Qualitative and Quantitative Soil Assays for *Phytophthora cinnamomi*

H. D. Shew and D. M. Benson

Graduate research assistant and associate professor, respectively, Department of Plant Pathology, North Carolina State University, Raleigh 27650.

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ABSTRACT

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Leaf disks of azalea and hybrid rhododendron and 4-wk-old seedlings of Fraser fir used as baits in a qualitative assay provided better recovery of *Phytophthora cinnamomi* from soil than did blue lupine radicles and Deodar cedar needles. A quantitative soil assay utilizing a wet-sieving technique and a selective agar medium (PCH) was developed for enumeration of population densities of *P. cinnamomi* in naturally and artificially infested soil. The selective PCH (pimaricin, chloramphenicol, and hymexazol) medium improved recovery of *P. cinnamomi* from soil in comparison to other selective media tested. A modified McCains wet-sieving procedure was used for assaying soil samples up to 50 g; samples up to 200 g were assayed by using a semiautomatic elutriator. *P. cinnamomi* colonies were macroscopically identifiable on PCH after 48–72 hr of incubation at 20 C in the dark. In assays of naturally infested soils, 87% of the colonies originated from chlamydospores free in the soil, 7% from pieces of colonized organic matter, and 6% from unidentified sources. Many chlamydospores from naturally infested soil had thick walls (mean thickness of 1.75 μm and a range of $<$1.0 to 2.6 μm).

Qualitative and quantitative recovery of *Phytophthora cinnamomi* Rands from soil has received considerable attention because this pathogen is of worldwide importance on many crops (25). Thus, many techniques have been developed for studying the ecology of *P. cinnamomi* in soil (20). Whole plants or plant parts have been used extensively as qualitative assays (baits) for *P. cinnamomi* in soil, with varying efficiency in recovery (2,3,6,15,16). In addition, selective agar media in combination with various assay procedures have been used to extend our knowledge of the ecology of *P. cinnamomi* and other *Phytophthora* spp. in soil by providing quantitative information on inoculum density and its fluctuations (5,10,14,18,22,24). Choice of a bait plant and a selective medium and assay procedure for recovery of a given *Phytophthora* sp., however, varies with the investigator because of availability of materials, ease of use, and sensitivity of a procedure with the soils present in the area. A wet-sieving selective medium procedure has been used for many years in the recovery of *P. cinnamomi* from soil (18). Disadvantages of the wet-sieving technique include the inability to assay large soil samples (6) and the possible loss of some *P. cinnamomi* propagules present in the soil through the smallest sieve used (14).

The development of sensitive, easy-to-use techniques was required to study the ecology and survival of *P. cinnamomi* in relation to the root rots of Fraser fir (*Abies fraseri* (Pursh.) Poir.) and azalea. Thus, the purpose of this study was to develop and compare assay techniques for the recovery of *P. cinnamomi* from soil, and to develop a procedure whereby large soil samples could be assayed for the presence of *P. cinnamomi*.

MATERIALS AND METHODS

Cultures and inoculum preparation. Cultures of *P. cinnamomi* used in the development of assay procedures were originally isolated from Fraser fir roots. Stock cultures were maintained on 5% clarified V-8 juice agar at 8 and 20 C.

In tests requiring artificially infested soil, chlamydospores were collected by blending and sieving *P. cinnamomi* cultures grown for 3 wk in 10% clarified lima bean broth. Desired inoculum densities were prepared by mixing known numbers of chlamydospores (free of other spores and hyphal fragments) with known weights of soil in a twin-shell blender. In other tests, naturally infested soil from either a Fraser fir nursery (sandy loam, 68.5% sand, 20.7% silt, and 9.5% clay) or an azalea bed (Cecil clay) was used as a source of *P. cinnamomi* inoculum.

