From Forest Nursery Notes, Summer 2009

143. Infective potential of sporangia and zoospores of *Phytophthora ramorum*.
Widmer, T. L. Plant Disease 93:30-35. 2009.

Infective Potential of Sporangia and Zoospores of *Phytophthora ramorum*

T. L. Widmer, Foreign Disease and Weed Science Research Unit, USDA/ARS, 1301 Ditto Avenue, Fort Detrick, MD 21702

ABSTRACT

Widmer, T. L. 2009. Infective potential of sporangia and zoospores of *Phytophthora ramorum*. Plant Dis. 93:30-35.

Phytophthora species produce sporangia that either germinate directly or release zoospores, depending upon environmental conditions. Previous *Phytophthora* spp. inoculation trials have used both sporangia and zoospores as the inoculum type. However, it is unknown what impact propagule type has on disease. Rhododendron leaf disks were inoculated with *P. ramorum* zoospores (75, 500, or 2,400 per disk), sporangia (75 per disk), or sporangia plus trifluoperazine hydrochloride (TFP) (75 per disk), a chemical that inhibits zoospore formation. Combining results from two different isolates, the highest concentration of zoospores (2,400 per disk) induced a significantly higher percentage of necrotic leaf disk area (96.6%) than sporangia (87.6%) and 500 zoospores per disk (88.7%). The sporangia plus TFP treatment had the lowest necrosis at 47.5%. Rooted rhododendron cuttings had a higher percentage of necrotic leaves per plant when inoculated with zoospores (3,000 or 50,000 per ml) or cysts (50,000 per ml) than with sporangia (3,000 per ml) with or without TFP. The percentage of necrotic leaf area was significantly higher when cysts or zoospores were inoculated at 50,000 per ml than sporangia without TFP and zoospores at 3,000 per ml. All treatments were significantly higher in the percentage of necrotic leaf area than the leaves treated with sporangia plus TFP. This demonstrates that the full inoculum potential may not be achieved when sporangia are used as the inoculum propagule.

Phytophthora species are known to be destructive and complex plant pathogens. They produce combinations of different sexual and asexual propagules depending upon the species. The most common and characteristic asexual propagule is the sporangium (9). Some species have caducous sporangia that detach easily from the sporangiophore by wind or water, giving them the capacity to travel several kilometers to new host plants (2). Sporangia germinate either directly or indirectly by releasing zoospores that subsequently encyst and germinate. These two different processes are influenced by temperature change, relative humidity, the age of sporangia, and substrate (21,28,31). Generally, at higher temperatures (>14 to 20°C, depending upon the species), direct germination occurs in which hyphae emerge through the wall of the sporangium (18). At lower temperatures, the sporangial cytoplasm cleaves and biflagellate zoospores are formed (16). The motile zoospores are released from the sporangium and recognize host tissue by chemical, electrical, and physical properties (36). The zoospores encyst, germinate, and penetrate susceptible host cells.

Phytophthora ramorum has a life cycle similar to that of other aerial-type *Phytophthora* spp., such as *P. capsici* and *P. infestans*. Wind and wind-driven rains are believed to spread the caducous *P. ramorum* sporangia within forest canopies (11) from which it is likely that zoospores are released from the sporangia and swim to new infection sites. Davidson et al. (7) observed abundant zoospores released from sporangia on infected bay laurel leaves under natural field conditions during rainstorms. In general, it is believed that infection of plant material via zoospores is the most important pathway in the disease cycle (18). However, the majority of previously published papers that involved screening hosts of *P. ramorum* used sporangia as the inoculum source (e.g., 25,32,35). Since a single *P. ramorum* sporangium can release, on average, 32 zoospores (*personal observation*), the actual number of potentially infective propagules derived from sporangia-based inoculum can be variable, depending upon the number of sporangia that actually release zoospores. It is generally assumed that the majority of sporangia will release zoospores; however, Judelson and Tani (20) reported that only 58% of *P. infestans* sporangia released zoospores when tested under artificial conditions. Factors including the solute potential and matric potential are known to affect sporangia releasing zoospores (27). It is not known what significance sporangia that have not released zoospores have via direct germination on disease development.

Studies involving other *Phytophthora* spp. sprayed zoospores to infect aerial plant parts to screen for resistance (5,12,13,25,34). Several exceptions found in the literature that used *P. ramorum* zoospores as inoculum for aerial plant parts dipped the leaves in the suspension (14,37,39). However, to the author's knowledge, no studies have actually been completed that directly compare infection by different propagule types of any *Phytophthora* spp. It is important to document if differences do exist between propagule types so that future experiments can be consistent while minimizing variability.

