

Quantitation of *Phytophthora cinnamomi* in Avocado Roots Using a Species-Specific DNA Probe

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We thank J. Menge for encouraging this project and for providing comments on the manuscript and H. Azad, H. Förster, and J. Hartin for supplying DNA samples.

Accepted for publication 20 April 1996.

ABSTRACT

Judelson, H. S., and Messenger-Routh, B. 1996. Quantitation of *Phytophthora cinnamomi* in avocado roots using a species-specific DNA probe. *Phytopathology* 86:763-768.

The proliferation of *Phytophthora cinnamomi* in avocado roots was quantified with a sensitive, species-specific DNA probe. The probe was isolated from a library of genomic DNA by identifying colonies that strongly hybridized to DNA of *P. cinnamomi* but not to DNA from other species of *Phytophthora*. The characterization of five such clones indicated that each detected the same repetitive sequence that was present in genomic DNA

at about 12,000 copies per haploid genome. Cross-hybridizing sequences were absent from DNA of other Oomycetes, bacteria, ascomycetes, basidiomycetes, or plants. The probes detected as little as 5 pg of *P. cinnamomi* DNA in dot- and slot-blot assays. The *P. cinnamomi* probe and a probe for avocado DNA were used to quantitate the growth of the pathogen in roots. The extent of colonization, judged by measuring the relative amounts of pathogen and host DNA, increased over time and with increasing amounts of inoculum.

Additional keywords: disease diagnosis, root rot.

Phytophthora cinnamomi Rands is a widely distributed and important pathogen of a large and diverse range of hosts, including forest, horticultural, and ornamental crops (37). It is considered primarily a soilborne root pathogen, causing the death of host tissue through fine-root necrosis, although the role of stem and bark infections in disease also has been considered (6). In California, about two-thirds of the acreage planted with avocado contains *P. cinnamomi*, resulting in yield losses estimated at 30% (5).

A variety of approaches have been evaluated for controlling *P. cinnamomi* in avocado and other hosts. These include use of resistant cultivars or rootstocks (10), biocontrol agents (11), soil amendments (36), and fungicides (5). Assays that measure the proliferation of *P. cinnamomi* are required to test the efficacy of these methods. Techniques for detecting the pathogen also are useful for diagnostic purposes and for surveying soil for the pathogen as an aid to the management of plantings.

Several approaches for the detection and quantitation of *P. cinnamomi* have been developed that vary in their sensitivity, specificity, and suitability to different applications. For example, a sensitive method for the immunodetection of zoospores was described that may be superior to traditional baiting methods for measuring levels of the fungus in soil (2). Immunoassays for measuring mycelia within host tissue have been developed, but their sensitivities are too low and their cross-reaction with other species too high for many applications (1). Other methods used for assessing the colonization of plants by *P. cinnamomi* include measuring lesions (10, 29) and testing root sections for fungal propagules by agar-plating techniques (1,6,8). These approaches are useful but imperfect because they may not be species-specific, lesions may reflect host response more than fungal proliferation, and high-resolution sampling for precise quantitation may be impractical. Also, root-plating techniques are difficult to relate to the degree of infection because sec-

tions that are heavily infected appear similar to areas containing lower numbers of propagules.

A specific DNA hybridization assay for *P. cinnamomi* would overcome many limitations of existing procedures for detecting and measuring this important pathogen. DNA probes have been developed previously for a variety of plant pathogens (18,25,35). In *Phytophthora*, DNA hybridization or polymerase chain reaction (PCR) has been used for species identification with pure cultures or baited tissue (12,13,14,19,30). However, such assays have not been used successfully to detect *Phytophthora* in tree roots or to quantify the colonization of any host tissue. This paper describes the cloning and characterization of fragments of repetitive DNA specific to *P. cinnamomi* and their use to detect and accurately measure levels of the fungus in roots of infected avocado.

