In Situ Production of Zoospores by Five Species of *Phytophthora* in Aqueous Environments for Use as Inocula

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Abstract

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The goal of this study was to develop a procedure that could be used to evaluate the potential susceptibility of aquatic plants used in constructed wetlands to species of *Phytophthora* commonly found in nurseries. V8 agar plugs from actively growing cultures of three or four isolates of *Phytophthora cinnamomi*, *P. citrophthora*, *P. cryptogea*, *P. nicotianae*, and *P. palmivora* were used to produce inocula. In a laboratory experiment, plugs were placed in plastic cups and covered with 1.5% nonsterile soil extract solution (SES) for 29 days, and zoospore presence and activity in the solution were monitored at 2- or 3-day intervals with a rhododendron leaf disk baiting bioassay. In a greenhouse experiment, plugs of each species of *Phytophthora* were placed in plastic pots and covered with either SES or Milli-Q water for 13 days during both summer and winter months, and zoospore presence in the solutions were monitored at 3-day intervals with the baiting bioassay and by filtration. Zoospores were present in solutions throughout

the 29-day and 13-day experimental periods but consistency of zoospore release varied by species. In the laboratory experiment, colonization of leaf baits decreased over time for some species and often varied among isolates within a species. In the greenhouse experiment, bait colonization decreased over time in both summer and winter, varied among species of *Phytophthora* in the winter, and was better in Milli-Q water. Zoospore densities in solutions were greater in the summer than in the winter. Decreased zoospore activities for some species of *Phytophthora* were associated with prolonged temperatures below 13 or above 30°C in the greenhouse. Zoospores from plugs were released consistently in aqueous solutions for at least 13 days. This procedure can be used to provide in situ inocula for the five species of *Phytophthora* used in this study so that aquatic plant species can be evaluated for potential susceptibility.

Species of *Phytophthora* cause some of the most frequently occurring and economically important diseases of ornamental crops throughout the world, and *Phytophthora* root and crown rots and foliage blights cause extensive losses during production in nurseries, particularly to woody landscape plants (1,6,21). Furthermore, exotic species of *Phytophthora* are a threat to natural ecosystems and already have had a devastating impact on the eucalyptus forests of Australia (caused by *Phytophthora cinnamomi*) and the coastal forests in California and southwestern Oregon (caused by *P. ramorum*) (23,25,29). In North America, the danger *P. ramorum* poses to red oak forests in the eastern United States is a major concern (17,25) because *P. ramorum* can survive temperatures of 2 to 26°C (9) and has been found on plants in nurseries and natural ecosystems from British Columbia to southern California (21). Complicating quarantine and management efforts for *P. ramorum* and other species of *Phytophthora* are the presence of several sources of inocula in nurseries, which include many different host plant species, infested soils, container substrates, and irrigation and production runoff water that can facilitate pathogen movement and dispersal (1,6,7,21,25).

Vegetated buffers and constructed wetlands are ecologically based treatment options that effectively remove both nutrient and

chemical contaminants from runoff water at ornamental plant nurseries (26,28). However, methods of water treatment that utilize wetland plants could become sources of inocula if the plant material used in these systems serve as an alternative host for species of *Phytophthora*. Thus, when installing vegetated buffers or constructed wetlands at a nursery, it would be prudent to utilize plant species that are not susceptible to and do not harbor *Phytophthora* spp.; however, only limited information is available on the susceptibility of wetland plants to these ubiquitous plant pathogens.

There is an abundance of literature pertaining to pathogenicity of and plant susceptibility to species of *Phytophthora* (6), with most studies employing long-standing methods of inoculation using laboratory-produced propagules (e.g., zoospores, oospores, and chlamydospores) or some type of colonized substrate (e.g., agar plugs, vermiculite [24], or rice grains [10]). These procedures have been developed primarily to evaluate susceptibility of plants growing in soil or a soilless substrate and may not be sufficient to characterize the potential for infection by zoospores in infested runoff at a nursery.

Therefore, the objective of this study was to develop a procedure to produce inocula of *Phytophthora* spp. in situ that would be similar to the inocula found in nursery effluent so that wetland plants could be exposed to a continuous supply of zoospores in an aqueous environment. This procedure then could be used to evaluate potential susceptibility of wetland plants to species of *Phytophthora* under experimental conditions. The procedure should be effective for multiple species of *Phytophthora* under the different environmental conditions that occur throughout the year. Preliminary results have been reported (22).