Phytophthora ramorum produces both chlamydospores and sporangia on tissue (6,33). In general, *P. ramorum* chlamydospores are regarded as long-term survival structures that may initiate the polycyclic epidemic, but their impact on sustaining an epidemic has not been shown. Although chlamydospores have been observed on leaf surfaces (6), they are not easily detached from the mycelium like sporangia. Thus, it is unlikely that chlamydospores are significant in increasing secondary inoculum for a polycyclic epidemic, and they were not included in this study. Sporangia, on the other hand, have been observed on the surface of leaf material and are caducous, thus playing a major role in spread within a plant and from plant to plant (6,18).

This study was conducted to determine what impact *P. ramorum* sporangia, zoospores, and cysts have on disease development of a susceptible host. This was done by comparing the necrotic leaf area caused by direct germination of sporangia or cysts.

MATERIALS AND METHODS

Pathogen isolates and inoculum preparation. Cultures of *P. ramorum* were obtained from P. W. Tooley (USDA-ARS, Fort Detrick, MD). *Phytophthora ramorum* isolates WSDA-1772, 5-C, and PRN-1 were used in different tests throughout the study. Isolate WSDA-1772 is an A2 mating

Corresponding author: Timothy Widmer
E-mail: tim.widmer@ars.usda.gov

Accepted for publication 17 September 2008.

doi:10.1094/PDIS-93-1-0030

type originally isolated from *Viburnum* 'Mariesii' in Oregon. Isolate 5-C is an A2 mating type originally isolated from *Camellia sasanqua* 'Bonanza' in California. Isolate PRN-1 is an A1 mating type originally isolated from *Rhododendron* sp. in The Netherlands. All isolates were maintained on 20% V8 agar at 20°C in the dark.

Zoospores were prepared by the method of Mitchell and Kannwischer-Mitchell (29). Five 4-mm plugs of each isolate were added to separate 60-mm plates containing sterile 20% V8 broth. The cultures were allowed to grow for 3 days at 20°C in the dark. The mycelium was rinsed three times in sterile 0.1 mM 2-[*N*-morpholino] ethanesulfonic acid (MES) buffer, pH 6.2 (herein referred to as MES buffer) and then placed back in the 20°C incubator in the dark overnight. Zoospores were induced to release from the formed sporangia by placing the cultures at 4°C for 30 min and incubating at room temperature. After 30 to 45 min, zoospores released and the concentration was determined by diluting the suspension in MES buffer, vortexing to induce encystment, and then counting on a hemacytometer. The zoospores were diluted to the final concentrations by carefully pipetting a specific amount in MES buffer. Motility of the zoospores was verified after dilution through a dissecting microscope.

For the leaf disk bioassay, sporangia were prepared by the method of Tooley et al. (35). Five-milliliter plugs from the edge of an actively growing *P. ramorum* culture on 20% V8 agar were removed and placed in at least two sterile 60-mm-diameter petri dishes. Enough MES buffer was poured into the dish to reach just below the top of the plugs. The plates were covered, but not sealed, and placed at 20°C in the dark overnight. Sporangia were released from the mycelium by sealing the plates with Parafilm and shaking the suspension. The suspensions of sporangia from multiple plates for each isolate were removed by pipette and combined into a 15-ml conical tube. The suspension was mixed, and one-half of it was transferred to another 15-ml conical tube. The sporangial concentration was determined for each suspension by mixing well and placing two 25-µl drops on a glass slide and counting all of the sporangia. A stock solution of 1.25 mM trifluoperazine hydrochloride (TFP; Sigma Chemical Co.) was prepared in sterile water. The TFP stock solution was added to one of the sporangial suspensions to make a final concentration of 12.5 µM. Sporangia can be prevented from forming zoospores by exposure to TFP without preventing direct sporangial germination (19).

For the whole plant bioassay, sporangia were prepared by transferring a plug of the *P. ramorum* isolate to 20% V8 agar plates and placing in an incubator at 20°C in the dark. Sporangia were harvested from the plate after approximately 2 weeks by pouring 5 ml of MES buffer onto the agar surface and gently swirling to release the sporangia from the mycelium. The suspension was pipetted off the surface into a 15-ml conical tube. After vortexing, half of the suspension was transferred to another 15-ml conical tube. The concentration of sporangia was determined as described above. The final concentration was adjusted to 3,000 sporangia per ml in MES buffer. A quantity of the TFP stock solution was added to one of the sporangial suspensions to make a final concentration of 12.5 µM.

Release and inhibition of zoospores from sporangial suspensions with or without TFP were conducted by exposing an aliquot of the suspensions to 4°C for 20 min and then incubating at room temperature for 45 min. Direct germination of sporangia exposed to TFP was observed through a compound microscope with a 10× objective lens on a 20% V8 agar plate containing a sample of the sporangial suspension after 24 h in the dark at 20°C. The effect of TFP on infection of rhododendron leaf disks was studied by placing 10 leaf disks (11-mm-diameter) abaxial side up on a plastic mesh screen on a moist filter paper in a petri dish. The epidermis of the abaxial side of each leaf disk was wounded slightly with a blunt scalpel. Thirty microliters of sterile distilled water or sterile 12.5 µM TFP solution was pipetted onto the wound site. A 3-mm mycelial plug was removed from the edge of an actively growing culture on V8 agar of *P. ramorum* isolates 5-C, WSDA-1772, and PRN-1 and placed mycelium side down in the solution over the wound site. The plates were sealed and placed in a 20°C incubator in the dark for 3 days. The disks were photographed and the percent necrotic area was calculated using ASSESS (American Phytopathological Society) software. The experiment was conducted twice.

