Quantitation and Detection of *Phytophthora cinnamomi* in Avocado Production Areas of South Florida

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ABSTRACT

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Several methods and several agar media were tested for recovery of *Phytophthora cinnamomi* (cause of Phytophthora root rot of avocado [*Persea americana*]) in avocado production areas in Florida. Greater recoveries of this pathogen from soil were achieved with McCain's and PARPH media than with four other previously described semiselective media ($P < 0.05$). Maximum recovery of *P. cinnamomi* from avocado roots, however, appeared to be less critical. Similar levels of the pathogen were recovered using one nonselective (PDA) and seven semiselective media or several different methods for treating roots prior to plating on media. The pathogen was recovered equally well from either soil or roots with PARPH. Therefore, either soil or roots may be used for detecting this pathogen in Florida avocado groves.

Additional keywords: avocado root rot, *Pythium* spp.

Phytophthora root rot (caused by *Phytophthora cinnamomi* Rands) is the most important disease of avocado (*Persea americana* Mill.) throughout the world (4,15,25). Root rot reduces root biomass and adversely affects root function by killing feeder roots (21,24). Affected trees may die or survive with reduced yields of normally sized fruit and nonspecific canopy symptoms that include chlorosis, necrosis, wilting of foliage, or defoliation (14,18,25).

Although a methodical survey for detecting *P. cinnamomi* in Florida avocado groves has never been conducted, this pathogen apparently occurs commonly in these areas. Burns et al (2) recovered *P. cinnamomi* from eight of 16 avocado groves sampled in south Florida in 1965, and we recovered the pathogen from eight of 10 groves sampled in 1987 (*unpublished*).

The effects of Phytophthora root rot in Florida have been underestimated in the past for several reasons: root rot seldom kills trees in Florida in the absence of flooding, and symptoms of the disease are not specific. The impairment of photosynthesis and stomatal conductance of avocado by root rot is accelerated by flooding (14,17). Flooding occurs periodically in Florida as a result of hurricanes and tropical storms. Rates of photosynthesis decline quickly in infected plants after the onset of flooding. Even in plants with low levels of root rot, wilting, defoliation, and death may occur within a week (14).

Photosynthesis and normal stomatal behavior of avocado are inversely related to root rot severity (14,17). However, canopies of root-rotted plants that have impaired photosynthesis may appear healthy. Also, canopy symptoms are not always found in trees with root rot in Florida (Ploetz, *unpublished*). Therefore, these symptoms are not reliable indicators for the presence of root rot.

The prediction of future problems with root rot associated with flooding may depend on methods for detecting *P. cinnamomi* in avocado production areas. In addition, studies on the ecology of *P. cinnamomi*, epidemiology of root rot, and the development of criteria for controlling the disease may depend upon a method for quantitating this pathogen. This study was conducted to evaluate

Table 1. Selective media used for the isolation of *Phytophthora cinnamomi* from soil and avocado roots

Medium	Authors, year, and basal medium	Selective ingredients
Flowers's	Flowers and Hendrix 1969 (5) Agar 20.0 g/L Sucrose 30.0 g/L Yeast extract 0.5 g/L Thiamine HCl 2 mg/L Mineral salts	Gallic acid 0.425 g/L Nystatin 100,000 units/L PCNB 25 mg/L Penicillin 80,000 units/L Rose bengal 0.5 mg/L
McCain's	McCain et al 1967 (9) Agar 40.0 g/L V-8 juice 100 ml	Nystatin 50 mg/L Vancomycin 100 mg/L PCNB 10 mg/L
Modified Kerr's	Hendrix and Kuhlman 1965 (6) Agar 40.0 g/L Sucrose 30.0 g/L Yeast extract 0.5 g/L Mineral salts	Nystatin 100,000 units/L PCNB 100 mg/L Streptomycin 50 mg/L Rose bengal 60 mg/L
PARPH	Mitchell et al 1986 (11) Cornmeal agar 17.0 g/L	Ampicillin 250 mg/L Hymexazol 50 mg/L PCNB 100 mg/L Pimaricin 10 mg/L Rifampicin 10 mg/L
PCH	Shew and Benson 1982 (19) Agar 15.0 g/L Yeast extract 0.3 g/L Thiamine HCl 1 mg/L Mineral salts	Chloramphenicol 10 mg/L Hymexazol 50 mg/L PCNB 26 mg/L Pimaricin 25 mg/L
PVPH	Tsao and Guy 1977 (23) Cornmeal agar 17.0 g/L	Hymexazol 50 mg/L PCNB 100 mg/L Pimaricin 10 mg/L Vancomycin 200 mg/L