Bioassay. Leaf disks (9-mm-diameter) of *Rhododendron obtusum* (Lindl.) Planch. 'Hershey Red' azalea and *R. catawbiense* Michx. 'Purple Splendour', 4- to 8-wk-old seedlings of Fraser fir, 3-day-old radicles of blue lupine (*Lupinus angustifolius* L.), and current-season needles of Deodar cedar (*Cedrus deodara* (D. Don) G. Don) were compared as baits for recovery of *P. cinnamomi* from naturally and artificially infested soil. Soil to be assayed was placed in either 150 ml (5- to 25-g samples) or 250 ml (26- to 50-g samples) beakers containing either 100 or 150 ml of deionized water, respectively, plus one drop of Tween-20. Three to five baits were floated in each beaker for either 1 or 2 days at room temperature. A test consisted of three beakers per treatment and all tests were repeated at least once. When baits were removed from the beakers, they were surface sterilized in either 0.5% NaOCl or 70% ethyl alcohol, blotted dry, and placed on either a modified pimaricin-penicillin G-polymyxin B medium (4) containing 10 μg/ml pimaricin or on the selective medium described below. Plates were observed daily for growth of *P. cinnamomi* from the baits.

Selective medium. An agar medium (PCH) was developed for the selective isolation of *P. cinnamomi* from soil. The basal medium contained (per liter of deionized water): KH_2PO_4, 1 g; $MgSO_4 \cdot 7\,H_2O$, 0.5 g; KCl, 0.5 g; $CaCl_2 \cdot 2H_2O$, 0.01 g; $FeSO_4 \cdot 7H_2O$, 0.02 g; thiamine HCl, 1 mg; yeast extract, 0.3 g; $NaNO_3$, 1 g; dextrose, 15 g; and agar, 20 g. The following ingredients were added to the basal medium after autoclaving (15 min, 121 C) and allowing the medium to cool to 45 C: pimaricin, 5 mg (Delvocid Instant 50%, Enzyme Development Corp., New York, NY 10001); pentachloronitrobenzene, 35 mg Terraclor 75% a.i. (Olin Corp., Little Rock, AR 72203); chloramphenicol, 10 mg added in 2 ml of 95% alcohol (Sigma Chemical Co., St. Louis, MO 63178); and hymexazol, 50 mg (Sankyo Co., Shiga-Ken, Japan). PCH medium was compared with several other *Phytophthora*-selective media (5,10,13,18,24) for recovery of *P. cinnamomi* from soil using the wet-sieving assay procedures described below. Both naturally and

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artificially infested soils were assayed. Three to five soil samples (5–10 g of soil per sample) constituted a test and each test was repeated at least once.

Assay procedures. Two procedures were developed for the quantitative enumeration of propagules of *P. cinnamomi* in soil. A modification of a wet-sieving procedure described by McCain et al (18) was used for assaying samples less than 50 g. From an initial bulked soil sample, three 10-g subsamples were oven-dried to determine percent moisture. Three additional subsamples up to 50 g each were used for determining fungal populations. Each sample was blended in 200 ml of deionized H_2O for 5–30 sec in a Waring Blendor at low speed. Blending time varied with soil structure and texture; moist clay soils required longer blending times to disrupt the soil aggregates. The entire soil suspension was poured through nested 20-cm-diameter sieves, 125-μm over 38-μm. The residue on the 125-μm sieve was washed with running tap water and discarded.

TABLE 1. Comparison of several selective media for the recovery of *Phytophthora cinnamomi* from soil[v]

Medium	Propagules recovered per gram of soil		
	Azalea soil	Fraser fir soil	Artificially infested soil
Test 1			
Modified Kerr's (10)[w]	5.9 ± 2.6[x]b[y]	1.8	0.3 ± 0.5 c
Gallic Acid (5)	2.1 ± 1.3 c	···	2.1 ± 1.4 ab
PVPH (24)	3.0 ± 1.3 c	1.8 ± 0.6 c	1.7 ± 1.0 b
McCain V-8 (18)	5.7 ± 2.1 b	2.9 ± 0.9 b	1.5 ± 0.4 b
PCH	8.2 ± 2.2 a	3.6 ± 0.8 a	2.9 ± 1.1 a
Test 2			
PARP (13)[z]	0.1 ± 0.2 b	3.1 ± 1.3 b	14.2 ± 1.2 b
PCH	3.5 ± 1.8 a	12. 6 ± 1.3 a	17.3 ± 1.0 a

[v] The azalea soil (clay) and Fraser fir soil (sandy loam) were naturally infested. The artificially infested soil in test 1 was clay loam (estimated inoculum density of five propagules per gram of soil) and in test 2, a sandy loam (estimated inoculum density of 20 propagules per gram of soil). Values are average of three-to-five reps and each test was repeated at least once. All values were obtained using the wet-sieving procedure.