Sporangial discharge. The percentage of sporangia that released zoospores was calculated using a procedure modified from Jones et al. (17). Detached sporangia were produced as described above for isolates WSDA-1772, 5-C, and PRN-1 (35). Two 0.5-ml aliquots of each sporangial suspension were transferred to a 24-well microtiter plate. The plate was placed at 4°C for 30 min and then transferred to an incubator at 20°C in the dark. After 4 h, 100 randomly selected sporangia were observed to assess the percentage of sporangia releasing zoospores (i.e., empty sporangia). The experiment was conducted three times.

The impact on the sporangia discharging zoospores after passing through a hand-pump sprayer was assessed by preparing two 10-ml aliquots of sporangia (isolate WSDA-1772) as described above in MES buffer. One aliquot was poured directly into a 60-mm petri plate. The other aliquot was transferred to a hand-pump sprayer and discharged through the sprayer into another 60-mm petri plate. The plates were covered, but not sealed, placed at 4°C for 1 h, returned to room temperature for 1 h, and observed under a dissecting microscope for release. The percentage of empty sporangia was determined as described above. The experiment was conducted three times.

Leaf disk bioassay. Tests were conducted with *P. ramorum* isolates WSDA-1772 and PRN-1. Individual healthy, mature leaves of *Rhododendron* 'Cunningham's White' were selected from at least 10 separate plants. The leaves were rinsed in water and blotted dry. Leaf disks were made with a cork borer (11-mm-diameter). The surface areas of the disks were not wounded and were placed abaxial side up on a plastic mesh screen on a moist filter paper in a petri dish. Ten disks were placed in a single petri dish, with two dishes for each treatment. Sporangial and zoospore suspensions were prepared as described above. If necessary, a volume of MES buffer was pipetted onto the disk surface to assure that the volume of liquid on each disk among the treatments was the same. The different treatment suspensions were pipetted onto the disk surface so that the liquid remained as a "bead" on the disk. The treatments were as follows: (i) MES buffer plus 12.5 µM TFP (negative control); (ii) 75 sporangia suspended in MES buffer; (iii) 75 sporangia suspended in MES buffer plus 12.5 µM TFP; (iv) 2,400 zoospores in MES buffer; (v) 500 zoospores in MES buffer; and (vi) 75 zoospores in MES buffer. The plates were covered, sealed, and placed at 20°C in the dark. Since zoospores are blocked from forming in sporangia at the concentration of TFP used in this experiment (19), a control with zoospores and TFP was not needed.

After 5 days, photographs of each of the disks were recorded and the percentage of necrotic leaf disk surface area was calculated for each disk using ASSESS and averaged together. The experiment was conducted four times for each concentration and propagule type of each *P. ramorum* isolate tested.

Comparing methods of cyst application. Due to mechanical agitation, active zoospores cannot be applied via a pressurized sprayer without the risk of encystment. However, it is more practical to apply the propagule with a pressurized sprayer. Since two application methods were used for the different propagule types, it was important to verify that the application method does not significantly impact the infection process and disease development. Zoospores of isolate WSDA-1772 were produced by the methods described above and diluted in two 15-ml aliquots of 50,000 per ml. Both aliquots were vigorously shaken on a vortex shaker

to induce encystment. Four *Rhododendron* 'Cunningham's White' cuttings in 5-cm pots with 10 to 15 leaves per plant were inoculated on both sides of the leaves with all of the prepared volume of cysts either by a hand-pump sprayer or by applying the cysts with a paintbrush. Fifteen milliliters is a sufficient volume to cover all of the leaves of four rhododendron plants to run-off. The plants were placed in a dew chamber with 100% relative humidity at 20°C in the dark. After 6 days, the plants were removed and the leaves detached from each plant. The leaves were scanned using an HP Scanjet 5500C model flatbed scanner (Hewlett-Packard, Palo Alto, CA). The percentage of necrotic leaf surface area for each leaf was calculated using ASSESS. The experiment was conducted three times.