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methods for detecting and quantitating this pathogen. Portions of this work were reported previously (13).

MATERIALS AND METHODS

Soil assays. Naturally infested soil from Collier County (Immokolee fine sand [FS], trial 2) or Dade County (Rockdale fine sandy loam [FSL], trials 4–7) and artificially infested Rockdale FSL amended with infested millet seed in trials 1 and 3 (14) were assayed for *P. cinnamomi* using previously described semiselective media (Tables 1 and 2). Naturally infested soil was recovered from the dripline of trees with canopy symptoms of root rot. Four subsamples (each about 1 L) were taken with hand trowels from each tree and were bulked and thoroughly mixed before use in an assay. Soil samples were processed within 16 hr of collection. Because Rockdale FSL consists of mainly large particles (>1 cm in diameter), this soil was wet sieved before use. Only the 2-mm to 0.045-mm fraction of Rockdale FSL was used in these assays. Nonfractionated Immokolee FS was used during trial 2.

All media were dispensed into 9-cm-diameter, plastic petri plates and stored in the dark at room temperature the day before use. In general, Flowers's (5), McCain's (9), modified Kerr's (6), PARPH (11), PCH (19), and PVPH (23) media were utilized per specifications published with descriptions of the media. With the exception of trial 1, each of these media was amended with 50 mg of hymexazol/L; only PARPH, PCH, and PVPH were amended with hymexazol for trial 1. Soil samples were suspended in sterile 0.25% water agar (about 0.3 g [oven-dry wt]/ml) and mixed for about 3 min with a magnetic bar and a stirring plate. While soil suspensions were stirring, 1.0 ml of given suspensions were recovered with wide-mouth pipettes, placed on each of five (trials 2 and 4–6) or 10 (trials 1, 3, and 7) plates of each medium, and dispersed evenly across the medium surface with the blunt end of a test tube. Soil suspensions were dried at 100 C to obtain dry weights used for the determination of propagule densities.

After soil was dispensed, media were incubated in the dark at 25 C for 3 days. Soil was then washed from plates and colonies of *P. cinnamomi* were counted. *P. cinnamomi* was identified on the basis of its characteristic granular appearance on agar media when backlit. Colonies of questionable identity or those that were obscured by other fungi were verified as *P. cinnamomi* with a compound microscope after observing coralloid hyphal thickenings that are formed by the pathogen (7,12). The identity of these colonies was verified further as *P. cinnamomi* by periodically examining subcultures of these colonies for nonpapillate sporangia. Sporangia were produced using V-8 broth and a mineral salts solution as described by Chen and Zentmyer (3). Recovery of *P. cinnamomi* was expressed as the number of propagules recovered per gram (oven-dry wt) of soil.

Root assays. Necrotic roots were taken from the dripline of avocado trees with canopy symptoms of root rot and tested with different media and methods for recovery of *P. cinnamomi*. Root segments used in root assays were about 1 cm long and usually included a root tip.

