Detection and Quantification of *Phytophthora cactorum* in Naturally Infested Soils

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ABSTRACT

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The optimum temperature for release of zoospores of *Phytophthora cactorum* grown on a culture medium was 8 C. In contrast, the most frequent *Pythium* isolated from naturally infested ginseng soils did not release zoospores at 4, 8, and 12 C. Based on these results, a procedure was developed to detect and quantify *P. cactorum* in naturally infested soil. Air-dried soil was moistened 1–3 days and flooded 2 days at 24 C. Soil was then exposed to 8 C for 2 hr to induce zoospore release. The soil supernatant was then plated onto a selective medium. Duration of the moistening period of 1–3 days did not affect recovery of the fungus. Temperatures of 16–24 C during this period did not significantly affect the recovery of the fungus, but a temperature of 28 C significantly reduced the recovery of the fungus. A soil sample from around an apple tree showing symptoms of crown rot and a soil sample from around a maple tree yielded 1,078 and 53 colony-forming units (cfu) of *P. cactorum* per gram dry soil, respectively. Thirty soil samples from ginseng gardens yielded a range of 7 to 61 cfu/g dry soil. Colonies of *Pythium* species were rarely observed. The number of colony-forming units obtained from the apple soil was not significantly different when the assay was replicated 15 times. The procedure developed here is simple, relatively quick, and a reproducible method for detection and quantification of *P. cactorum* in naturally infested soils.

Since 1960, baiting has been the most frequent method used to detect and isolate *Phytophthora cactorum* (Lebert & Cohn) Schroeter from naturally infested soils (10,16,21). Banihashemi and Mitchell (1) used safflower seedlings for detection and quantification of *P. cactorum* in apple soils. Molot and Beyries (12) placed soil into a small hole in immature strawberry fruit to isolate *P. cactorum* from strawberry soil. Recently, Jeffers and Aldwinckle (6) used apple cotyledons to bait *P. cactorum* from soil obtained from apple orchards in New York. Drilias and Kuntz (3) used sugar maple cotyledons to detect *P. cactorum* from soils.

Baiting soils to detect and isolate *P. cactorum* has the following major limitations. Baiting requires the availability of the bait and also subsequent isolation of the pathogen from infected bait tissues on culture media. When soil is heavily infested with *Pythium* spp., the bait frequently will be invaded more rapidly with *Pythium* spp. than with *P. cactorum*, making recovery of *P. cactorum* difficult. Masago et al (9) and Tsao and Guy (20) reported that *Pythium* spp. could be selectively inhibited on a selective medium amended with hymexazol. Later, it was found that hymexazol was inhibitory to *P. cactorum* and also not inhibitory to all the *Pythium* spp. (5,7,19). The objective of the present study was to determine the optimum temperature for release of zoospores of *P. cactorum* and to use the temperature response in isolation and quantification of *P. cactorum* in naturally infested soils. The preliminary result of this study has been reported (14).

MATERIALS AND METHODS

Soils naturally infested with *P. cactorum* were collected from ginseng gardens in Wisconsin. Soil samples also were obtained from around the crown of an apple tree showing symptoms of phytophthora crown rot disease and from around the crown of a symptomless maple tree in an old research orchard in Madison, WI. Soil samples were approximately 2 kg and were collected from the top 15 cm of soil by a 2.5-cm-diameter sampling tube in September 1984. The soil samples were stored in polyethylene bags at 5 C in the dark. Each soil sample was sieved through a 2-mm-mesh screen and thoroughly mixed before use.

To enhance detection of the pathogen, soil moisture was manipulated before the assay according to the method of Stack and Millar (18). Soil subsamples were air dried 2–3 days at room temperature (23–27 C), and then 10 g of air-dried soil was placed in a 90-cm-diameter glass petri plate, moistened by adding 4–7 ml of distilled water, depending on moisture-holding capacity of the soil, and incubated 3 days at room temperature and 50 $\mu Em^{-2}sec^{-1}$ illumination. Soil was then flooded with 10 ml of distilled water for 2 days unless otherwise specified.