Whole plant bioassay. Uniform *Rhododendron* 'Cunningham's White' rooted cuttings in 5-cm pots, obtained from a commercial nursery, were trimmed back to leave approximately 10 to 20 leaves per plant. Zoospores were prepared as described above and adjusted to one 15-ml aliquot of 3,000 zoospores per ml and two 15-ml aliquots of 50,000 zoospores per ml in MES buffer. These concentrations were chosen because an inoculum level of 3,000 sporangia per ml, found to be adequate for disease development (35) and used in other studies (32), could have the potential to release almost 100,000 zoospores. Hansen et al. (14) found that an inoculum dose above 12,000 zoospores per ml did not change significantly the percent necrosis of rhododendron leaves. Therefore, a concentration of 50,000 zoospores per ml was chosen based upon a conservative estimation that no less than 50% of the sporangia would release zoospores *in planta* and would not affect necrosis. One aliquot of the 50,000 zoospore per ml suspension was shaken vigorously using a vortex shaker to induce encystment. The remaining 50,000 zoospore per ml aliquot was left undisturbed to keep the zoospores motile. Fifteen milliliter sporangial suspensions with and without TFP were prepared as described above to a final concentration of 3,000 sporangia per ml.

Five plants were inoculated with a 15-ml suspension for each of the propagule types and concentrations. A hand-pump sprayer was used to inoculate the plants with the cysts, sporangia, and sporangia plus TFP. The zoospores were applied by application to each of the leaves with a paintbrush. Inoculum was applied to both sides of the leaves of each plant as evenly as possible, distributing the total volume for all five plants. Five noninoculated plants treated with MES buffer alone were included as controls.

The plants were placed in a dew chamber with 100% relative humidity at 20°C in the dark. After 5 days, the plants were removed and the leaves were detached from each plant. The leaves were stored at 4°C until they could be processed further, usually within 2 days. The leaves were scanned using an HP Scanjet 5500C model flatbed scanner. The percentage of necrotic leaf surface area for each leaf, including the controls, was calculated using ASSESS. For the inoculated plants, a leaf was considered infected if it had a higher percentage of necrotic leaf surface area than the control average. The experiment was conducted three times.

Statistical analyses. All statistical analyses were conducted using SAS for Windows (version 9.1; SAS Institute, Inc., Cary, NC). For the leaf disk bioassay, based on initial ANOVA, there was no trial effect and so the data were combined. Data were transformed using arcsine square root. Transformed data were analyzed by ANOVA using PROC MIXED. Treatments were considered fixed effects and isolates were considered random. Treatment means were separated using the pairwise option (PDIFF) in PROC MIXED ($P = 0.05$). Nontransformed means are presented for comparison. A two-factor ANOVA was used to analyze differences between isolates.

For the whole plant bioassay, based on initial ANOVA, there was no trial effect and so the data were combined. The percentage of infected leaves and percentage of necrotic leaf surface area were arcsine square root transformed prior to analysis to stabilize variance and then subjected to general linear model (GLM) procedure for ANOVA. Appropriate transformed means were separated using Duncan's multiple range test ($P = 0.05$). Nontransformed means are presented for comparison.

RESULTS

Zoospores were not observed in any of the sporangial suspensions containing TFP, whereas abundant zoospores were released after chilling in the non-TFP-treated suspensions. No differences were observed in direct germination of treated and nontreated sporangial suspensions on V8 agar plates, or in the colony growth resulting from the direct germination (data not shown). TFP did not affect infection of leaf material as demonstrated by no observed significant differences in necrotic leaf disk surface area when the mycelial plugs were immersed in a TFP solution (data not shown).

Sporangial discharge. All three isolates produced abundant sporangia. The percentage of sporangia that released zoospores after 4 h was 92, 94, and 93% for WSDA-1772, 5-C, and PRN-1, respectively. Passage of sporangia through a hand-pump sprayer did not significantly ($P = 0.6374$) affect zoospore release.

Leaf disk bioassay. There was not a significant difference in the percentage of necrotic leaf disk surface area between the isolates used ($P = 0.298$) nor an interaction between isolate and treatment ($P = 0.083$), so the data could be combined. The sporangia plus TFP was significantly lower than all other treatments (Table 1). Inoculation with the highest zoospore concentration (2,400 per disk) resulted in significantly higher necrosis than the other treatments. The 75 sporangia per disk without TFP treatment was not significantly different than exposing the disks to 500 zoospores. For the control disks inoculated with MES buffer alone, 2.4% of the disk surface area was necrotic.

Comparing methods of cyst application. There was no statistical difference in leaf infection using either method of application of *P. ramorum* cysts ($P = 0.358$). In both methods, 100% of the leaves were infected, with 69 and 65% of the leaf area necrotic by method of spraying and hand painting, respectively.

Whole plant bioassay. The plants inoculated with the cysts and the motile zoospores, regardless of concentration, had a significantly higher percentage of necrotic leaves per plant than the sporangia or sporangia plus TFP treatments (Table 2). The mean percentage of leaf necrotic area was significantly higher in the zoospore and cyst treatment at a concentration of 50,000 per ml. Zoospores at 3,000 per ml were equal to the sporangial suspension

Table 1. Mean percentage of necrotic leaf disk surface area of *Rhododendron* 'Cunningham's White' inoculated with *Phytophthora ramorum*[v]

Treatment[w]	Propagule concentration[x]	Mean ± SE[y]
Zoospores	2,400	96.6% ± 0.61 A[z]
Zoospores	500	88.7% ± 0.16 B
Zoospores	75	80.5% ± 0.27 C
Sporangia – TFP	75	87.6% ± 1.28 B
Sporangia + TFP	75	47.5% ± 3.03 D

[v] Data from isolates WSDA-1772 and PRN-1 were not statistically different and were combined ($P = 0.298$).

