concentration). The data for the two trials was combined, \( n = 10 \). Before analysis, zoospore concentration and the number of lesions data were transformed using a logarithmic (base 10) transformation with a “1” added to the value for the number of lesions to retain the ability to analyze low values.

**RESULTS**

**Laboratory assays.** There were no significant differences between the number of CFU/ml that formed from aliquots taken from the non-pressurized or pressurized suspensions when pressures of 35, 210 and 420 kPa were used in the assays testing the effect of various pressures on zoospore viability (Fig. 4A and B); however, when a pressure of 630 kPa was used, there was a significant reduction of the number of CFUs that formed from the pressurized suspensions in trial 1 only (Fig. 4A). A single pressure of 210 kPa was used in the assays testing the inoculum threshold of *P. ramorum* zoospores on detached rhododendron leaves. There were no significant differences in the number of CFU/ml that formed from the pressurized and non-pressurized suspensions (data not shown). The numbers of CFUs that formed from the pressurized suspensions were higher than that of the non-pressurized suspensions in some cases (Fig. 4B) but the differences were not significant.

There was a trend of fewer lesions forming as the pressure increased in lab assays testing the effect of pressure on *P. ramorum* zoospore infectivity, but the number of lesions per set of ten leaves was only significantly lower between the control (35 kPa) and the highest pressure tested in trial 1 (Fig. 5A). There was not a significant difference in the number of lesions in the
leaf sets exposed to lower concentrations of zoospores in the inoculum threshold assays (Fig. 5B); however, the number of lesions per leaf set increased significantly at higher zoospore concentrations. A linear regression showed a strong relationship between zoospore concentration and the number of lesions per leaf set when both variables were log_{10} transformed (r^2=0.7685, df=38, p<0.0001) (Fig. 6). The regression line equation was used to estimate an inoculum threshold of 51 P. ramorum zoospores/ml required for infection of detached rhododendron leaves.

**Quantification of blank sets in the simulated nursery study.** The number of CFU/ml that formed from plating aliquots of non-pressurized and pressurized blank sets of P. ramorum zoospore suspension showed a similar trend for direct plating (Fig. 7A) and filtration (Fig. 7B). There was a significant decrease in the number of direct plate CFU/ml that formed from the pressurized inoculum at the high concentration (Fig. 7A) while the rest of the relationships were not significantly different from one another. Direct plate and filtration methods recovered approximately 10% of the target zoospores at the high, medium and low concentrations.

There was a significant decrease in the amount of recovered DNA between the non-pressurized and pressurized zoospore suspensions at the medium concentration (Fig. 8A). The amount of recovered DNA in the low, medium and high concentrations had an approximate 10-fold difference, similar to that of the target zoospore concentrations. The BOB method was able to detect P. ramorum zoospores at all inoculum concentrations (Fig. 8B) with approximately 80% bait colonization at the low level and 100% at the medium and high levels. There were no significant differences between the pressurized and non-pressurized blanks for the BOB method at any concentration.
Low levels of zoospores were detected in the blanks at the zero inoculum level of the pressurized and non-pressurized zoospore suspension by the BOB method (< 2% of BOB baits were colonized) and in the pressurized zoospore suspension by direct plating and filtration (< 1 CFU/ml). Detection of zoospores was probably due to cross-contamination at a very low inoculum concentration.

**Plant infection in the simulated nursery study.** A single *P. ramorum* foliar infection was found on one camellia plant during the first sampling period, 2 weeks after plants were inoculated, at the highest inoculum concentration (Fig. 3). The foliage of all other test plants was found to be negative for *P. ramorum*. While between 10 to 30% of the inoculated plants showed foliar symptoms (data not shown), these were likely due to infection by fungal pathogens such as *Botrytis* and *Pestalotia* spp, which were both seen in the isolation plates.

Soil leachate baiting at the end of the 10 weeks resulted in positive detections of *P. ramorum* from seven rhododendron, five viburnum and three camellia plants at the highest inoculum concentrations. No *P. ramorum* was detected in the leachate from any plants inoculated with the low or medium levels of inoculum. The soil leachate tested positive for *P. ramorum* in one rhododendron inoculated with the zero inoculum treatment, which might have resulted from cross-contamination as described above; or the possibility exists that inoculum may have washed off the inoculated foliage of an adjacent plant and into the soil of the control rhododendron during an irrigation event.