In a first set of root assays, Difco potato-dextrose agar (PDA) plus the six semiselective media tested for recovery of *P. cinnamomi* from soil were evaluated for recovery of the pathogen from roots (Table 3); all semiselective media were amended with 50 mg of hymexazol/L. Roots were thoroughly washed with tap water, and, with one exception, were then immersed for 30 sec in 95% ethanol, rinsed once in sterile, deionized water, blotted dry on sterile paper towels, and placed on one of the semiselective media or PDA (PDA1). These plates were incubated for 3 days in the dark at 25 C before they were observed for growth of *P. cinnamomi*. Roots were also processed and incubated on PDA in a second manner (PDA2) as described by McMillan and Tepper (10). These root segments were washed under flowing tap water for 1 hr, surface-disinfested for 5 sec in 1% NaClO, and rinsed 10 times in sterile, distilled water. Root segments were blotted dry on sterile paper towels (trials 2 and 3) or not blotted dry (trial 1) before placement on the medium surface. These plates were incubated in the dark at 20 C for 3 days before they were scored for growth of *P. cinnamomi*.

In a second set of root assays, surface-disinfestation (95% ethanol for 30 sec) or no surface-disinfestation, overnight washes in running tap water or no washes, and PARPH amended (PARPH1) or not amended with 100 mg of iprodione (Rovral 50% WP)/L (20) were tested for recovery of *P. cinnamomi*. In the first two trials in this set of root assays, root segments from naturally (trial 1) and artificially (trial 2) infested Rockdale FSL were assayed; a 2 × 2 × 2 factorial combination of the above treatments was tested. In a third trial, roots from a naturally infested avocado grove were utilized in a 2 × 2 factorial testing of tap water washes and surface-disinfestation with ethanol; roots were plated on

Table 2. Quantitation and detection of *Phytophthora cinnamomi* in soil with selective agar media

Trial[t]	Tree[u]	Medium[s]/mean propagule densities (per g soil [oven dry wt])					
		Flowers's	Kerr's	McCain's	PARPH	PCH	PVPH
1	1	25.0 ab[v]	0 b	6.3 b	37.5 a	0 b	0 b
2	1	0 a	0 a	0 a	0 a	0 a	0 a
	2	0 b	0 b	0 b	2.5 a	0.5 b	0 b
	3	0 b	0 b	0 b	2.6 a	0.5 b	0 b
	4	0 a	0 a	0 a	0 a	0 a	0 a
3	1	1.0 bc	0.5 bc	3.3 b	9.5 a	0 c	2.5 bc
4	1	0 a	0 a	2.1 a	2.1 a	0 a	0 a
	2	0 a	0 a	0 a	0 a	0 a	0 a
5	1	0 a	0 a	0 a	0.9 a	0 a	0 a
	2	0 a	0 a	0 a	0 a	0 a	0 a
6	1	+[w]	+	7.8 a	6.3 a	0 b	...[x]
	2	+	+	14.5 a	15.9 a	6.2 b	...
	3	+	+	0.6 b	2.8 a	0 b	...
7	1	+	+	3.3 a	3.3 a	0 a	2.5 a
	2	+	+	30.7 a	38.0 a	0 b	1.5 b
	3	+	+	4.2 a	2.1 ab	0 b	0 b
Quantitation[y]		2.6 b	0.1 b	4.5 ab	7.7 a	0.5 b	0.5 b
Detection[z]		2/10 b	2/10 b	9/16 ab	12/16 a	3/16 b	3/13 b

[s] Flowers's = Flowers's (5) medium, Kerr's = modified Kerr's medium (6), McCain's = McCain's (9) medium, PARPH = PARPH medium (11), PCH = PCH medium (19), PVPH = PVPH medium (23). For all trials except 1, media were amended with 50 mg hymexazol/L; only PARPH, PCH, and PVPH were amended with hymexazol during trial 1.

[t] For trials 2 and 4–7, each trial represents one avocado grove naturally infested with *P. cinnamomi*; artificially infested soil from pot studies was used for trials 1 and 3. The 2-mm to 0.045-mm fraction of Rockdale soil was assayed for trials 1 and 3–7, and nonfractionated Immokolee fine sand was assayed for site 2.

[u] For trials 2 and 4–7, trees with canopy symptoms of root rot were chosen for assays; soil was recovered from the dripline of these trees.