MATERIALS AND METHODS

Extraction of DNA. DNA was extracted from *Achlya ambisexualis* (strain 87-1), *P. infestans* (strain 1306), and the *P. cinnamomi* isolates mentioned in Figure 1, as described previously (17), using mycelia grown in peptone-yeast extract-glucose media (3), clarified rye (4), and clarified V8-juice (V8C; [24]) broth, respectively. DNA from *Penicillium digitatum* (grown on V8 media) and *Agaricus bisporus* (Alpha-Beta Supermarket, Riverside, CA) was extracted by the potassium ethyl xanthogenate (PEX) method (16). Other laboratories provided DNA from *Gliocladium virens* KA230, *P. cactorum* 3406, *P. capsici* 412, *P. citricola* 1821, *P. citrophthora* 1200, *P. cryptogea* 3650, *P. dreschleri* 1795, *P. fragariae* 6569, *P. meadii* 3500, *P. megakarya* 1664, *P. megasperma* 6979, *P. palmivora* 1787, *P. parasitica* 6622, *P. sojae* 7076, *Pseudomonas syringae*, *Pythium irregulare* 3804, and *Trichoderma harzianum* K6.

Avocado DNA was obtained by several procedures. Root DNA usually was extracted by a modification of the Murray and Thompson method (21). Roots (2 g) were ground under liquid nitrogen and incubated at 65°C for 20 min with 8 ml of prewarmed 2% CTAB (cetyltrimethylammonium bromide hydroxide), 0.1 M Tris, pH 8.0, 20 mM Na₂EDTA, 1.4 M NaCl, and 1% β-mercaptoethanol.

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The resulting slurry was extracted twice with 10 ml of chloroform/isoamyl alcohol (24:1) and incubated at 4°C for 2 h with 125 µg of yeast tRNA, 20 ml of 1% CTAB, 50 mM Tris, pH 8.0, 10 mM Na₂EDTA, and 0.5% β-mercaptoethanol. The nucleic acids were recovered by centrifuging at 7,500 × g for 5 min and were dissolved in 0.5 ml of 10 mM Tris, pH 8.0, 1 mM Na₂EDTA, and 1 M NaCl. DNA was reprecipitated by adding 1 ml of ethanol and centrifuging for 10 min at 12,000 × g. The pellet was washed in 70% ethanol, dissolved in 100 µl of 10 mM Tris, pH 8.0, plus 1 mM Na₂EDTA, and incubated with 10 µg of ribonuclease A for 30 min at 37°C. DNA concentrations were estimated by spotting samples on a gel of 0.8% agarose containing 0.5 µg of ethidium bromide per ml, with bacteriophage λ DNA as concentration standards. As described in the text, leaf and some root DNA samples were ex-

tracted or further purified by protocols with PEX (16), sodium dodecyl sulfate (SDS) plus proteinase K (18), guanidium salts (DNAzol, Life Technologies, Bethesda, MD), polyethylene glycol precipitation (26), or commercial DNA affinity matrices (QIAEX silica resin, Qiagen Ltd., Chatsworth, CA; Wizard ion exchange resin, Promega, Madison, WI).

Construction of the library and analysis of clones. DNA manipulations were performed by standard techniques (26). Total genomic DNA from *P. cinnamomi* M254 was digested with *Hind*III and *Xba*I, ligated to similarly digested pUC18, and transformed into *Escherichia coli* strain DH5α. The resulting transformants were replicated to nitrocellulose filters that were hybridized to ³²P-labeled total genomic DNA of *P. parasitica* 1751 and *P. cinnamomi* M254, as described below, to identify *P. cinnamomi*-specific clones. For 40 candidate clones, specificity was characterized further by spotting approximately 200 ng of plasmid DNA on seven replicate nylon membranes (Hybond N⁺, Amersham Corp., Arlington Heights, IL), which were hybridized to radiolabeled genomic DNA from other species of *Phytophthora*, as described below.

Hybridization assays. ³²P-labeled probes obtained from the clones described above were hybridized to nitrocellulose filters for colony hybridizations or to nylon membranes for dot, slot, and gel blots, as described previously (17). For slot and dot blots, DNA samples in 0.2 ml of 1× SSPE (180 mM NaCl, 10 mM sodium phosphate, pH 7.7, 1 mM EDTA) were boiled for 5 min, cooled on ice, and applied by mild suction to membranes prewetted with 1× SSPE; after rinsing the wells with 0.2 ml of 1× SSPE, the membranes were removed from the manifold, floated for 5 min on 0.4 M NaOH plus 0.8 M NaCl, and rinsed three times in 3× SSPE prior to hybridization. After hybridization, filters were washed with a final high-stringency wash of 0.1× SSPE, 0.3% SDS, and 0.1% sodium pyrophosphate at either 65°C (blots shown in Figures 1, 2, 4, and 5) or 68°C (blot shown in Figure 3). The filters were exposed to film with intensifying screens, except for the experiment shown in Figure 1, in which screens were not used. In some experiments, the insert from pCR-S1 was used as a control for nonspecific hybridization; this plasmid contains a fragment of *P. infestans* DNA that does not hybridize to DNA of other Oomycetes or plants.