Effect of temperature on mycelial growth and release of zoospores. Isolates of *P. cactorum* and an unidentified *Pythium* sp. that were used in these tests were recovered from ginseng garden soil by baiting with ginseng leaf disks.

To measure mycelial growth, agar disks 5 mm in diameter were taken from the edge of a 5-day-old culture of *P. cactorum* and a 2-day-old culture of a *Pythium* sp. growing on V-8 agar (200 ml of V-8 juice, 3.0 g of $CaCO_3$, 20 g of agar, and 800 ml of distilled water) and placed in the center of fresh V-8 agar plates. Three replicate plates for each fungus were incubated at temperatures from 4 to 32 C with 4-deg increments. Radial growth of mycelium was measured after 2 and 4 days of incubation.

The effect of temperature on production and release of zoospores was measured by flooding agar disks (5 mm in diameter) from a 5-day-old culture of each fungus grown on V-8 agar in a petri plate containing sterilized distilled water and incubating the plates at 24 C and 50 $\mu Em^{-2}sec^{-1}$ illumination. Three days later, five agar disks on which many sporangia had formed were transferred to 60-cm-diameter petri plates containing 12 ml of sterilized distilled water. These plates were preconditioned 2 hr at the desired temperature before transferring the disks. Three replicate plates were used for each temperature. The zoospore suspension from each plate was collected in a test tube after a 2-hr incubation at the desired temperature and then shaken on a Vortex mixer to induce the encystment of zoospores. The number of zoospores released was counted by using a hemacytometer.

In vitro zoospore production and germination. The pathogen was grown on V-8 agar 7 days at 24 C and 50 $\mu Em^{-2}sec^{-1}$ illumination. The culture was cut into 5-mm strips. Half of the strips from each plate were removed and placed in a sterile petri plate containing 20 ml of sterile distilled water. One hour later, water was decanted and replaced with 20 ml of fresh sterile distilled

water and incubated 2 days at 24 C and 50 $\mu Em^{-2}sec^{-1}$ illumination. Water was replaced with sterile distilled water cooled to 8 C and incubated at 8 C for 2 hr. Up to 10^6 zoospores/ml was obtained. One-half milliliter of zoospore concentrations of 3×10^3, 3×10^2, and 3×10^1 was plated on pentachloronitorbenzene-benomyl-neomycin-chloramphenicol (PBNC) medium (15) (three plates per concentration). Two days later, plates were examined microscopically for germinated zoospores.

Isolation of *P. cactorum* in naturally infested soil. Soils were air dried, placed in a petri plate (10 g/plate) so that a portion of the plate was not covered with soil, and then moistened. The uncovered portion of the plate was used as a site to add or remove water from the plate. Moist soil was incubated 3 days at room temperature and 50 $\mu Em^{-2}sec^{-1}$ illumination and then flooded 2 days with sterile distilled water. Four replicate plates were used for each soil. To induce zoospore release, the excess water was removed and replaced with 10 ml of distilled water cooled to 8 C, and the plates were then incubated at 8 C for 2 hr. The zoospore suspension (approximately 9 ml) was transferred to a test tube with a glass pipette and shaken on a Vortex mixer. Each suspension was diluted to 10^{-1} and 0.5 ml of the original, and this diluted suspension was spread on plates of PBNC medium and incubated at room temperature. Three PBNC plates were used for each dilution sample. After 3–7 days, plates were examined for the presence of colonies of *P. cactorum* as characterized by large numbers of papillated sporangia formed on PBNC medium.

RESULTS

Effect of temperature on mycelial growth and release of zoospores. *Phytophthora cactorum* grew at temperatures from 8 to 32 C with maximum radial growth at 24 C (Table 1). The *Pythium* grew faster than *P. cactorum* at all temperatures with maximum mycelial growth at 32 C. The zoospores of *P. cactorum* were released at all temperatures from 4 to 24 C. The number of zoospores released at 8 C was significantly greater than those released at any other temperature. In contrast, *Pythium* did not release zoospores at 4, 8, and 12 C. Although the number of zoospores of the *Pythium* produced at 20 C was greater than at 16 or 24, the difference was not significant.