[w] Propagule type of *P. ramorum* suspended in 0.1 mM 2-[*N*-morpholino]ethanesulfonic acid (MES) buffer, pH 6.2, used to inoculate rhododendron plants. Trifluoperazine hydrochloride (TFP) was added (sporangia + TFP) or not added (sporangia – TFP) to suspensions of sporangia before application to leaf disks.

[x] Initial number of propagules per leaf disk.

[y] Mean percentage of necrotic leaf disk surface area was calculated by ASSESS software.

[z] Values within a column followed by the same letter are not significantly different from each other according to the pairwise difference option (PDIFF) in PROC MIXED at the 0.05 level.

without TFP treatment, and both were statistically greater than the sporangial suspension with TFP.

DISCUSSION

The result trends from this study were similar whether whole plants or leaf disks were used. The data show that without zoospore release, direct germination of *P. ramorum* sporangia is not as effective in causing necrosis as an equivalent number of zoospores. This supports the recommendation that zoospores or cysts rather than sporangia should be used as the inoculum propagule when screening potential hosts against *P. ramorum*, as has been utilized for other *Phytophthora* spp. (5,24,34).

One drawback mentioned with the use of sporangia as the inoculum type is the uncertainty that all sporangia will release zoospores. In this study, the vast majority (average of 93%) of sporangia released zoospores after 4 h when produced at 20°C in liquid culture, exposed to 4°C for 1 h, and returned to 20°C. This is higher than the number observed for *P. infestans*, which ranged from 58% (20) to 74% (19), and *P. parasitica*, which discharged 73% of the time (38). For all of these results, including the present study, these tests were conducted under ideal conditions that are unlikely on a leaf surface. The phylloplane is a complex microenvironment influenced by microorganisms and leaf exudations. One aspect of leaf exudations is the solute potential that is dependent upon environmental and genetic factors (23,26). MacDonald and Duniway (27) demonstrated that the solute potential affects zoospore release from sporangia. In one study, it was demonstrated that the host plant affected the release of *Plasmopara viticola* zoospores from sporangia (22). Temperature of the culture growth also plays an important role in zoospore release. In this study, cultures were grown only at one temperature, 20°C. When Davidson et al. (7) produced sporangia on California bay laurel (*Umbellularia californica*) disks at different temperatures, large differences between trials in zoospore release after 30 min exposure at 4°C were observed at the 25°C treatment. These factors demonstrate that different aspects affect zoospore release and that the full inoculum potential may not be realized when using sporangia alone as the inoculum propagule.

For whole plants, the number of necrotic leaves per plant was higher when zoospores were applied as the inoculum source, but the percentage of necrotic leaf area was not different from that of cysts. This lack of difference between zoospores and cysts is important because there are advantages to using these propagules as the inoculum type. First, a hand-pump sprayer is ideal for applying large amounts of inoculum in a quick and efficient manner that can easily cover all plant parts. In this experiment, the method for applying cysts to leaves, by using either a hand-pump sprayer or a paintbrush, did not affect the percentage of necrotic leaf area. This comparison could not be done with zoospores because it cannot be assured that all zoospores will remain motile when subjected to conditions of agitation when passing through a sprayer. In addition, care must be taken as to the type of pump sprayer used in application. The application pressure and use of carbon dioxide affected the survival of *Phytophthora nicotianae* zoospores (3). Secondly, inoculum levels can be more carefully controlled and applied homogenously using cysts compared to sporangia because of the uncertainty as to how many or if any zoospores are released from the sporangia. As mentioned above, not all sporangia may release zoospores depending upon different factors. Therefore, if sporangia are applied, actual inoculum potential due to the number of actual infective propagules released may vary within an experiment. Using cysts as the inoculum type would reduce the error of variable laboratory conditions affecting zoospore release on the host, assuring that the full potential of infective propagules are in contact with the host. In this study, higher variability in the mean percentage of necrotic leaf disk surface area (Table 1) and in the percentage of necrotic leaves per plant (Table 2) was observed when sporangia were used as the inoculum type compared to zoospores or cysts. Lastly, it is more time-consuming to produce abundant quantities of sporangia in concentrations useful for larger-scale inoculation trials than cysts. Typical methods require from 1 to 2 weeks to produce sporangia for inoculum and may require centrifugation for adequate concentrations (35). In contrast, high concentrations of zoospores can be achieved in liquid cultures in 4 days.