Hybridization intensities were determined by scanning films at 600-dpi optical resolution with a Hewlett-Packard (Palo Alto, CA) ScanJet3C device and analyzing the data with a NIH Image 1.58 (written by W. Rasband, National Institutes of Health, Bethesda, MD) running on a Macintosh 7100/66 computer. Intensities were translated to absolute amounts of DNA by comparison to internal standard curves.

Infection assays. Avocado seedlings (cv. Topa Topa) were grown in UC No. 4 soil mix in pots in a greenhouse for 10 months and

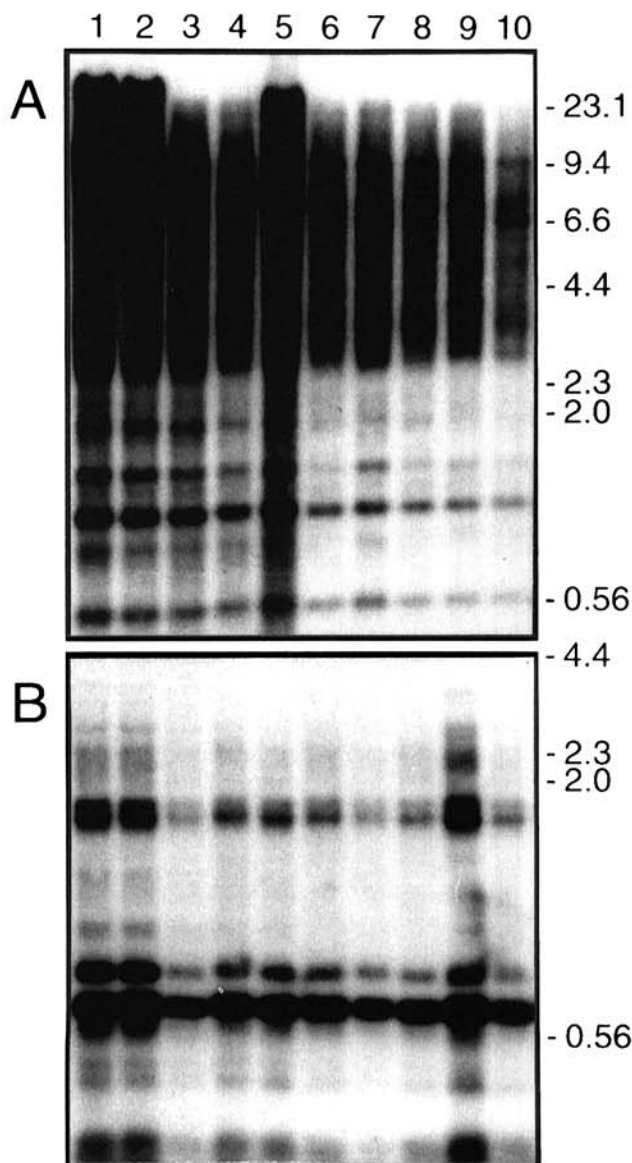


Fig. 1. Hybridization of a high-copy probe to DNA of *Phytophthora cinnamomi*. One microgram of *P. cinnamomi* DNA was digested with **A**, *Hind*III or **B**, *Hae*III, resolved by agarose gel electrophoresis, transferred to a nylon membrane, hybridized with a radiolabeled 4.2-kb insert from pPC19, and exposed to film for 2 h. Lane 1, A1 isolate 6493 from *Rhododendron* spp. in China; lane 2, A1 isolate 3657 from *Castanopsis* spp. in Papua New Guinea; lane 3, A1 isolate 6379 from *Ananas comosus* in Taiwan; lane 4, A2 isolate 2428 from *Persea americana* in California; lane 5, A1 isolate 2021 from *Camellia japonica* in California; and lanes 6 through 10, A1 isolates M283, M282, M280, M262, and M254, respectively, from avocado in California. Size markers derived from *Hind*III-digested DNA of bacteriophage λ are indicated on the right.