Materials and Methods

***Phytophthora* spp.** Five species of *Phytophthora* commonly found in nurseries in the southeastern United States were selected for this study: *P. cinnamomi*, *P. citrophthora*, *P. cryptogea*, *P. nicotianae*, and *P. palmivora* (1,4,18,19). Three isolates of each species

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(all from a permanent collection maintained in the laboratory of S. N. Jeffers at Clemson University) were used for all experiments conducted. All 15 isolates were recovered from diseased ornamental plants in South Carolina that were submitted to the Clemson University Plant Problem Clinic between 1997 and 2010 (Table 1). A fourth isolate of *P. citrophthora* also was used; this isolate was collected from the roots of *Sagittaria latifolia*, a wetland plant growing in a vegetated runoff channel at a nursery in Georgia (Table 1). Active cultures of all isolates were maintained on PARPH-V8, a medium selective for species of *Phytophthora* (7).

Isolate characterization. The identities of the 16 isolates of *Phytophthora* spp. used in this study were confirmed using selected phenotypic characteristics (oospore production and sporangium morphology) and molecular diagnostics (restriction fragment length polymorphism [RFLP] and sequence analysis of the nuclear ribosomal internal transcribed spacer [ITS] region).

Morphological characteristics. To produce oospores, isolates were grown on super-clarified V8 juice agar (scV8A; 14) at 20°C in the dark for 6 weeks. Isolates that produced oospores were classified as homothallic and those that did not produce oospores were classified as heterothallic. To produce sporangia, isolates were grown on 10% V8 juice agar (V8A; 100 ml of V8 juice, 1 g of CaCO₃, 15 g of Bacto agar, and 900 ml of distilled water) for 3 to 5 days in the dark at 20°C. Plugs (5 mm in diameter) from the advancing margin of a colony were transferred to a 60-mm-diameter petri dish and covered with 1.5% nonsterile soil extract solution (SES; 13). Petri plates were placed under continuous fluorescent light at room temperature (22 to 25°C) and sporangia were examined after 12 to 24 h.

Molecular diagnostics. Mycelium mats of each isolate were grown in 10% clarified V8 broth (cV8B; 100 ml of buffered and clarified V8 juice [7] and 900 ml of distilled water) for 5 days at 20°C in the dark, and then mats were harvested by vacuum filtration, rinsed with distilled water, and frozen at -20°C (3). Total DNA was extracted using a modified protocol for the DNeasy Plant Mini Kit (Qiagen Inc.), and polymerase chain reaction (PCR) was conducted using universal primers ITS 4 and ITS 6 (2,3). A RFLP fingerprint for each isolate was obtained using restriction enzymes *AluI* and *MspI* (5). In addition, PCR products from all isolates were purified and then submitted to the Clemson University Genomics Institute for Sanger sequencing, and sequences were compared and matched with sequences of isolates deposited in the *Phytophthora* Database at Pennsylvania State University (20).

Zoospore production. For all experiments, isolates initially were grown on cV8A to ensure culture purity and then on V8A at 25°C in the dark for 3 to 4 days to produce actively growing colonies for sporangium production. A disinfested cork borer was used to cut agar plugs (5 mm in diameter) containing actively growing hyphae from the advancing edge of a colony. V8A plugs were placed on the bottom of containers and covered with an aqueous solution to stimulate natural production of sporangia and subsequent release of zoospores.

Laboratory experiment. A laboratory experiment was conducted to determine the consistency and duration of zoospore release from colonized V8A plugs. The experiment utilized a randomized complete block design with one replicate of each treatment in each of three blocks. Treatments consisted of V8A plugs from an isolate of one of the five species of *Phytophthora*. Nine agar plugs from one isolate were placed on the bottom of a 260-ml clear plastic cup. Each cup was a replicate; thus, there were three replicate cups per treatment. This experiment was conducted twice as trials one and two.

Each cup was filled with 150 ml of SES (7,13) and placed on one of three trays (blocks) in racks in the laboratory at ambient light ($4.55 \pm 1.0 \mu\text{mol m}^{-2}\cdot\text{s}^{-1}$) and temperature. Average daily temperatures were 21.6 ± 0.9 to $26.4 \pm 0.8^\circ\text{C}$ in trial 1 and 20.5 ± 0.4 to $24.1 \pm 0.5^\circ\text{C}$ in trial 2. Cups were monitored for 28 days after the experiment was initiated; therefore, each trial lasted 29 days. On the second day of each trial, six leaf disks (5 mm in diameter) from *Rhododendron catawbiense* 'English Roseum' were floated on the surface of the SES in each cup. In trial 1, leaf disks were removed and replaced every 2 days; in trial 2, leaf disks were removed and replaced every 3 days. After removal, leaf disks were blotted dry and embedded in PARPH-V8 selective medium, and plates were held at 25°C in the dark for 24 to 30 h. Leaf disks were examined microscopically ($\times 30$ to 70) and the presence of zoospores in solutions was confirmed based on mycelium growth from colonized leaf disks. The extent of colonization was quantified using a rating scale (0 to 5) based on the percentage of the perimeter of each disk from which mycelium grew (Table 2).