In vitro zoospore production and germination. Zoospores of *P. cactorum* produced in vitro were plated on PBNC medium at concentrations of 3×10^3, 3×10^2, and 3×10^1 zoospores/ml. Two days later, microscopic examination of plates revealed that approximately 50% of the zoospores germinated on PBNC medium.

Isolation of *P. cactorum* in naturally infested soil. Colonies of *P. cactorum* on PBNC medium were easily identified (Fig. 1). The colonies were characterized by a less dense growth in the center than in the margins of the colonies. Mycelium of *P. cactorum* formed in the medium and large numbers of papillated sporangia formed on the surface of the medium. Colonies of bacteria and fungi such as *Alternaria* and *Pythium* were occasionally observed on PBNC medium. Colonies of *Pythium* grew faster than colonies of *P. cactorum*. When colonies of *P. cactorum* and *Pythium* spp. overlapped, colonies of *P. cactorum* were still distinguishable by the more compact mycelium growth and formation of many sporangia on the medium.

The number of colony-forming units (cfu) of *P. cactorum* produced in soil obtained from around the roots of the apple tree was 1,078 cfu/g of dry soil compared to a range of 7–61 cfu/g of dry soil produced in 30 different soils collected from ginseng gardens (Table 2). No colonies of *Pythium* were obtained from the apple orchard soil and very few colonies of *Pythium* were observed on the agar plates prepared from soils from ginseng gardens. The soil obtained from near the crown of the maple tree produced 53 cfu/g of dry soil of *P. cactorum* and 9.5 cfu/g of dry soil of *Pythium* spp.

The effect of duration of moistening and the effect of duration of flooding on recovery of *P. cactorum* were tested using the soil subsamples obtained from the ginseng garden and apple orchard. Three soil subsamples were each moistened for 1, 2, or 3 days

TABLE 1. Effect of temperature on radial growth of *Phytophthora cactorum* and *Pythium* sp. on V-8 agar and release of zoospores from V-8 agar disks flooded and preconditioned 2 hr at the indicated temperature

Incubation temperature (C)	Mycelial growth (mm)			Number of zoospores released $\times 10^4$/ml	
	P. cactorum	*Pythium* sp.[a]			
	4 days	2 days	4 days	*P. cactorum*	*Pythium*
4	0	0	0	0.33 ± 0.04[b]	0
8	13	0	14	4.16 ± 0.44	0
12	19	12	34	1.60 ± 0.49	0
16	34	34	85	1.50 ± 0.22	0.54 ± 0.23[b]
20	44	53	>[c]	0.97 ± 0.20	1.16 ± 0.33
24	49	68	>	0.25 ± 0.07	0.70 ± 0.23
28	31	75	>	ND[d]	ND
32	19	84	>	ND	ND

[a] The most common *Pythium* isolated from ginseng soil naturally infested with *P. cactorum*.
[b] Means for three replicated plates ± standard errors.
[c] Overgrown plates.
[d] Test not performed.