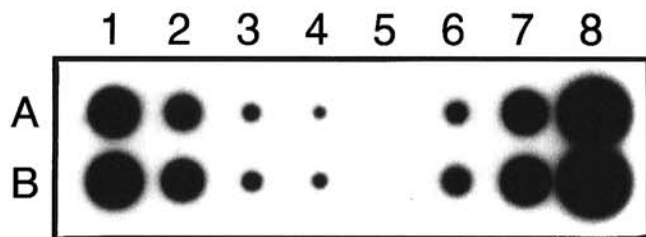


Fig. 2. Reconstruction experiment used to determine the copy number of the repeated sequence. DNA from *Phytophthora cinnamomi* isolate M254 and the 460-bp high-copy fragment from pPC19 was applied to a nylon membrane and hybridized to the fragment labeled with ³²P. Hybridization intensities and relative copy numbers were determined by densitometry as described in text. Columns 1, 2, 3, and 4 contained 9.2, 0.92, 0.092, and 0.0092 ng of the 460-bp band, respectively; columns 5, 6, 7, and 8 contained 0, 2, 20, and 200 ng of *P. cinnamomi* DNA, respectively. Rows A and B contained duplicate samples. The signal from the 0.092-ng pPC19 sample (column 3) was equivalent to that which would result if the repeated sequence was present in 5,000 copies per 10⁸-bp haploid genome.

were watered with 14% Hoagland's solution (15) as needed. *P. cinnamomi* M254 was grown on V8C at room temperature. To induce sporangia, plugs of the culture were removed aseptically with a cork borer (4 mm diameter) and placed in half-strength V8C for 2 days. The resulting mycelial mats were washed three times with sterile distilled water, placed in a water extract of avocado soil (1:100 wt/wt) for 2 days under fluorescent light to allow for the formation of sporangia, and washed three times with sterile distilled water. The mycelial mats were cooled at 4°C for 20 min and placed at room temperature for 1 h. The cultures were

microscopically examined for swimming zoospores, which were quantified with a Hawksley eelworm counter. To prepare the plants for infection, their roots were gently washed clean of soil particles, and the plants were placed in 4 liters of tap water perfused with air. Zoospores were added to the water to a final concentration of 35, 350, or 3,500/ml. After 2, 5, and 8 days, feeder roots were harvested manually and stored at -80°C prior to the extraction of DNA, as described above. All assays were performed in duplicate with replicated plants; data presented represent the average of duplicates plus/minus the range.

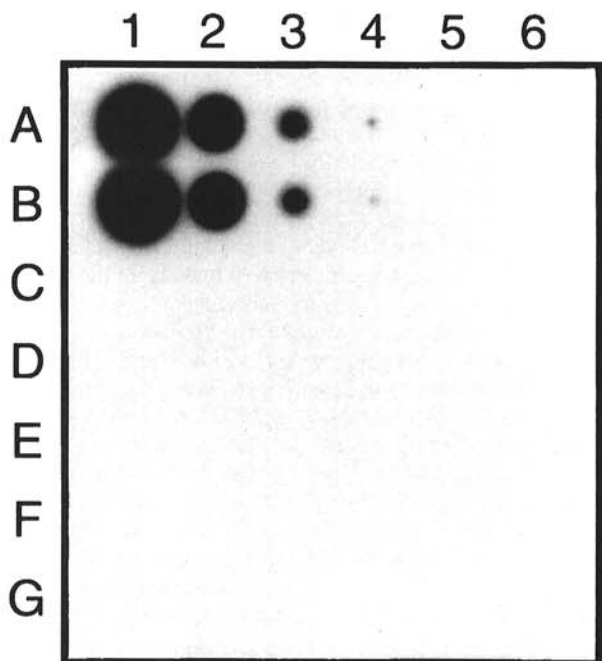


Fig. 3. Dot-blot hybridization of the pPC19 probe to DNA from different species of *Phytophthora*. DNA from *P. cinnamomi* M254 was applied to positions A1 and B1 (50 ng), A2 and B2 (5 ng), A3 and B3 (0.5 ng), A4 and B4 (50 pg), and A5 and B5 (5 pg). Other positions either contained no DNA (A6 and B6) or 100 ng of DNA from *P. cactorum* (C1 and D1), *P. parasitica* (C2 and D2), *P. citricola* (C3 and D3), *P. infestans* (C4 and D4), *P. fragariae* (C5 and D5), *P. dreschleri* (C6 and D6), *P. cryptogea* (E1 and F1), *Pythium irregulare* (E2 and F2), *Achlya ambisexualis* (E3 and F3), *Trichoderma harzianum* (E4 and F4), *Gliocladium virens* (E5 and F5), *Penicillium digitatum* (E6 and F6), *Agaricus bisporus* (G1 and G2), *Pseudomonas syringae* pv. *syringae* (G3 and G4), or avocado leaves (G5 and G6). The filter was hybridized to the 460-bp high-copy portion of pPC19 as indicated in text and exposed to film for 20h. Other *Phytophthora* species tested, but not shown in the figure, included *P. capsici*, *P. citricola*, *P. citrophthora*, *P. meadii*, *P. megasperma*, *P. megakarya*, *P. palmivora*, and *P. sojae*—all of which failed to hybridize with the probe.