Greenhouse experiment. Zoospore production in aqueous environments was evaluated in a greenhouse to determine the impact of environmental conditions at different times of the year because future experiments utilizing this procedure will be conducted in a greenhouse to evaluate wetland plants for potential susceptibility to these species of *Phytophthora*. The same five species and isolates of *Phytophthora* were used and two types of aqueous solution were

Table 1. Sources of 16 isolates of five species of *Phytophthora* used in this study^y

Species, isolate number	Host plant	Isolated from	County ^z	Location
<i>Phytophthora cinnamomi</i>				
SC.02-0912	<i>Itea virginica</i> 'Little Henry'	Roots	Pickens	Landscape
SC.02-1054	<i>Rosa banksiae</i>	Roots	Lexington	Landscape
SC.10-0053	<i>Viburnum obovatum</i>	Roots	Hampton	Landscape
<i>P. citrophthora</i>				
SC.97-0673	<i>Rosa</i> hybrid 'Livin Easy'	Roots	Berkeley	Nursery
SC.07-0248	<i>Rosa</i> hybrid 'Home Run'	Roots	York	Nursery
SC.07-0303	<i>Heuchera</i> hybrid 'City Lights'	Crown	Aiken	Nursery
S.lat 3.5	<i>Sagittaria latifolia</i>	Roots	Grady, GA	Nursery
<i>P. cryptogea</i>				
SC.03-0222	<i>Dicentra</i> hybrid 'King of Hearts'	Roots	York	Nursery
SC.05-0491	<i>Sedum spurium</i>	Stem	Yor	Nursery
SC.06-0989	<i>Euphorbia amygdaloides</i>	Roots	Aiken	Nursery
<i>P. nicotianae</i>				
SC.05-0690	<i>Hibiscus paramutabilis</i> $\times$ <i>syriacus</i> 'Lohengrin'	Stem	Edgefield	Nursery
SC.06-0496	<i>Perovskia</i> sp.	Roots	York	Nursery
SC.07-1391	<i>Rosa</i> hybrid 'The Fairy'	Roots	Berkeley	Nursery
<i>P. palmivora</i>				
SC.98-0092	<i>Pinckneya pubens</i>	Roots	Aiken	Nursery
SC.98-0177	<i>Juniperus</i> sp.	Roots	Charleston	Landscape
SC.02-0875	<i>Nerium oleander</i>	Roots	Georgetown	Landscape

^y Isolates from South Carolina were recovered from samples submitted to the Clemson University Plant Problem Clinic. Isolate S.lat 3.5 was isolated by G. A. Ridge. All isolates are maintained in a permanent collection at Clemson University.

^z All counties are in South Carolina, except one in Georgia (GA).

compared. The experiment utilized a randomized complete block design with three replicate containers for each treatment, with one replicate in each of three blocks.

Zoospore production by the five species of *Phytophthora* was compared in two aqueous solutions: Milli-Q (EMD Millipore Corporation) water and SES. Each 15 liters of either solution was amended with 0.30 g of 20-2-20 nitrate-special water-soluble fertilizer (Southern Agricultural Insecticides, Inc.). Large batches of SES were made by mixing 225 g of soil in 15 liters of Milli-Q water for 1 h; the soil used for SES was tested with a baiting bioassay before use to ensure that it was free of *Phytophthora* spp.

The effect of season on zoospore production was investigated by conducting two trials in summer and two trials in winter months (i.e., four trials in all). The duration of each greenhouse trial was 13 days, based on results from the laboratory experiment. The two summer trials were conducted from 21 August to 3 September 2011 and from 6 to 18 September 2012. The two winter trials were conducted from 1 to 14 December and 8 to 22 December 2011.

Fifteen 1.9-liter nursery containers (Amerikan Nursery Products, Inc.) were filled with each solution, and 9 or 12 V8A plugs with active mycelium, all of one species, were placed in the bottom of a container: 3 plugs from each of three isolates of *P. cinnamomi*, *P. cryptogea*, *P. nicotianae*, and *P. palmivora* and from each of four isolates of *P. citrophthora*. On the second day of each trial, all containers were assayed for zoospores by floating six rhododendron leaf disks (5 mm in diameter) on the surface of the aqueous solution. After 3 days, leaf disks were removed, blotted dry, and embedded in PARPH-V8 medium, and isolation plates were held at 25°C in the dark for 1 day. The presence of zoospores in each container was verified by examining leaf disks for growth of hyphae of *Phytophthora* spp., and bait colonization was quantified using the 0-to-5 rating scale described previously (Table 2). After disks were removed, six fresh leaf disks were added to each container, and this process was continued over the 13-day exposure period.