[v] Within a row, means are separated on the basis of Duncan's multiple range test at $P < 0.05$.

[w] + = Excessive bacterial and fungal background contaminants; detection of colonies of *P. cinnamomi* on these plates impossible.

[x] PVPH was not used in trial 6.

[y] Quantitation = mean propagule densities for all trials in which a given medium was tested.

[z] Detection = number of soil samples (trees) for which *P. cinnamomi* was detected/total number of soil samples assayed.

PARPH. Roots in the second set of root assays were incubated at 25 C for 3 days before they were examined for growth of *P. cinnamomi*.

Sixty root segments were assayed for each medium in the first set of root assays, and for each treatment combination in the second set of root assays. In each set of root assays, six root segments were placed on each of 10 plates for each treatment or treatment combination; each plate was considered a replicate. Analyses of variance for integer values (i.e., values ranged from 0–6 for replicates) were conducted prior to the calculation of proportions presented in Table 3.

Comparison of recovery of *P. cinnamomi* from soil vs. roots. Soil and roots were each recovered from the dripline of a total of 24 symptomatic trees in naturally infested avocado groves on eight different occasions and were tested on PARPH for recovery of *P. cinnamomi* to determine if the pathogen was recovered more frequently from soil or roots (Table 4). Soil assays were conducted as described above. Ten plates were used for each tree sampled. Root segments used in root assays were surface-disinfested for 30 sec with 95% ethanol, rinsed once with sterile, deionized water, and blotted dry with sterile paper towels before placement on PARPH. Sixty root segments were used for each sample. Soil and roots were incubated for 3 days in the dark at 25 C before plates were examined for growth of *P. cinnamomi*.

RESULTS

Soil assays. The PARPH medium was the most reliable of six selective media that were tested to quantitate and detect *P. cinnamomi* in soil. Propagule densities recovered from soil by PARPH were, in all cases, as high or higher than those recovered by any of the other media used ($P < 0.05$) (Table 2). However, mean recovery of *P. cinnamomi* from all 16 soil samples with PARPH was not different from that obtained with McCain's medium ($P < 0.05$).

In four of 16 soil samples, we failed to detect *P. cinnamomi* using PARPH. However, the pathogen was also not detected with any of the other semiselective media tested with these samples. Although McCain's medium was also a reliable medium for detecting *P. cinnamomi* in soil, we did not detect the pathogen with this medium in three samples in which the pathogen was detected with PARPH (Table 2). This difference was not significant when detection for all 16 samples was considered (Table 2).

Root assays. No medium gave consistently superior recovery of *P. cinnamomi* from avocado roots (Table 3). Recovery of the pathogen with PARPH was greater than that achieved for some (tree 2) or for all other media (tree 1) tested in trial 3 ($P < 0.05$). However, differences were not detected in two other trials in this series (Table 3). Mean quantitation and detection for all eight root samples in the series were similar for all media (Table 3).

In the second series of root assays, no treatment or treatment combination resulted in consistently greater recovery of *P. cinnamomi* from roots. There were no significant interactions in trials 1 and 2 between the different root treatments and PARPH or PARPHI ($P < 0.05$), and no differences were detected when pooled comparisons were made between results for the two media. There were significant interactions between results for the wash and ethanol treatments in trial 1, but not trial 2; appropriate pooled comparisons were made on these bases. Ethanol surface-disinfestation without an overnight tap water wash was responsible for higher recoveries of *P. cinnamomi* than other treatments in trial 1, but no differences were detected in trial 2. Ethanol surface-disinfestation and overnight tap water washes had no effect on recovery of *P. cinnamomi* in trial 2, and in trials 2 and 3, respectively.

Recovery of *P. cinnamomi* from soil vs. roots. The frequencies of detection of *P. cinnamomi* from soil and avocado roots with the PARPH medium were very similar (Table 4). In paired samples taken from the same trees, the pathogen was detected in 19 of 24 root samples and 18 of 24 soil samples.