[w] Number in parentheses is reference to original description of the medium.

[x] Propagules per gram recovered plus standard error of the mean.

[y] Values in a column followed by the same letter are not significantly different. $P = 0.05$ (test 1 and test 2 were analyzed separately).

[z] Hymexazol at 50 μg/ml was added to the medium for use in the soil assays.

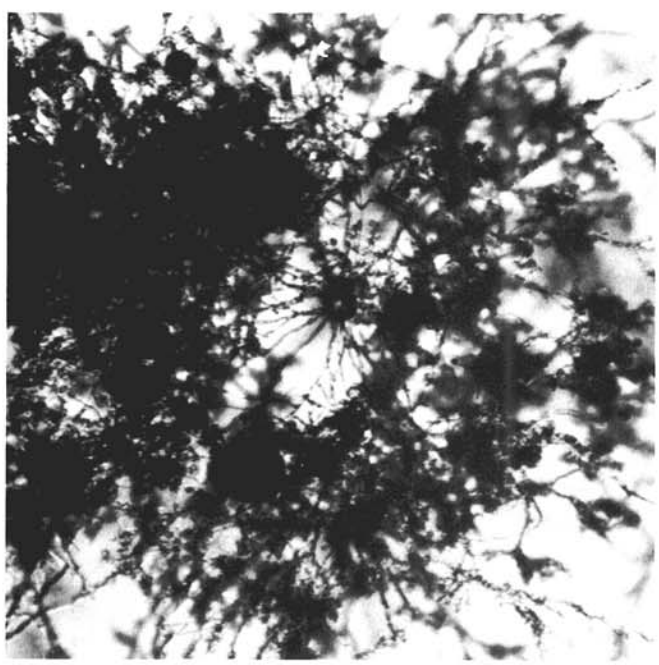

Fig. 1. Colony of *Phytophthora cinnamomi* developed from germinating chlamydospore on PCH medium.

The residue on the 38-μm sieve was rinsed with tap water and collected in 20–30 ml of deionized water in a 50-ml beaker. The suspension collected from each sample was then distributed onto 5–15 freshly poured 9-cm-diameter petri plates of PCH medium, and incubated for 48–72 hr at 20 C in the dark. Soil was rinsed from the agar surface of the plates with running tap water and colonies of *P. cinnamomi* were counted.

The semiautomatic elutriator NC-El (1,19) was used to extract fungus propagules from 50- to 200-g soil samples. Water and airflow rates to each of four elutriation columns were set at 80 ml/sec and 30–40 cm^3/sec, respectively. Organic matter and soil particles larger than 425 μm were collected on an initial 15-cm-diameter sieve. Water and debris passing through the 425-μm sieve were run through a sample splitter, and one-fifth of the suspended particles between 38 and 425 μm were collected on a 20-cm-diameter 38-μm sieve. The residue on the 38-μm sieve was suspended in deionized water and dispensed on PCH as described previously. The debris collected on the 425-μm sieve was blended in deionized water for 1 min at high speed in a Waring Blendor and then dispensed on the PCH medium. Elutriation was completed in 6–10 min; sandy loam soil required less elutriation time than the clay soil.

RESULTS

Bioassay. Initial tests compared Fraser fir seedlings and blue lupine radicles for recovery of *P. cinnamomi* from the naturally infested sandy loam soil. Fraser fir seedlings significantly ($P = 0.05$) increased recovery of *P. cinnamomi* compared to the blue lupine radicles; 81% of the Fraser fir seedlings and 33% of the lupine radicles yielded *P. cinnamomi* colonies on the selective medium.