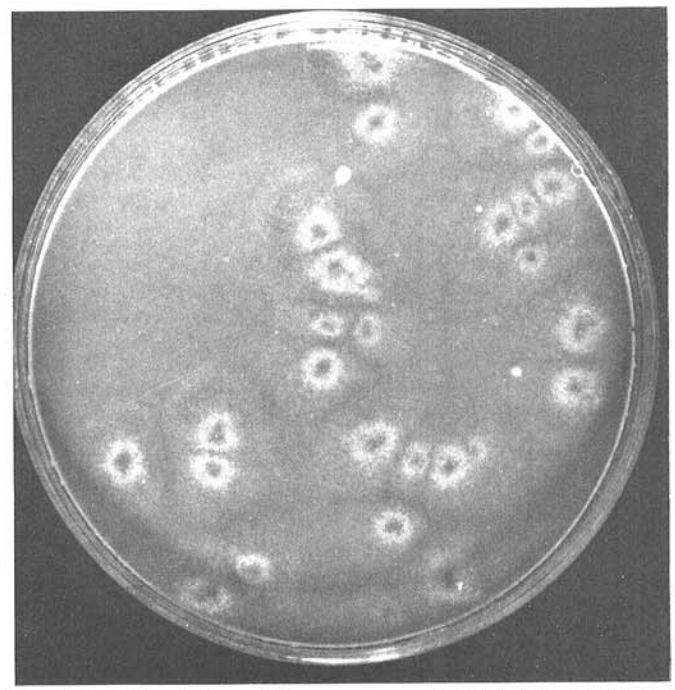

Fig. 1. Colonies of *Phytophthora cactorum* isolated from a naturally infested soil sample obtained from a ginseng garden on pentachloronitorbenzene-benomyl-neomycin-chloramphenicol medium. These colonies have a less dense growth in the center than in the margins of the colonies.

TABLE 2. Recovery of *Phytophthora cactorum* and *Pythium* spp. in naturally infested soil incubated at cold temperature to induce zoospore production

Soil[a] from:	Colony-forming units[b] per gram of dry soil	
	P. cactorum	*Pythium* spp.
Apple	1,078	None
Ginseng	7–61[c]	Rare
Maple	53	9

[a] Soil was air dried, moistened, and flooded. Then water was replaced with distilled water cooled to 8 C, and soil was incubated 2 hr at 8 C.
[b] Colonies of *P. cactorum* or *Pythium* on pentachloronitorbenzene-benomyl-neomycin-chloramphenicol medium.
[c] Range of colony-forming units in 30 different soil samples obtained from 30 different diseased patches in ginseng gardens. Four soil subsamples from each soil type were used.

followed by flooding for 2 days. There was 6, 8, and 5 cfu/g of soil from ginseng for 1-, 2-, and 3-day moistening periods, respectively. The soil from apple yielded 1,104, 989, and 1,209 cfu/g for 1-, 2-, and 3-day moistening periods, respectively. When soil subsamples were flooded for 2, 4, or 6 days after a 1-day moist period, there was 56, 5 and 6 cfu/g present in the soil from ginseng gardens, respectively. The soil from apple orchards yielded 908, 112, and 114 cfu/g for 2, 4, and 6 days flooding, respectively.

When soil was incubated at 16, 20, 24, or 28 C during the 3-day moist period, the number of colony-forming units of *P. cactorum* produced from ginseng soil for 20 and 24 C was significantly higher than the number of colony-forming units for 16 C. The fungus was not detected when the soil from ginseng gardens was incubated at 28 C. There was no significant difference in the number of colony-forming units produced from the apple soil incubated at 16, 20, and 24 C. However, the number of colony-forming units was significantly less when this soil was incubated at 28 C (Table 3).

The reproducibility of this technique was examined using 15 10-g soil subsamples obtained from around the apple tree. A range of 826 ± 212 to 1,240 ± 260 cfu/g was obtained, which was not significantly different.

DISCUSSION

Detection and isolation of slow-growing *Phytophthora* spp., including *P. cactorum*, in naturally infested soils on soil dilution plates is commonly difficult. This difficulty is likely due to rapid growth of fast-growing *Pythium* spp. on selective culture media. More recently, baits have been used as zoospore traps to isolate *Phytophthora* spp. from soil (19). Unfortunately baits are readily colonized by *Pythium* spp., making isolation of *P. cactorum* difficult or impossible (2,8).

The results obtained from in vivo and in vitro tests in this study indicated that *P. cactorum* releases zoospores at temperatures from 4 to 24 C (Table 1). However, the zoospores are rarely released at 24 C if the supernatant water is not replaced. The optimum temperature for zoospore release was 8 C which is 16 degrees below the optimum for radial growth of mycelium. Similarly, *Pythium aphanidermatum* was found to have maximum mycelial growth at 35 C, whereas the optimum for zoospore release was at 20 C (13). This relationship between the optimum temperature for mycelial growth and the optimum temperature for the release of zoospores may exist in some other Pythiaceous fungi.