DISCUSSION

P. cinnamomi was commonly recovered from artificially and naturally infested Rockdale FSL with either McCain's medium or PARPH (Table 2). In addition, although it was used only four times with Immokolee FS, it would appear that PARPH is superior to McCain's and the other media tested for recovery of *P. cinnamomi* from this soil type.

At times, quantitation of *P. cinnamomi* in soil with McCain's medium and PARPH was difficult due to background contamination by species of *Pythium* that were insensitive to hymexazol. Microscopic examination of plates heavily contaminated with these fungi was necessary for some soil and root samples, and some colonies of *P. cinnamomi* were probably not detected due to these contaminants.

Shew and Benson (19) and Tsao (22) observed that naturally infested soils should be used during the development of a selective medium for recovering species of *Phytophthora* from naturally infested soil. Apparently, four of the six semiselective media tested in our work

Table 3. Quantitation and detection of *Phytophthora cinnamomi* in avocado roots with selective agar media

	Recovery[w]									
	Trial 1		Trial 2			Trial 3				
Medium[x]	Tree 1	Tree 2	Tree 1	Tree 2	Tree 3	Tree 1	Tree 2	Tree 3	Q[y]	D[z]
1	0 a	0.06 a	0.12 a	0.25 a	0.03 a	0.07 b	0.17 ab	0.13 a	0.10 a	7 a
2	0.05 a	0.02 a	0.08 a	0.13 a	0.02 a	0.10 b	0.12 ab	0.12 a	0.08 a	8 a
3	0.02 a	0.04 a	0.07 a	0.13 a	0.05 a	0.08 b	0.12 ab	0.10 a	0.08 a	8 a
4	0.04 a	0.02 a	0.07 a	0.22 a	0.05 a	0.25 a	0.23 a	0.18 a	0.13 a	8 a
5	0.05 a	0 a	0.05 a	0.23 a	0.12 a	0.08 b	0.15 ab	0.13 a	0.10 a	7 a
6	0.02 a	0.05 a	0.05 a	0.15 a	0.07 a	0.08 b	0.13 ab	0.10 a	0.08 a	8 a
7	0 a	0.05 a	0.08 a	0.17 a	0.03 a	0.12 b	0.13 ab	0.15 a	0.09 a	7 a
8	0.03 a	0.03 a	0.08 a	0.23 a	0.02 a	0.10 b	0.05 b	0.20 a	0.09 a	8 a

[w] Necrotic feeder roots were recovered from the dripline of symptomatic avocado trees in three avocado groves (trials 1–3). Recovery = proportion of 60 1-cm root segments from which *P. cinnamomi* was recovered. Within a column, proportions are separated on the basis of Duncan's multiple range test at $P < 0.05$.

[x] 1 = Flowers's (5) medium, 2 = modified Kerr's (6) medium, 3 = McCain's (9) medium, 4 = PARPH medium (11), 5 = PCH medium (19), 6 and 7 = Difco potato-dextrose agar, 8 = PVPH medium (23). With one exception, root segments were surface-disinfested for 30 sec in 95% ethanol, rinsed once in sterile, deionized water, blotted dry on sterile paper towels, and placed on a medium before incubation for 3 days without light at 25 C. Root segments for medium 7 (PDA) were prepared and incubated at 20 C as described by McMillan and Tepper (10).

[y] Q = quantitation: mean recoveries of *P. cinnamomi* for all root samples (trees) assayed for a given medium.

[z] D = detection: number of root samples (trees) for which *P. cinnamomi* was detected per eight total root samples.

performed as well with naturally infested soil as with artificially infested soil (Table 2). Although we detected *P. cinnamomi* only in artificially infested soil with Flowers's and modified Kerr's media, it is possible that this was due to the relative insensitivities of these media in detecting this pathogen because artificially infested soil in these assays had higher levels of *P. cinnamomi* than the naturally infested soil.