Attempts were made in this experiment to maintain a constant inoculum volume, regardless of the inoculum type. This is important because Biles et al. (4) showed that disease incidence increased with larger volumes, although the total number of zoospores was the same. In that same experiment, however, the volume did not affect lesion size. In the present study, it appeared, but was not confirmed, that the liquid suspension of propagules more uniformly covered the leaf surfaces when applied with the hand-pump sprayer than with the paintbrush. Although not examined in detail in this study, this is probably due to the finer particle droplets emitted from the sprayer and the waxy composition of the leaf surfaces. Despite this apparent lack of coverage, no statistical differences were observed in leaf infection and necrosis when the application methods were compared using cysts. However, when zoospores were applied using a brush there was an increase in the number of necrotic leaves per plant, but not in the percentage of necrotic leaf area, when compared to the application of cysts at the same concentration (50,000 per ml) via the hand-pump sprayer. This demonstrates that the apparent lack of coverage either was not real or did not negatively affect infection. One explanation for this difference may be that motile zoospores were attracted to more susceptible infection sites on other leaves. *Phytophthora nicotianae* zoospores were shown to preferentially target the grooves between adjacent epidermal cells, demonstrating that zoospore motility is an important process in finding favorable infection sites (15).

A possible explanation for the cyst inoculum yielding a similarly high percentage of necrotic leaf area as the zoospore inoculum, despite the lack of motility, is that the inoculum concentration may have

Table 2. Mean percentage of necrotic *Rhododendron* 'Cunningham's White' leaves per plant and mean percentage of necrotic leaf area inoculated with different propagules and concentrations of *Phytophthora ramorum* isolate WSDA-1772

Treatment[v]	Propagule concentration per ml[w]	Mean % (±SE) of necrotic leaves per plant[x]	Mean % (±SE) of necrotic leaf area[y]
Sporangia – TFP	3,000	76.1% ± 5.09 C[z]	37.6% ± 3.62 B
Sporangia + TFP	3,000	65.7% ± 4.91 C	25.1% ± 3.46 C
Cysts	50,000	89.0% ± 2.91 B	56.6% ± 3.51 A
Zoospores	50,000	97.3% ± 1.89 A	66.2% ± 3.07 A
Zoospores	3,000	88.9% ± 3.39 B	41.9% ± 4.98 B

[v] Propagule type of *P. ramorum* suspended in 0.1 mM 2-[*N*-morpholino]ethanesulfonic acid (MES) buffer, pH 6.2, used to inoculate rhododendron plants. Trifluoperazine hydrochloride (TFP) was added (sporangia + TFP) or not added (sporangia – TFP) to suspensions of sporangia before application to plants.

[w] Propagule concentration in 15 ml applied to five rhododendron plants for each repetition.

[x] A leaf was considered necrotic if the percentage of necrosis, as determined by ASSESS software, was greater than the control average (1.3%). Percentage of leaves infected per plant was calculated by subtracting the number of infected leaves from the total number of leaves and dividing by the total number of leaves.

[y] Mean percentage of necrotic leaf area was calculated by dividing the total area of necrosis for an individual infected leaf into the total area of that leaf as calculated by ASSESS software.

[z] Mean values within a column followed by the same letter are not significantly different ($P = 0.05$) according to Duncan's multiple range test.

been above a threshold needed for disease development that compensated for the lack of motility and attraction toward open wounds. Tooley et al. (35) found that disease developed on bearberry (*Arctostaphylos uva-ursi*) and mountain laurel (*Kalmia latifolia* 'Minuet') when inoculated with as few as 100 *P. ramorum* sporangia per ml, which if all discharged would equal approximately 3,000 zoospores. This study showed that necrosis occurred on leaf disks when inoculated with only 75 zoospores. In a field study involving *P. capsici* (30), inoculum density was shown not to be significant in the final disease incidence, although the data did show an impact of inoculum density on the area under the disease progress curves (AUDPC). The difference in AUDPC in that study was related most likely to the higher disease incidence at the initial recording of data.

Information obtained from this study helps us understand the epidemiology of this pathogen in the field. Previous studies demonstrated that under laboratory conditions, sporangia are produced at temperatures ranging from 5 to 30°C with an optimum temperature range of 16 to 22°C (7,8). Therefore, daily temperature fluctuations between 10 and 22°C, along with a minimum time of 6 to 12 h of free standing water (10), should promote sporangia formation and zoospore release, resulting in a higher rate of infection. In contrast, 25°C might be a threshold temperature for direct germination of sporangia versus zoospore production (7). Thus, based upon results from the present study, new infections by *P. ramorum* could be higher when field temperatures are below 25°C due to an increase in zoospore release. This model fits what was observed in the field in California. A study by Anacker et al. (1) revealed that symptomatic leaf count of *U. californica* was related to mean minimum annual temperature and the mean annual precipitation at the field plot level. *Phytophthora ramorum* populations, as measured by colony forming units, in the soil and leaf litter increased in the cooler middle to late rainy seasons (7). These higher populations are very likely due to an increase in zoospore release.