In subsequent tests, azalea and rhododendron leaf disks and the Fraser fir seedlings increased ($P = 0.05$) recovery of *P. cinnamomi* from both the sandy loam and clay soils compared to the cedar needle baits. For example, when 10 g of the sandy loam soil (inoculum density of two propagules per gram of soil as determined by the wet-sieving selective medium technique) was placed in 100 ml of deionized water and baited, 100, 100, 83, and 34% of the azalea leaf disks, rhododendron leaf disks, Fraser fir seedlings, and cedar needles, respectively, yielded colonies of *P. cinnamomi* on the selective medium. With all baits except the cedar needles, an incubation time of 24 hr was superior to 48 hr because of a subsequent increase in the number of secondary organisms present (mostly bacteria and *Pythium* spp.), without an increase in the number of baits yielding *P. cinnamomi* after 48 hr.

Selective medium. The PCH medium improved recovery of *P. cinnamomi* in soil assays of naturally and artificially infested soils compared to other media tested (Table 1). Recovery of *P. cinnamomi* was increased 28–74% in the clay soil and 19–75% in the sandy loam soil with the PCH medium (Table 1).

The PCH medium effectively suppressed the growth of all undesired organisms (bacteria and fungi) in almost all soils tested. The low concentration of chloramphenicol in conjunction with the low pH of 5.2 for the PCH medium adequately suppressed soil bacteria when used with the wet-sieving procedure. When a surface soil dilution plating procedure (placing aliquots of unsieved soil suspensions directly on the agar surface) was used with the PCH medium, it was sometimes necessary to add 10 μg/ml chloramphenicol or 50 μg/ml penicillin-G and 5 μg/ml pimaricin to the soil suspension for optimum recovery of *P. cinnamomi*. In most soils, hymexazol efficently reduced growth of *Pythium* and *Mortierella* spp.; however, even at 75 ppm, hymexazol did not prevent growth of all *Pythium* spp. in all of our soil assays.

Mycelial growth rate of *P. cinnamomi* was reduced on PCH compared to cornmeal and V-8 agar media. The slower growth rate on PCH was an advantage of the medium since distinct colonies developed. Colonies were macroscopically identifiable on PCH after 48–72 hr of incubation at 20 C. *P. cinnamomi* colonies often consisted of a tight cluster of mycelium around the germinating propagule as well as the characteristic coralloid mycelial growth into the agar medium (Fig. 1). This growth pattern was most common when a colony originated from a *P. cinnamomi*

chlamydospore, the propagule most frequently recovered in assays of naturally infested soils.

Of 233 colonies of *P. cinnamomi* examined from assays of naturally infested clay and sandy loam soils, 87% originated from chlamydospores free in soil, 7% from organic matter (propagule unknown), and 6% had an unidentifiable origin. Many, but not all, of the chlamydospores from the naturally infested soil were thick-walled (Fig. 2). Thickness of the chlamydospore wall was determined following germination on PCH medium, with a mean wall thickness of 1.75 μm and a range of 1 to 2.6 μm observed.

Assay procedures. The wet-sieving and elutriation procedures were compared for the recovery of *P. cinnamomi* from the sandy loam soil. Average recovery in two tests, four reps per test (each rep consisted of a 100-g soil sample for elutriation and a 10-g soil sample for wet sieving) was 25 propagules per 10 g of soil with elutriation and 21 propagules per 10 g of soil with wet sieving, and was not significantly different.

Airflow reduced the time required to elutriate a soil sample and aided in breaking up soil aggregates. A sample was elutriated until the water flowing from the elutriation column contained few if any suspended particles; this required 6–10 min with all soils tested.

DISCUSSION

Dance et al (3) listed the characteristics of an ideal bait in a bioassay for *Phytophthora*. These characteristics include substrate availability, ease of use, high sensitivity, and susceptibility to all or at least most *Phytophthora* spp. Leaf disks of azalea and rhododendron satisfied the first three criteria very well for use in recovery of *P. cinnamomi* from soil. The leaf disks were not tested for recovery of other *Phytophthora* spp.

Since the PCH medium was developed for use with a wet-sieving procedure that removes most bacteria and small-spored fungi (<38 μm), the concentrations of the antibiotics and fungicides in the medium were low (18). The PCH medium can be effectively used in the direct plating of soil suspensions onto the surface of the medium which would allow for the recovery of all *P. cinnamomi* propagules present in the soil, but may require the addition of antibiotics to the soil suspension.