Flowers's and modified Kerr's media were relatively inflexible with regard to the amount of certain soils that could be assayed effectively for *P. cinnamomi*. Excessive bacterial and fungal contamination developed with some soils when about 0.3 g (dry wt) of soil was added to a plate. Consequently, it was difficult or impossible to identify colonies of *P. cinnamomi* in these situations (10-fold dilutions of these soils [0.03 g] resulted in more easily read plates). McCain's, PARPH, PCH, and PVPH media were more adaptable in this regard.

The PCH medium used in the present work has been used previously to recover *P. cinnamomi* from soil and roots in North Carolina (1,8,16,19), and in one report, it performed better than PARPH for recovering this pathogen from soil (19). Reasons for the poor performance of PCH in the present soil assays are not clear (Table 2). Although Shew and Benson (19) suggested that maximum recovery of *P. cinnamomi* from soil with PCH may be pH dependent, the different pHs of PCH and PARPH (5.2 and 5.7, respectively) were probably not responsible for the different levels of recovery noted for these media in our work. Alternatively, it is possible that soil must be suspended in water rather than dilute water agar in order for PCH to perform satisfactorily. However, based on a limited number of studies conducted with these media and tap water and 0.25% water agar (Ploetz, *unpublished*), this appears unlikely. Apparently, PCH and PARPH perform differently with different soils.

In contrast to the recovery of *P. cinnamomi* from soil with semiselective media, no superior medium was identified for recovering this pathogen from avocado roots (Table 3; *data not shown*). Solel and Pinkas (20) described inhibiting the growth of *Pythium* spp., which were insensitive to hymexazol and hindered detection of *P. cinnamomi*, by amending the medium used for detection (PARPH) with iprodione. In our work, the addition of iprodione to PARPH had no effect on the performance of this medium. Also, pretreatment of roots with ethanol, NaClO, or tap water had no effect on recovery of this pathogen. Therefore, time-consuming preparation of selective media or treatment of roots prior to plating on culture media appears to be unnecessary for recovery of *P. cinnamomi* from symptomatic avocado roots in Florida.

During the present study, *P. cinnamomi* was as readily detected in soil as in avocado roots (Table 4). Therefore, soil or roots are equally reliable sources of this pathogen in Florida when one is concerned only with detecting the fungus in a given area. For this objective, one need only test roots for colonization by the pathogen on PDA. Ecological or epidemiological studies, however, may require knowledge of soilborne populations of the fungus, thereby necessitating the use of semiselective media. In any event, *P. cinnamomi* may be detected in avocado roots in Florida by using PDA, or in artificially and naturally infested soil by using semiselective media.

Table 4. Comparison of soil and avocado roots as sources for recovery of *Phytophthora cinnamomi*

Trial	Tree	Source[w] Soil	Source[w] Roots
1	1	0[x]	0.01[y]
2	1	6.3	0.07
	2	15.9	0.22
	3	2.8	0.05
3	1	3.3	0.25
	2	38.0	0.23
	3	2.1	0.18
4	1	0	0.10
	2	2.7	0
	3	0	0.03
	4	0.7	0.02
5	1	0	0
	2	5.3	0.04
	3	3.7	0.13
	4	2.0	0.13
6	1	0.4	0.2
	2	2.7	0.03
	3	2.5	0.10
7	1	0	0
	2	6.3	0.03
	3	1.7	0.07
8	1	0	0
	2	0.4	0
	3	7.8	0.08
Detection[z]			
Soil		18/24	
Roots			19/24

[w] Avocado trees in each trial in naturally infested groves in Rockdale soil. Soil and roots taken from the dripline of symptomatic trees.

[x] Propagules of *P. cinnamomi* per g (oven-dry wt) of soil (2-mm to 0.045-mm fraction) detected on PARPH (11).

[y] Proportion of approximately 60 1-cm-long root segments from which *P. cinnamomi* was recovered on PARPH.

[z] Proportion of samples in which *P. cinnamomi* was detected.

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