In addition to the baiting bioassay, zoospore densities in solutions were monitored directly by filtration in three of the four trials (12): the second summer trial and both winter trials. Two 25-ml aliquots of water from each pot were filtered on days 4, 7, and 13 and each aliquot was passed through a 47-mm-diameter membrane filter with 5- μ m pores (Durapore SVLP04700; Millipore Corporation) under vacuum. Filters were inverted on PARPH-V8 medium and plates were held at 20°C in the dark. Approximately 100 ml of aqueous solution was added to each container after aliquots were collected to replace liquid removed for filtration and lost to evaporation. After 2 days, filters were removed, the agar surface was rinsed under running tap water, and colonies of *Phytophthora* spp. were counted. Zoospore densities were quantified as the number of CFU in 25 ml.

Statistical analyses. Data were analyzed using factorial analysis of variance (ANOVA) techniques to determine whether the main effects and interactions of isolate, species of *Phytophthora*, solution (Milli-Q water and SES), and exposure period (i.e., time = days after inocula were added) influenced the percentages of leaf

Table 2. Rating scale used to quantify the amount of the perimeter of a rhododendron leaf disk colonized by *Phytophthora* spp.

Value	Perimeter colonized (%) ^y	Midpoint value (%) ^z
0	None	0
1	1–25	12.5
2	26–50	38.5
3	51–75	63.5
4	76–99	88.5
5	100	100

^y Estimated amount of the perimeter on a leaf disk, 5 mm in diameter, from which hyphae of *Phytophthora* spp. grew. Leaf disks, which were used to bait *Phytophthora* spp. in aqueous solutions, were blotted dry and then embedded in PARPH-V8 selective medium; isolation plates were placed at 25°C in the dark for 24 to 30 h. Leaf disks were observed at $\times 20$ to 40.

^z Midpoint value for each range that was used in statistical analyses.

disk perimeters colonized. The ANOVA tests were adjusted for random effects (blocks and disks) and repeated measures over time. If interactions were found to be significant, simple effects of individual treatments were evaluated. When main effect or simple effects were found to be significant, treatment means were separated using Fisher's least significant difference. All hypothesis tests were conducted with $\alpha = 0.05$. Data were analyzed using JMP v.10.0 software (SAS Institute).

Results

In both the laboratory and greenhouse experiments, colonization of leaf disk baits floating on the surface of aqueous solutions served as a direct indication that zoospores had been released from sporangia produced on the agar plugs placed on the bottom of containers (i.e., plastic cups and nursery containers, respectively); agar plugs did not float and remained submerged throughout each trial. In addition, the mean percentage of the perimeter of leaf disks colonized was used as an indirect measure of zoospore activity in a solution. Therefore, quantitative comparisons among treatments could be made and the activity of zoospores over time could be evaluated. In the greenhouse experiment, data on leaf disk colo-

Fig. 1. Colonization of leaf disk baits while exposed for 29 days to zoospores released from agar plugs of three or four isolates of five species of *Phytophthora*. Data are means of six replicates from two trials. Percentage of the perimeter of each leaf bait that was colonized was estimated every 2 (trial 1) or 3 (trial 2) days. Specific isolates for each species are those listed in Table 3.

nization was corroborated with zoospore density (i.e., direct enumeration of zoospores in 25-ml aliquots of solution using a filtration assay).

Isolate identification. Morphological characteristics and molecular diagnostics confirmed the identities of all 16 isolates used in this study (Table 1). None of the isolates produced oospores in single-strain culture on scV8A after incubation for 6 weeks at 20°C in the dark. Therefore, all isolates were classified as heterothallic, which is consistent with the species identification of each isolate.

Laboratory experiment. Each of the 16 isolates produced zoospores and these zoospores were able to infect and colonize leaf baits throughout the 29-day exposure period used in all trials (Fig. 1). There was a significant interaction between the experimental factors exposure period (= time) and species (Table 3), indicating that bait colonization among the species of *Phytophthora* was not consistent over time. Likewise, colonization varied significantly over the 29-day exposure period and among isolates within a species (Table 3). These differences were evident when bait colonization by individual isolates was plotted over time (Fig. 1). Colonization of leaf baits was most variable among the isolates of *P. citrophthora* and *P. nicotianae* and did not differ significantly among the isolates of *P. cinnamomi*, *P. cryptogea*, and *P. palmivora* (Table 3; Fig. 1).