RESULTS

Identification of high-copy probes for *P. cinnamomi*. A library of 16,000 clones of DNA from isolate M254 was screened for colonies that strongly hybridized to radiolabeled DNA of *P.*

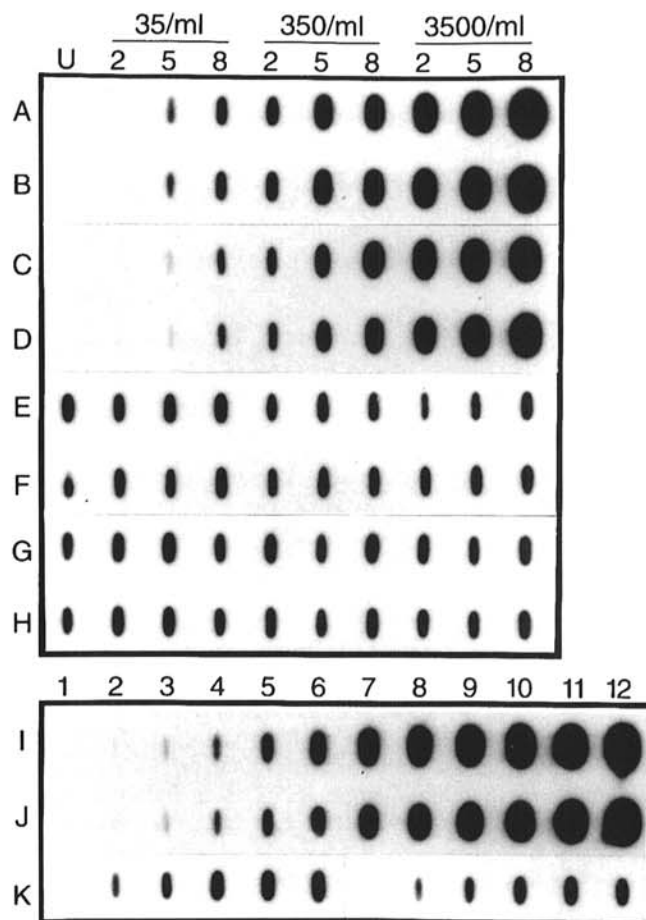


Fig. 5. Assay for *Phytophthora cinnamomi* and avocado DNA extracted from roots. DNA samples (about 100 ng) were applied to a nylon membrane with a slot-blot apparatus and hybridized to ³²P-labeled probes prepared from the 460-bp repetitive fragment of pPC19 or total genomic DNA of avocado. U, uninfected roots; 2, 5, and 8, roots at 2, 5, or 8 days after placement in suspensions of 35, 350, or 3,500 zoospores per ml. Rows A and B are samples from one series of plants hybridized with the pPC19 fragment, with A and B representing duplicate samples from the same DNA extraction. Rows C and D are the same as A and B but were hybridized with radiolabeled avocado DNA. Rows E and F are duplicate DNA samples from a second series of plants hybridized with the pPC19 fragment. Rows G and H are the same as C and D but were hybridized with the avocado probe. Rows I and J are duplicate standard curves of *P. cinnamomi* DNA hybridized to the pPC19 fragment, with each well containing 100 ng of avocado DNA plus varying amounts of fungal DNA: lane 1, 4.88 pg; lane 2, 9.75 pg; lane 3, 19.5 pg; lane 4, 39 pg; lane 5, 78 pg; lane 6, 156 pg; lane 7, 313 pg; lane 8, 625 pg; lane 9, 1.25 ng; lane 10, 2.5 ng; lane 11, 5 ng; or lane 12, 10 ng. Row K is the standard curve of avocado DNA hybridized with the avocado probe, with wells containing lanes 1 and 7, 0 ng; lanes 2 and 8, 25 ng; lanes 3 and 9, 50 ng; lanes 4 and 10, 100 ng; lanes 5 and 11, 150 ng; and lanes 6 and 12, 200 ng of DNA from uninfected avocado roots.