The results presented in this study confirm the importance of zoospore release by sporangia on the leaf surface in regard to infection and subsequent disease symptoms. Although sporangia that do not release zoospores still infect leaves and cause leaf necrosis, the number of leaves that are infected per plant and the mean percentage of necrotic leaf area are lower than an equivalent number of zoospores. This knowledge could lead to research that is more efficient in developing methods, either through chemicals or through integrated pest management options, to manage and limit the spread of this pathogen.

ACKNOWLEDGMENTS

I thank Nina Shishkoff, Paul Tooley, and the anonymous reviewers for their reviews and helpful suggestions to improve this manuscript.

LITERATURE CITED

1. Anacker, B. L., Rank, N. E., Hüberli, D., Garbelotto, M., Gordon, S., Harnik, T., Whitkus, R., and Meentemeyer, R. 2008. Susceptibility to *Phytophthora ramorum* in a key infectious host: Landscape variation in host genotype, host phenotype, and environmental factors. New Phytol. 177:756-766.
2. Aylor, D. E. 2003. Spread of plant disease on a continental scale: Role of aerial dispersal of pathogens. Ecology 84:1989-1997.
3. Banko, T. J., Richardson, P. A., and Hong, C. X. 2006. Effects of zoospore concentration and application pressure on foliage blight of *Catharanthus roseus* caused by *Phytophthora nicotianae*. Plant Dis. 90:297-301.
4. Biles, C. L., Bruton, B. D., Wall, M. M., and Rivas, M. 1995. *Phytophthora capsici* zoospore infection of pepper fruit in various physical environments. Proc. Okla. Acad. Sci. 75:1-5.
5. Colon, L. 1994. Resistance to *Phytophthora infestans* in *Solanum tuberosum* and wild *Solanum* species. Ph.D. thesis. Wageningen Agricultural University, Wageningen, The Netherlands.
6. Davidson, J. M., Rizzo, D. M., Garbelotto, M., Tjosvold, S., and Slaughter, G. W. 2002. *Phytophthora ramorum* and sudden oak death in California: II. Transmission and survival. Pages 741-749 in: Fifth Symposium on California Oak Woodlands. R. Standiford and D. McCreary, eds. USDA Forest Service, General Technical Report PSW-GTR-184, Albany, CA.
7. Davidson, J. M., Wickland, A. C., Patterson, H. A., Falk, K. R., and Rizzo, D. M. 2005. Transmission of *Phytophthora ramorum* in mixed-evergreen forest in California. Phytopathology 95:587-596.
8. Englander, L., Browning, M., and Tooley, P. W. 2006. Growth and sporulation of *Phytophthora ramorum* in vitro in response to temperature and light. Mycologia 98:365-373.
9. Erwin, D. C., and Rebeiro, O. K. 1996. Phytophthora Diseases Worldwide. American Phytopathological Society, St. Paul, MN.
10. Garbelotto, M., Davidson, J. M., Ivors, K., Maloney, P., Hüberli, D., Koike, S. T., and Rizzo, D. M. 2003. Non-oak native plants are main hosts for sudden oak death pathogen in California. Calif. Agric. 57:18-23.
11. Goheen, E. M., Hansen, E., Kanaskie, A., Osterbauer, N., Parke, J., Pscheidt, J., and Chastagner, G. 2006. Sudden oak death and *Phytophthora ramorum*: A guide for forest managers, Christmas tree growers, and forest-tree nursery operators in Oregon and Washington. Oregon State Univ. Ext. Publ. EM 8877.
12. Grenville-Briggs, L. J., Aurova, A. O., Bruce, C. R., Williams, A., Whisson, S. C., Birch, P. R. J., and van West, P. 2005. Elevated amino acid biosynthesis in *Phytophthora infestans* during appressorium formation and potato infection. Fungal Gen. Biol. 42:244-256.
13. Hansen, E. M., Hamm, P. B., and Roth, L. F. 1989. Testing Port-Orford-cedar for resistance to *Phytophthora*. Plant Dis. 73:791-794.
14. Hansen, E. M., Parke, J. L., and Sutton, W. 2005. Susceptibility of Oregon forest trees and shrubs to *Phytophthora ramorum*: A comparison of artificial inoculation and natural infection. Plant Dis. 89:63-70.
15. Hardham, A. R. 2007. Cell biology of plant-oomycete interactions. Cell. Microbiol. 9:31-39.
16. Hardham, A. R., and Hyde, G. J. 1997. Asexual sporulation in the Oomycetes. Adv. Bot. Res. 24:353-398.
17. Jones, E. S., Breese, W. A., and Shaw, D. S. 2001. Inoculation of pearl millet with the downy mildew pathogen, *Sclerospora graminicola*: Chilling inoculum to delay zoospore release and avoid spray damage to zoospores. Plant Pathol. 50:310-316.
18. Judelson, H. S., and Blanco, F. A. 2005. The spores of *Phytophthora*: Weapons of the plant destroyer. Nat. Rev. Microbiol. 3:47-58.
19. Judelson, H. S., and Roberts, S. 2002. Novel protein kinase induced during sporangial cleavage in the oomycete *Phytophthora infestans*. Eukaryotic Cell 1:687-695.
20. Judelson, H. S., and Tani, S. 2007. Transgene-induced silencing of the zoosporogenesis-specific *NIFC* gene cluster of *Phytophthora infestans* involves chromatin alterations. Eukaryotic Cell 6:1200-1209.
21. Jung, T., Nechwatal, J., Cooke, D. E. L., Hartmann, G., Blaschke, M., Oßwald, W. F., Duncan, J. M., and Delatour, C. 2003. *Phytophthora pseudosyringae* sp. nov., a new species causing root and collar rot of deciduous tree species in Europe. Mycol. Res. 107:772-789.
22. Kiefer, B., Riemann, M., Büche, C., Kassemeyer, H. H., and Nick, P. 2004. The host guides morphogenesis and stomatal targeting in the grapevine pathogen *Plasmopara viticola*. Planta 215:387-393.
23. Kumar, A., and Singh, D. P. 1998. Use of physiological indices as a screening technique for drought tolerance in oilseed *Brassica* species. Ann. Bot. 81:413-420.
24. Lee, B. K., Kim, B. S., Chang, S. W., and Hwang, B. K. 2001. Aggressiveness to pumpkin cultivars of isolates of *Phytophthora capsici* from pumpkin and pepper. Plant Dis. 85:497-500.
25. Linderman, R. G., and Davis, E. A. 2007. Evaluation of *Phytophthora ramorum* in nursery crop tissue culture propagation. Online. Plant Health Progress doi:10.1094/PHP-2007-0822-01-RS.
26. Loach, K. 2006. Leaf water potential and the rooting of cuttings under mist and polythene. Physiol. Plant. 40:191-197.
27. MacDonald, J. D., and Duniway, J. M. 1978. Influence of the matric and osmotic components of water potential on zoospore discharge in *Phytophthora*. Phytopathology 68:751-757.
28. Minogue, K. P., and Fry, W. E. 1981. Effect of temperature, relative humidity, and rehydration rate on germination of dried sporangia of *Phytophthora infestans*. Phytopathology 71:1181-1184.
29. Mitchell, D. J., and Kannwischer-Mitchell, M. E. 1992. *Phytophthora*. Pages 31-38 in: Methods for Research on Soilborne Phytopathogenic Fungi. L. L. Singleton, J. D. Mihail, and C. M. Rush, eds. American Phytopathological Society, St. Paul, MN.
30. Ristaino, J. B. 1991. Influence of rainfall, drip irrigation, and inoculum density on the development of Phytophthora root and crown rot epidemics and yield in bell pepper. Phytopathology 81:922-929.
31. Sato, N. 1994. Effect of some inorganic salts and hydrogen ion concentration on direct germination of the sporangia of *Phytophthora infestans*. Ann. Phytopathol. Soc. Jpn. 60:441-447.
32. Shishkoff, N. 2007. Susceptibility of some *Lilac* cultivars and other members of the Oleaceae to *Phytophthora ramorum*. Online. Plant Health Progress doi:10.1094/PHP-2007-1101-02-RS.
33. Shishkoff, N. 2007. Persistence of *Phytophthora ramorum* in soil mix and roots of nursery ornamentals. Plant Dis. 91:1245-1249.
34. Stewart, H. E., Flavelle, P. H., McCalmont, D. C., and Wastie, R. L. 1983. Correlation between glasshouse and field tests for resistance