During the 29-day exposure period, leaf disk colonization among the three or four isolates of each of the five species of *Phytophthora* varied. Variability in the amount of leaf bait colonized increased over time and was most evident after 14 days for isolates of *P. citrophthora*, *P. cryptogea*, and *P. nicotianae*. Leaf disk colonization was much less variable over time among isolates of *P. cinnamomi* and *P. palmivora*; however, colonization of leaf baits by *P. cinnamomi* varied greatly over the 29-day experiment, ranging from 100 to 15% of the perimeter colonized (Fig. 1). The ability of the five species of *Phytophthora* to release zoospores and colonize leaf baits varied somewhat when averaged over the entire exposure period but this difference was not significant ($P = 0.087$, Table 3).

Greenhouse experiment. Based on results from the laboratory experiment, zoospore release from agar plugs was most consistent over the first 14 days; therefore, an exposure period of 13 days was used in this experiment. Leaf baits were infected by zoospores from all five species of *Phytophthora* throughout the 13-day exposure periods in trials conducted under both summer and winter

conditions. However, leaf bait colonization by *P. nicotianae* and *P. palmivora* decreased steadily throughout the exposure periods during trials conducted in the winter (Fig. 2). During this experiment, the average air temperature in the greenhouse in winter was 13.5°C and in summer was 24.7°C. Trials 1 and 2 in the summer had mean solution temperatures of 28.1 ± 0.1 and 25.1 ± 0.1 °C during the day and 25.4 ± 0.1 and 22.3 ± 0.1 °C at night, respectively. Trials 1 and 2 in the winter had mean solution temperatures of 15.5 ± 0.2 and 16.1 ± 0.2 °C during the day and 10.8 ± 0.1 and 10.7 ± 0.1 °C at night, respectively.

When bait colonization data over time (Fig. 2) were examined by ANOVA (Table 4), there were no interactions among the three experimental factors (species, time, and solution) in either summer or winter trials. However, there were differences in bait colonization over time in both winter ($P = 0.020$) and summer ($P = 0.002$) trials for all species of *Phytophthora* combined. There was also a difference in bait colonization among *Phytophthora* spp. in the two trials conducted in the winter ($P = 0.029$). Time was a significant factor in this experiment, with bait colonization decreasing over the 13-day exposure period in both summer and winter trials (Table 4). Colonization of leaf disks for all species combined over time was consistently better in Milli-Q water than in SES (Fig. 2); however, this difference was not statistically significant for either the summer or winter trials (Table 4). The difference between Milli-Q water and SES was most notable for *P. citrophthora* in the winter, when colonization of leaf baits floated on Milli-Q water was nearly 100% over the 13 days, whereas colonization of baits in SES varied between 40 and 60% (Fig. 2). During winter trials, leaf bait colonization by *P. nicotianae* was significantly lower than that by *P. cinnamomi* and *P. cryptogea*, and colonization by *P. palmivora* was significantly lower than that by *P. cinnamomi*, *P. citrophthora*, and *P. cryptogea* (Table 4).

In addition to colonization of leaf baits, zoospore release in treatment solutions was verified by direct enumeration of CFU in 25-ml aliquots using a filtration assay. Zoospores of all five species were present throughout the 13-day exposure periods in trials conducted in both summer and winter, although more zoospores usually were present in the summer (Fig. 3). When filtration data were analyzed (Table 5), only one of the possible interactions among the three experimental factors (species, time, and solution) was significant (species-solution in summer, $P < 0.001$). In the summer, zoospore densities were greater in Milli-Q water, decreased over time, and were highest for *P. palmivora*. In

Table 3. Differences among isolates within each of five species of *Phytophthora* for ability to release zoospores and colonize rhododendron leaf disks over a 29-day exposure period (= time) in the laboratory experiment^a

Species, effects	Perimeter colonized (%) ^y				Two-way ANOVA ^z		
	Isolate 1	Isolate 2	Isolate 3	Isolate 4	df	F value	P > F
<i>Phytophthora cinnamomi</i>	58 de	55 de	66 cde				
<i>P. citrophthora</i>	85 abc	49 e	61 cde	97 a	...	...	...
<i>P. cryptogea</i>	72 bcde	83 abc	76 abcd	...	...	...	...
<i>P. nicotianae</i>	97 a	90 ab	57 de	...	...	...	...
<i>P. palmivora</i>	99 a	99 a	99 a	...	...	...	...
Main effects							
Species	...	...	...	...	4	13.75	0.087
Isolates within a species	...	...	...	...	11	5.24	0.047
Time	...	...	...	...	17	7.90	<0.001
Trial	...	...	...	...	1	0.67	0.450
Error _A	...	...	...	...	15	...	...
Two-way interactions							
Species × time	...	...	...	...	68	2.04	0.005
Isolates within a species × time	...	...	...	...	187	0.62	0.986
Error _B	...	...	...	...	11	...	...