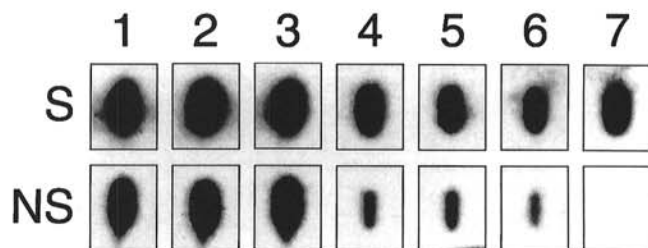


Fig. 4. Evaluation of methods for extracting DNA. Approximately 50 ng of DNA from avocado roots was fixed to duplicate nylon filters with a slot-blot apparatus. The filters were hybridized with 5×10^6 cpm of ³²P-labeled avocado DNA (row S) or 10^8 cpm of a nonspecific probe (pCR-S1, a probe specific for *Phytophthora infestans*, row NS). DNA was obtained, as described in text, by the potassium ethylxanthogenate method (PEX; column 1), the proteinase K-phenol method (column 2), the guanidium isocyanate method (column 3), the PEX method plus binding to silica resin (column 4), the PEX method plus binding to ion-exchange resin (column 5), the proteinase K-phenol method plus polyethylene precipitation (column 6), or cetyltrimethylammonium bromide hydroxide extraction (column 7).

cinnamomi but not DNA of *P. parasitica*. This was based on the premise that the intensity of hybridization was related to the abundance of the cloned sequences in the genome. Approximately 25% of the colonies showed moderate to strong hybridization to the *P. cinnamomi* probe and 15% of these hybridized poorly to the *P. parasitica* probe. As a further test for specificity and copy number, DNA from 40 such clones was hybridized to radiolabeled DNA of *P. cinnamomi*, *P. infestans*, *P. cryptogea*, *P. cactorum*, *P. citricola*, *P. dreschleri*, and *P. fragariae*. Half of the clones appeared to be specific for *P. cinnamomi*, and the five with the strongest specific hybridization signals (pPC19, pPC20, pPC30, pPC36, and pPC39) were selected for further study.

Hybridization studies revealed that each of the five clones detected the same high-copy sequence that was present throughout the species. For example, when probed with the insert from pPC19, intense hybridization was observed to DNA from each of 10 isolates of *P. cinnamomi* (Fig. 1). Five of these isolates (6493, 3657, 6379, 2428, and 2021) were tested because they represented the range of diversity within the species based on an isozyme survey (23), whereas the others (M254, M262, M280, M282, and M284) were representative of strains colonizing avocado in California. In the *Hind*III digests, hybridization was observed to a broad range of bands, whereas a predominant 630-bp band was detected in the *Hae*III digests. This suggested that the probe detected a dispersed repeated sequence evolved from an ancestor containing the *Hae*III fragment. The intensity of hybridization was in proportion to the amount of DNA per lane based on ethidium bromide staining, indicating that the repeated sequence was present in roughly equal amounts in each isolate. The four plasmids, in addition to pPC19, contained the same repetitive sequence, because they resulted in similar banding patterns when hybridized to genomic DNA and they cross-hybridized to each other (data not shown).

The distribution of the repetitive sequence within the plasmids was characterized to learn more about its structure and to develop probes with maximum specificity. The inserts were fractionated with *Hinc*II plus *Pvu*II and hybridized to radiolabeled DNA from isolate M254 to ascertain which bands contained the high-copy sequence. Strong hybridization was observed to 1 of 2 bands of the insert from pPC19, 2 of 7 from pPC20, 1 of 10 from pPC30, 2 of 7 from pPC36, and 1 of 5 from pPC39. This suggested that the repetitive sequence did not generally exist in long tandem arrays. It also indicated how to increase the sensitivity of the probe by enriching for the repetitive sequence. Because the repeated element within the 4.2-kb insert of pPC19 was limited to a 460-bp fragment, a probe synthesized from the smaller fragment would be expected to be more sensitive than one made from the entire insert. Subsequent experiments used probes derived from such subfragments.