**DISCUSSION**

**Zoospore viability:** Overall, *P. ramorum* zoospore viability was not significantly reduced by pressurization and subsequent spraying of the zoospore suspension out of the MISA for air
pressures up to 630 kPa. There was only one incident when the number of CFU/ml of the pressurized zoospore suspension was significantly lower than the CFU/ml of the non-pressurized zoospore suspensions in the laboratory assays (Fig. 4A and B). Ahonsi et al. (1) found the number of CFUs that formed from non-pressurized zoospore suspensions of *Phytophthora nicotianae* did not differ significantly from those that were pressurized with air for 8 min at a pressure of 840 kPa. It was observed that most of the zoospores in the pressurized suspensions in the current research were encysted when viewed under a microscope. It is possible that the pressurization of the zoospore suspension in the MISA caused the zoospores to encyst, offering additional protection from damage as they passed through the spray nozzle. Encysted zoospores may have germinated at the same rate as motile zoospores when aliquots of the pressurized solution were plated. Because encysted zoospores tend to sink to the bottom of the solution, the significant difference seen in Fig. 4A may be due to the lack of thorough mixing of the pressurized zoospore solution to ensure homogeneity of the aliquots taken for CFU quantification.

Subjecting zoospore suspensions to increasing air pressures may have had a lesser effect on the number of lesions that formed on wounded detached rhododendron leaves than zoospore concentration (Fig. 5A and B). There was a significant decrease in the number of lesions that formed from zoospore suspensions pressurized at 630 kPa and those that formed from suspensions pressurized at 35 kPa in trial 1, but the same result was not observed in trial 2. The zoospore concentration was higher in trial 2 than in trial 1 (773 verses 1,415 zoospores/ml). The possibility exists that the higher concentration of zoospores in trial 2 might have diminished the effects of increasing pressurization on zoospore infectivity. More testing would need to be
conducted to determine if increasing air pressure lowered zoospore infectivity at lower zoospore concentrations.

An inoculum threshold concentration of 51 zoospores/ml was required for infection of detached wounded rhododendron leaves when leaves were incubated at 20°C and relative humidity was high. Similar inoculum thresholds were found for *P. ramorum* zoospores in studies where incubation conditions were comparable. Tooley et al. (37) found threshold estimates for low levels of infection by *P. ramorum* on detached leaves of *Rhododendron* x ‘Cunningham’s White’ to be 1.80 sporangia/ml (approximately 60 zoospores). Similarly, Widmer (44) reported *Rhododendron* x ‘Cunningham’s White’ leaf disks developed 80% necrotic surface area at a concentration of 75 zoospores/leaf disk. Mitchell et al. (23) found that a soil drench containing 50 motile *Phytophthora cryptogea* zoospores per plant was able to initiate infection on the roots of watercress (*Nasturtium officinale*), while Granke and Hausbeck (9) found an inoculum concentration of 100 *Phytophthora capsici* zoospores/ml caused infection on detached non-wounded cucumber (*Cucumis sativus*) fruit floated in spiked water.

Infection by *P. ramorum* occurred at a much higher concentration of zoospores, between 1,000 and 10,000 zoospores/ml, in the simulated nursery study. Tjosvold et al. (35) and Werres et al. (43) found low rates of foliar infections on potted rhododendrons that had been spray-inoculated with irrigation water containing low densities of *P. ramorum* zoospores, 0.001 and 74 zoospores/ml, respectively. Plants were watered multiple times with infested water throughout the spring and summer season in both studies while plants in this research were spray-inoculated twice at the beginning of the test. Multiple applications of infested water may have facilitated infection by *P. ramorum* zoospores in the previous two studies.
Many factors influence the onset of disease for *P. ramorum* hosts growing outdoors and include leaf wetness, temperature and ultraviolet light. When comparing the results of the lab and simulated nursery studies, these factors may explain why the laboratory results showed a much lower inoculum threshold and a much higher occurrence of disease. Humidity and temperature were optimal for infection in the lab assays (8). Temperatures were favorable for *P. ramorum* infection of plants in the simulated nursery study (Fig. 3) and inoculated plants were periodically misted; however, the leaves may have dried before the 6 to 12 h of free standing water required for infection (8) were met. Zoospores sprayed on the upper leaves may have been exposed to UV radiation, which has been shown to damage the zoospores of several *Phytophthora* spp. (16).