Other researchers found that air drying and moistening soil samples increased the chance of success in detection and isolation of the pathogen from soil. Sneh and McIntosh (17) demonstrated that air drying soil samples eliminated the active propagules of the pathogen in soil. On the other hand, it has been shown that sporangia are formed more readily in moist soil than in saturated soil (4,17), suggesting that moistening the soil samples after they have been kept dry should enhance sporangium formation. The success of the technique presented here is based on the incubation of moist and then flooded soil at 24–26 C to stimulate sporangium formation and maturation while preventing zoospore release. Then to obtain synchronized release of zoospores, soil was moved to 8 C for 2 hr. For all soils tested here, this method effectively reduced release of zoospores of *Pythium* spp. (Table 2) and effectively increased release of zoospores of *P. cactorum*.

When the assay was done with 15 soil subsamples from the soil sample obtained from around an apple tree, the counts of colony-forming units were not significantly different, indicating that this technique is reproducible.

The previous methods in which baiting is essential for detection and isolation of *P. cactorum* (2,3,5,16,21) are primarily qualitative isolation techniques. To make a quantitative estimate, the serial dilution end-point method (1) and the most probable number method (11) have been adopted to these techniques to quantify propagules of *P. cactorum* in soil. Nevertheless, these methods require dilution of soil samples. In contrast, the method presented here does not require dilution of the soil sample and it estimates inoculum density of the pathogen in naturally infested soils in terms of colony-forming units per gram of dry soil. Theoretically, using the method here, as low as four zoospores released in a gram of soil can be recovered. However, because the exact number of germinating oospores and the potential of each oospore for producing zoospores in a soil are not known, the exact sensitivity of this method cannot be calculated.

The technique developed here appears to have the following advantages: no bait is necessary and the pathogen is directly isolated and quantified on a selective culture medium; it is a simple method that requires a few steps of manipulation of soil moisture and soil temperature; interference by fast-growing *Pythium* spp. is greatly reduced due to the difference in temperature optima for release of zoospores by *Pythium* and *P. cactorum*; and it is reproducible.

The following procedure is recommended to detect and quantify *P. cactorum* based on the results obtained here. Store soil samples in polyethylene bags at 5 C until used. Sieve soil subsamples through a 2-mm-mesh screen and thoroughly mix before use. Air dry three soil subsamples 2–3 days at room temperature (23–27 C). Place 10-g subsamples of dried soil in each of three 90-cm-diameter glass petri plates. Moisten by adding 4–7 ml of distilled water and incubate 1–3 days at 24 C and 50 $\mu Em^{-2}sec^{-1}$ illumination. Flood the soil with 10 ml of distilled water for 2 days. Remove excess water and replace with 10 ml of sterilized distilled water cooled to 8 C. Incubate the plates at 8 C for 2 hr. Transfer the zoospore suspension from above the soil to a sterile test tube with a glass pipette. Shake the zoospore suspensions in the tubes on a Vortex mixer 2–3 min. Spread three 0.5 ml of the suspension from each tube on each of three PBNC medium and incubate at room temperature for 5 days. Examine the plates for the presence of colonies of *P. cactorum* characterized by large numbers of papillated sporangia formed on the culture medium. If a soil sample has many zoospores, then the zoospore suspensions should be diluted before transfer.

TABLE 3. Effect of temperature during the soil-moistening period on recovery of *Phytophthora cactorum* in naturally infested soils[a]

Temperature, C	Colony-forming units[b] per gram of dry soil	
	Soil from ginseng garden[c]	Soil from apple tree[d]
16	35[e]	868[e]
20	62	849
24	51	1,062
28	0	687
$LSD_{0.05}$	15	232

[a] Soil was air dried, moistened for 3 days at 16, 20, 24, or 28 C, and then flooded for 2 days at 24 C.
[b] Colonies of *P. cactorum* on pentachloronitorbenzene-benomyl-neomycin-chloramphenicol medium produced by reflooding soil with distilled water cooled to 8 C and incubated at 8 C for 2 hr.
[c] Soil was obtained from a *Phytophthora*-diseased area in a ginseng garden.
[d] Soil was obtained from around the roots of an apple tree with Phytophthora crown rot disease.
[e] Means of three replicated soil plates.

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