to foliage blight caused by *Phytophthora infestans*. Potato Res. 26:41-48.
35. Tooley, P. W., Kyde, K. L., and Englander, L. 2004. Susceptibility of selected ericaceous ornamental host species to *Phytophthora ramorum*. Plant Dis. 88:993-999.
36. Tyler, B. M. 2002. Molecular basis of recognition between *Phytophthora* pathogens and their hosts. Annu. Rev. Phytopathol. 40:137-167.
37. Vettraino, A. M., Hüberli, D., Swain, S., Smith, A., and Garbelotto, M. 2006. A new report of *Phytophthora ramorum* on *Rhamnus purshiana* in northern California. Plant Dis. 90:246.
38. von Broembsen, S. L., and Deacon, J. W. 1997. Calcium interference with zoospore biology and infectivity of *Phytophthora parasitica* in nutrient irrigation solutions. Phytopathology 87:522-528.
39. Wagner, S., Kaminski, K., and Werres, S. 2005. Inoculation trials with *Phytophthora ramorum* on Moorland species. Pages 520-522 in: Proc. Sudden Oak Death 2nd Symposium: The State of Our Knowledge. U.S. Dep. Agric. For. Serv., Pacific Southwest Res. Stn., Gen. Tech. Rep. PSW-GTR-196. Albany, CA.