^a Three or four isolates (numbered 1 through 4) were used for each species. Agar plugs were placed in 260-ml plastic cups and covered with 150 ml of nonsterile soil extract solution.

^y Mean percentage of the perimeter of leaf baits colonized by an isolate of *Phytophthora* sp. for all sample times combined. Means of all 16 isolates were compared, and those with a letter in common are not significantly different based on Fisher's protected least significant difference (LSD) with an LSD value of 24 ($\alpha = 0.05$).

^z Results for two trials were combined, with three replicates of each treatment per trial. Data were analyzed by two-way analysis of variance (ANOVA) with trial as a random effect and repeated measures over time.

the winter, zoospore densities were similar in both solutions, did not vary significantly over time, but did vary among species. *P. citrophthora* and *P. cryptogea* produced more zoospores under winter conditions in the greenhouse than did the other three species. Colonization of leaf disk baits and zoospore densities were not correlated ($P > 0.05$) in any of the four trials when correlation coefficients (r) were calculated.

Discussion

In this study, a standard method of producing sporangia and small amounts of zoospores of *Phytophthora* spp. for taxonomic

studies (6,7,13) was used to produce a continuous supply of zoospores in aqueous solutions for up to 29 days. It is known that sporangia are produced on V8A plugs placed in solutions such as water and SES and that zoospores are released in 24 to 48 h (6,7,13) but the duration of zoospore release from a single set of flooded agar plugs has not been studied. In our study, colonization of rhododendron leaf disks floating on the surface of aqueous solutions was used as direct evidence that zoospores were present in these aqueous solutions. Likewise, the amount of the perimeter of the disks colonized was used as a measure of zoospore activity because zoospores are known to be both negatively geotropic and

Fig. 2. Percent colonization of the perimeter of leaf disk baits during exposure for 13 days to zoospores of each of five species of *Phytophthora* in a greenhouse. Agar plugs from three or four isolates of a species were combined and placed in a container containing 1.5% nonsterile soil extract solution (SES) or Milli-Q water during summer and winter months. Data are means $\pm$ standard errors ($n = 6$) from two trials.

chemotactic, at least in laboratory studies (6). Therefore, once these propagules were released from sporangia into solution at the bottom of the containers, they rose to the surface and sought out susceptible host tissue (i.e., the rhododendron leaf disks that were used as baits). Therefore, a reduction in the percentage of the leaf disk perimeter colonized was attributed to either decreased zoospore release from agar plugs or reduction in the activity of the zoospores in solution. Interestingly, leaf disk colonization (percentage of perimeter colonized) and zoospore density (number of zoospores in 25 ml of solution) were not significantly correlated when correlation coefficients were calculated. Therefore, number of zoospores in solution was closely related to amount of leaf bait tissue colonized. This probably is due to differences in virulence and aggressiveness among the isolates of the various species and also differences among species. These differences would affect the number of zoospores needed to infect leaf disks and the subsequent ability of an isolate to colonize leaf tissue. Overall, leaf bait colonization was a better measure of zoospore activity because it took into account both numbers of zoospores present in solution and the aggressiveness and virulence of individual isolates.

Bait colonization among isolates within a species of *Phytophthora* varied in the laboratory experiment, and this most likely was due to differences in zoospore density, differences in virulence and aggressiveness among isolates, or a combination of both. Zoospore densities were not determined in the laboratory experiment. Test isolates originated from a variety of host plants, and variation in virulence among isolates within a species of *Phytophthora* has been reported. For example, 40 isolates of *P. nicotianae* from an-

nual vinca varied in virulence when different cultivars of this host were inoculated (15), a worldwide collection of isolates of *P. capsici* varied in virulence when inoculated on the fruits of tomato and various cucurbits (8), and isolates of *P. ramorum* varied in virulence when coast live oak and California bay laurel were inoculated (11).