Finally, detached leaves were wounded before inoculum was applied while the leaves of the plants in the simulated nursery study were not. Wounded leaf tissue has been shown to develop more consistent infection by *Phytophthora* spp. in detached leaf assays (3, 20).

*P. ramorum* propagules were detected in the soil leachate of several pots that received the high inoculum treatment using rhododendron leaf disk baits at the end of 10 weeks. *P. ramorum* zoospores have been shown to infect rhododendron and viburnum roots (28, 32), infected roots of *Viburnum tinus* can produce sporangia (31) and infected rhododendron plants sometimes display symptomless above-ground parts (40). Recovered inoculum in the soil leachate may have been produced by infected roots in this study; however, the roots were not tested to confirm colonization by *P. ramorum*.

Direct plate and filtration methods gave comparable results for the number of CFU/ml that formed from the pressurized and non-pressurized zoospore solutions (Fig. 7A and B). Both methods recovered approximately 10% of the motile zoospores in the working solutions; a similar germination rate was reported by Kong et al. (17) and J. Hwang and S. Oak (personal
communication) for filtration and direct plating of *P. ramorum* zoospores. Although there was a significant decrease in the amount of CFUs that formed in the pressurized zoospore solution at the high zoospore concentration, results from filtration, qPCR and BOB did not show a significant difference between the pressurized and non-pressurized CFUs at this concentration. Because encysted zoospores tend to sink to the bottom of the solution, the significant difference may be due to the lack of properly mixing of the pressurized zoospore solution to help ensure an even distribution of motile and encysted zoospores before removing an aliquot to plate.

There was a significant decrease in the amount of recovered DNA in the pressurized zoospore solution at the medium concentration for qPCR, although this result was not repeated for the other quantification methods. As above, the significant difference could be due to improper mixing of the solution before removing an aliquot to filter. An estimation of the number of recovered zoospores is possible using the genome size of 0.065 x 10^9 bp for *P. ramorum* proposed by Tyler et al. (38) and the conversion factor of Dolezel et al. (7). Assuming there is one copy of the genome in each *P. ramorum* zoospore, the estimated number of zoospores for the non-pressurized and pressurized concentrations are determined by dividing the amount of recovered DNA (ng) by the content of DNA in one zoospore (0.0000665 ng). The estimated number of zoospores recovered from the non-pressurized and pressurized solutions are 106 and 108 (low), 1,212 and 773 (medium) and 9,470 and 8,417 (high). The estimations fall within the range of target zoospores, indicating that the qPCR method was able to accurately quantify the number of zoospores in pressurized and non-pressurized solutions.

The BOB method showed a great level of sensitivity and was able to detect *P. ramorum* zoospores at all zoospore concentrations (Fig. 8B), including low zoospore concentrations in the zero inoculum level. The colonization rate of the baits was 80% at the low zoospore
concentration and 100% at the medium and high concentrations, indicating the method lost precision at higher zoospore concentrations. Preliminary research results suggested that rhododendron leaf disk colonization ranged between 90 to 100% for *P. ramorum* zoospore concentrations between 50 to 100 CFU/ml (data not shown). The BOB method may not be able to accurately quantify zoospore concentrations above 400 zoospores/ml.

The MISA was designed to deliver pre-measured quantities of zoospore inoculum to plants in a manner simulating an overhead irrigation system in a nursery or greenhouse setting. Polyvinyl chloride (PVC) pipe and fittings used to assemble the chamber are available at most hardware stores that sell plumbing supplies and the material to construct the applicator cost less than US $20. Expansion couplings come in sizes that range from 1.27 to 15.24 cm (0.5 to 6 in), so applicators that hold a wide range of volumes of inoculum could be constructed. The MISA could be used in inoculum threshold studies, studies that examine the effect of various application pressure and spray mister-types on inoculum viability and infectivity, and in studies that examine host resistant or susceptibility to a specific pathogen. While the results of the current study would indicate pressurization and passage of *P. ramorum* zoospores through the spray mister did not lower zoospore viability or infectivity, tests would need be run to determine what effects, if any, pressurization and passage through a spray mister might have on the pathogen of interest.