Although hymexazol reduced or eliminated growth of *Pythium* and *Mortierella* spp. (17,24) in most of our soil assays, assay results from some samples of the clay and sandy loam soil indicated that hymexazol had little effect on some of the *Pythium* spp. that were present. Similar results were reported by other researchers (7). Chloramphenicol at 10 μg/ml, in conjunction with a low pH of 5.2, was sufficient to inhibit bacterial growth when used in combination with wet sieving. Chloramphenicol was used in other *Phytophthora*-selective media for recovery of *Phytophthora* spp. from soil (11,22). Sneh (22) developed a medium containing 60 μg/ml chloramphenicol. When chloramphenicol at 60 μg/ml was added to the PCH medium, propagules germinated but growth of mycelium was sparse and not characteristic of *P. cinnamomi*.

A pH of 4.5–5.0 was reported to be optimum for recovery of *P. cinnamomi* (10). Adjusting the pH of PCH, either lower or higher than the initial 5.2, reduced recovery of *P. cinnamomi* in our assays. The optimum pH for recovery of *P. cinnamomi* may thus be dependent on the assay medium being used. Optimum pH range for germination of *P. cinnamomi* chlamydospores (the most common propagule of *P. cinnamomi* in azalea and fir soils) is 5.0–7.0 (25).

The presence of thick-walled chlamydospores of *P. cinnamomi* has been reported from culture (21) and in nature (9). Many of the chlamydospores observed in our soil assays from naturally infested soil had thick walls (mean thickness, 1.75 μm). Chlamydospores of *P. cinnamomi* produced in culture typically do not have an inner wall (8,25). The thick wall may enhance long-term survival of *P. cinnamomi* in soil, and/or may be a response to substrate or to biotic stresses present in soil. Thick-walled chlamydospores of *P. palmivora* had different nutritional requirements for germination than thin-walled spores (12). If the same is true for *P. cinnamomi* chlamydospores, it would support the conclusion of Tsao (23) that development of selective media should be based on isolation or recovery of the organism from naturally infested soil. The PCH medium was developed based on improved recovery of *P. cinnamomi* from naturally infested soil.

The use of a selective medium in conjunction with the elutriation procedure permits the assay of large soil samples for *P. cinnamomi*. This may be advantageous because the inoculum density of many *Phytophthora* spp. in soil is very low and both the elutriation and wet-sieving procedures effectively concentrate *P. cinnamomi* propagules (18).

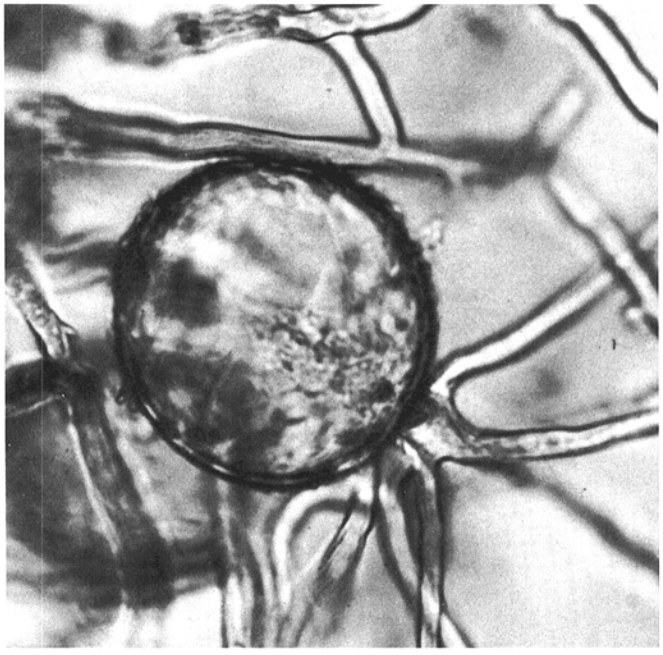

Fig. 2. Thick-walled chlamydospore of *Phytophthora cinnamomi* isolated from naturally infested soil and germinating on PCH medium.

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