Leaf disk colonization by *P. cinnamomi* varied considerably over the 29-day exposure period in the laboratory experiment but bait colonization values among isolates of this species at specific sample times were similar. This uniform variation in zoospore activity among isolates may lie in the inherent biology of *P. cinnamomi*. *P. cinnamomi* is known to be a poor producer of sporangia in aquatic laboratory environments (6). It is possible that the three isolates of this species responded synchronously and produced sporangia and zoospores intermittently but consistently. More importantly, it is of interest that *P. cinnamomi* produced zoospores consistently in all trials of the two experiments conducted in this study, despite its reputation of limited sporangium production.

During the 29-day exposure periods in the laboratory experiment, all isolates of the five species of *Phytophthora* released zoospores that consistently infected rhododendron leaf baits. However, after 14 days, a general decline in leaf bait colonization and increased variation among isolates within species were observed. Therefore, the exposure period in the greenhouse experiment was reduced to 13 days. The greenhouse experiment was conducted in both summer and winter months to evaluate the effects of seasonal conditions on zoospore activity. Both bait colonization for some species and zoospore densities of all species were lower in winter

Table 4. Differences among five species of *Phytophthora* in ability to release zoospores and colonize rhododendron leaf baits in two aqueous solutions over a 13-day period (= time) in the greenhouse experiment, which was conducted in summer and winter months^x

Factor, level	Perimeter colonized (%) ^y		Three-way ANOVA ($P > F$) ^z	
	Summer	Winter	Summer	Winter
Species				
<i>Phytophthora cinnamomi</i>	95.5	92.6 a	...	...
<i>P. cryptogea</i>	76.8	92.5 a	...	...
<i>P. citrophthora</i>	90.6	76.1 ab	...	...
<i>P. nicotianae</i>	91.2	60.2 bc	...	...
<i>P. palmivora</i>	99.2	42.0 c	...	...
LSD	ns	30.3	...	...
Time (days)				
4	100.0 a	88.1 a	...	...
7	94.0 ab	77.1 ab	...	...
10	89.7 b	57.9 b	...	...
13	79.0 c	67.6 b	...	...
LSD	10.2	19.2	...	...
Solution				
Milli-Q water	94.1	80.5	...	...
Soil extract solution	87.2	64.8	...	...
LSD	ns	ns	...	...
ANOVA				
Species	...	...	0.656	0.029
Time	...	...	0.002	0.020
Solution	...	...	0.060	0.068
Species × time	...	...	0.394	0.352
Species × solution	...	...	0.519	0.284
Time × solution	...	...	0.141	0.100
Species × time × solution	...	...	0.950	0.100

^x Three or four isolates were used for each species. Agar plugs were placed in 1.9-liter plastic containers and covered with nonsterile soil extract solution or Milli-Q water.

^y Mean percentage of the perimeter of leaf baits colonized by a species of *Phytophthora*. Data for summer and winter were analyzed separately. Means in each column with a letter in common are not significantly different based on Fisher's protected least significant difference (LSD; $P = 0.05$); ns = not significantly different.

^z Results for two trials were combined, with three replicates of each treatment per trial. Data were analyzed by three-way analysis of variance (ANOVA) with trial as a random effect and repeated measures over time.

Table 5. Differences among five species of *Phytophthora* in numbers of zoospores (measured as CFU) in 25 ml of treatment solution over a 13-day period in the greenhouse experiment, which was conducted in summer and winter months^x

Factor, level	CFU ^y		Three-way ANOVA ($P > F$) ^z	
	Summer	Winter	Summer	Winter
Species				
<i>Phytophthora cinnamomi</i>	18.6 b	4.9 b	...	...
<i>P. citrophthora</i>	29.0 b	12.0 a	...	...
<i>P. cryptogea</i>	20.0 b	13.0 a	...	...
<i>P. nicotianae</i>	20.0 b	6.5 b	...	...
<i>P. palmivora</i>	64.1 a	2.4 b	...	...
LSD	12.4	4.1	...	...
Time (days)				
4	41.0 a	8.3	...	...
7	md	8.2	...	...
13	19.7 b	6.7	...	...
LSD	7.8	ns	...	...
Solution				
Milli-Q water	45.6 a	8.3	...	...
Soil extract solution	15.1 b	7.2	...	...
LSD	7.8	ns	...	...
ANOVA				
Species	...	...	<0.001	0.007
Time	...	...	<0.001	0.963
Solution	...	...	<0.001	0.474
Species × time	...	...	0.113	0.694
Species × solution	...	...	<0.001	0.140
Time × solution	...	...	0.112	0.837
Species × time × solution	...	...	0.096	0.290

^x Three or four isolates were used for each species. Agar plugs were placed in 1.9-liter plastic containers and covered with nonsterile soil extract solution or Milli-Q water.