The spray device used in this study was able to apply a specific amount of inoculum under variable pressures to containerized plants and detached leaves in a way that simulated over-head irrigation. The pressurization and spraying of *P. ramorum* zoospores out of the inoculum chamber did not significantly affect zoospore viability or infectivity on wounded detached rhododendron leaves. The inoculum threshold was found to be 51 zoospores/ml for
infection on wounded detached leaves and between 1,000 and 10,000 zoospores/ml for non-wounded whole plants in a simulated nursery study. Direct plating, filtration and qPCR methods were able to quantify *P. ramorum* zoospores at the low, medium and high concentrations and the BOB method was able to quantify zoospores densities between 1 and 100 zoospores/ml.
ACKNOWLEDGEMENTS

This research was possible with funding from the United States Department of Agriculture Animal and Plant Health Inspection Service. Cooperative Agreement 12-8130-0178-CA. Assistance from the staff at NORS-DUC and from members of the WSU Puyallup Ornamental Plant Pathology program at the Puyallup Research and Extension Center is acknowledged.
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Fig. 1. Measured-Inoculum Spray Applicator (MISA) diagram and materials list.
Fig. 2. In the simulated nursery study conducted at NORS-DUC, each main plot was surrounded by a “stovepipe” made of a sheet of ridged plastic shaped into a cylinder that acted as an irrigation chamber. *P. ramorum* zoospores of various concentrations were applied to the foliage of rhododendron, camellia and viburnum plants using a MISA to simulate overhead irrigation.

*Photos by Marianne Elliott*
Fig. 3. Precipitation, maximum and minimum daily temperatures during the 10 week simulated nursery study conducted at NORS-DUC. Foliage from one camellia plant tested positive for \textit{P. ramorum} and occurred at the highest inoculum concentration during the first sampling interval on 5/22/2014.
**Fig. 4.** The survival of *P. ramorum* zoospores in a non-pressurized and pressurized zoospore suspension (35, 210, 420 and 630 kPa) as measured by the number of germinating zoospores (CFU/ml) in trial 1 (A) and trial 2 (B). Zoospore concentrations were 773 (trial 1) and 1,415 (trial 2) zoospores/ml. The asterisk symbol (*) represents a significant difference between the number of CFU/ml in the non-pressurized and pressurized zoospore suspensions. Error bars represent standard error of the means, n = 15.
Fig. 5. Results of an assay testing the effect of various air pressures on zoospore infectivity (A) and (B) the results of an assay conducted to determine the inoculum threshold for infection of wounded detached rhododendron leaves. Two zoospore concentrations were used in (A): 773 zoospores/ml and 1,415 zoospores/ml for trial 1 and trial 2, respectively. Treatments labeled with the same letter did not differ significantly by PROC MIXED analysis. Error bars represent standard error of the means, n = 5.
Fig. 6. A linear regression between the log_{10} transformed variables *P. ramorum* zoospores/ml and the number of lesions/set of ten leaves, where n=10. Based on the regression line equation, an estimated density of 51 zoospores/ml was needed to cause infection on detached *Rhododendron* x ‘Nova Zembla’ leaves.
Fig. 7. The verification of *P. ramorum* zoospore density in three suspensions (high: 1,000 – 10,000; medium: 100 – 1,000; and low: 1 – 100 zoospores/ml) used to inoculate potted rhododendron, viburnum and camellia plants in a simulated nursery study. Methods used for verification were direct plating (A) and filtration of a zoospore suspension (B). The asterisk symbol (*) represents a significant difference between the number of CFU/ml in the non-pressurized and pressurized zoospore suspensions. Error bars represent standard error of the means, n = 3.
**Fig. 8.** The verification of *P. ramorum* zoospore density in three suspensions (high: 1,000 – 10,000; medium: 100 – 1,000; and low: 1 – 100 zoospores/ml) used to inoculate potted rhododendron, viburnum and camellia plants in a simulated nursery study. Methods used for verification were qPCR performed on zoospores trapped on a filter (A) and the BOB method (B). The asterisk symbol (*) represents a significant difference between the number of CFU/ml in the non-pressurized and pressurized zoospore suspensions. Error bars represent standard error of the means, n = 3.