^y Number of CFU present in a solution was determined by filtration. Data for summer and winter were analyzed separately. Means in each column with a letter in common are not significantly different based on Fisher's protected least significant difference (LSD; $P = 0.05$); md = missing datum because water samples were not collected at 7 days in the summer trials; ns = not significantly different.

^z Results for two trials were combined, with three replicates of each treatment per trial. Data were analyzed by three-way analysis of variance (ANOVA), with trial as a random effect and repeated measures over time.

trials than in summer trials. Temperatures in the greenhouse were lower in the winter than in the summer, and this resulted in lower solution temperatures, which may have affected production of sporangia or activity of zoospores. In particular, bait colonization by *P. nicotianae* and *P. palmivora* declined through the 13-day exposure periods in the winter trials, and bait colonization in SES by *P. citrophthora* was relatively low throughout the winter trials. The optimum growing temperatures for *P. nicotianae* and *P. palmivora* are 25 to 30°C (6,27) and the average temperature of container solutions during our winter trials was only 13°C.

The gradual decline of leaf disk colonization by *P. cryptogea* during the summer trials in the greenhouse may also be attributed to solution temperature. Bait colonization for *P. cryptogea* during

summer trial 1 was significantly different from that in summer trial 2 (*data not presented*). The decline in ability to infect leaf baits was only observed during the first summer trial, when the average temperature of container solutions was 28°C during the day and 25°C at night, with extended periods of solution temperatures above 30°C. Previously, MacDonald and Duniway (16) reported that prolonged exposure to temperatures $\geq 30^{\circ}\text{C}$ resulted in a consistent decline in the ability of sporangia of *P. cryptogea* to discharge zoospores. The optimum temperature for growth for *P. cryptogea* is 20 to 25°C, with the upper limits for zoospore release being 33°C (6,16). Consequently, excessive daytime solution temperatures during the first summer trial may have adversely affected zoospore release by *P. cryptogea*. Mean daytime water tempera-

Fig. 3. Numbers of zoospores produced by five species of *Phytophthora* and present in 25 ml of treatment solution during summer and winter months in the greenhouse experiment. Solutions were sampled twice in one summer trial ($n = 3$) and three times in two winter trials ($n = 6$) over a 13-day period. Numbers were determined by filtration and data are mean numbers of CFU $\pm$ standard errors. Scales for the y-axes for winter and summer graphs are different.

tures during the second summer trial were 25°C during the day and 22°C at night, and no decline in leaf disk colonization by *P. cryptogea* was recorded during this trial.

The type of treatment solution seemed to affect the number of zoospores present in these solutions, with greater densities of zoospores present in Milli-Q water than in SES. This was particularly true for *P. palmivora* during the summer trials; however, leaf bait colonization was not adversely affected. This disparity leads one to wonder how much inoculum must be present to cause infection. During the winter, infection of leaf baits was more variable than in summer and lower numbers of zoospores were present in both solutions; therefore, reduced zoospore availability may have contributed to this increased variability.

It is unclear why both zoospore density and bait colonization were reduced in SES compared with Milli-Q water. SES commonly is used to induce sporangium formation in the laboratory (6,7,13); therefore, we presumed it would work as well as, or better than, Milli-Q water in the greenhouse environment; however, this was not the case. Some potential explanations could involve either competition or interaction with the microbial population or a negative influence of salts or other ions in the SES. Because Milli-Q is ultrafiltered water, there were no or very few microbes and few chemical contaminants present to affect sporangium formation and zoospore survival.

This research was conducted to develop a method to inoculate wetland plants used in vegetative buffers and constructed wetlands so that these plants could be evaluated for potential susceptibility to species of *Phytophthora* present in runoff water at nurseries growing ornamental plants in the southeastern United States. We have successfully developed a routine inoculation procedure that can be used in future studies investigating potential susceptibility. Plants can be exposed to zoospores of the five species of *Phytophthora* used in this study (*P. cinnamomi*, *P. citrophthora*, *P. cryptogea*, *P. nicotianae*, and *P. palmivora*) but other species of *Phytophthora* easily could be included. At least three isolates of each species and three V&A plugs from actively growing cultures of each isolate should be used because isolates were shown to vary in ability to colonize baits. Isolates of the same species can be pooled but it would be interesting to determine whether isolates of different species also could be combined to streamline the procedure. Fresh agar plugs should be added to each container every 14 days throughout the test period to maintain adequate levels of inocula. Wetland plant species should be exposed to species of *Phytophthora* during both summer and winter months because zoospores of all species were most abundant during summer but cooler winter temperatures may be more favorable to some species of *Phytophthora*.

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