The abundance of the repeated sequence within *P. cinnamomi* was estimated by a reconstruction experiment. A dot blot containing varying amounts of *P. cinnamomi* DNA and the 460-bp high-copy fragment of pPC19 was hybridized with the labeled fragment (Fig. 2). By comparing the hybridization signals, the sequence was calculated to be present potentially at $11,700 \pm 2,800$ copies per haploid genome, assuming it was present once on the 460-bp fragment and based on a size of 10^8 bp for the haploid genome of *P. cinnamomi*; the haploid genome size is unknown, but 10^8 was picked because it is between the sizes of the genomes of *P. infestans* (2.5×10^8 bp; [32]) and *P. sojae* (6.3×10^7 bp; [20]). A similar experiment with pPC20 provided an estimate of 13,300 \pm 3,400 copies.

Specificity and sensitivity of the probe. Hybridization assays confirmed that the 460-bp fragment from pPC19 was highly specific to *P. cinnamomi* and capable of detecting small amounts of genomic DNA (Fig. 3). Fifty picograms of *P. cinnamomi* DNA was detected readily in a dot-blot assay after overnight exposure of the hybridization membrane to film, whereas no hybridization was observed against 100 ng of DNA from 14 species of *Phy-*

tophthora, including members of each of the six taxonomic classes proposed by Waterhouse et al. (34), other Oomycetes such as *Achlya ambisexualis* and *Pythium irregulare*, ascomycete and basidiomycete fungi, bacteria, and avocado leaves. After a 1-week exposure, 5 pg of *P. cinnamomi* DNA was readily detected (data not shown), as well as weak binding to the 100-ng sample of *P. cryptogea* DNA (i.e., at 1/20,000 of the strength of the homologous signal). Similar results were obtained with pPC20 and pPC39.

Extraction of DNA from roots. Due to the specificity and sensitivity of the probes, they appeared to be potentially useful for assaying for *P. cinnamomi* in plant tissue. To develop such an assay, previously demonstrated procedures for extracting DNA from plants and fungi were tested on avocado roots. Several methods were evaluated because difficulties in isolating high-quality DNA from root and soil-containing material have been reported (25,31). Each procedure enabled the recovery of undegraded DNA capable of hybridizing to radiolabeled probes in dot and slot blots. However, DNA extracted by a proteinase K-phenol procedure, a guanidium detergent method, and a xanthate extraction procedure displayed unacceptable levels of nonspecific hybridization. Hybridization was assessed by comparing the relative binding to the plant DNA samples of 5×10^6 cpm of a 32 P-labeled probe made from total avocado DNA (Fig. 4, row S) and 10^8 cpm of a probe for a *P. infestans*-specific sequence (Fig. 4, row NS). The *P. infestans* probe was used because previous experiments had demonstrated its lack of affinity to *P. cinnamomi* and plant DNA; therefore, it would indicate levels of nonspecific hybridization to the samples. The nonspecific binding of the *P. infestans* probe could be reduced but not eliminated by further purification with DNA-binding resins or polyethylene glycol precipitation. A CTAB method provided the most satisfactory results in terms of the lowest levels of nonspecific hybridization. The addition of carrier tRNA was required to obtain reliable yields of DNA (2 μ g/g of roots) by this method.

Quantitation of *P. cinnamomi* in avocado. A slot-blot assay was used to measure the relative amount of *P. cinnamomi* DNA in roots 2, 5, and 8 days after being treated with suspensions of 35, 350, or 3,500 zoospores per ml (Fig. 5). DNA was extracted from these samples and from uninfected avocado by the CTAB method and fixed to nylon membranes, along with standards containing known amounts of *P. cinnamomi* and avocado DNA. These membranes were hybridized with the pPC19 probe, exposed to film, stripped of the pPC19 probe, and then similarly treated with radiolabeled DNA from avocado. The resulting data were analyzed to determine the absolute amounts of *P. cinnamomi* and avocado DNA in each sample by comparing the hybridization intensity of each experimental sample to the standard curves. The ratios between amounts of *P. cinnamomi* and avocado DNA were calculated as a measure of fungal proliferation (Fig. 6); this treatment of data also served to correct for differences in DNA quality that might affect the binding of DNA to the membrane or radiolabeled probes. Hybridizations with a DNA sequence specific to *P. infestans* confirmed the lack of significant nonspecific hybridization in the various DNA preparations (data not shown).

P. cinnamomi DNA was detected in each of the infected samples. The level of DNA was roughly proportional to the inoculum, with ratios of fungal to avocado DNA of about 5, 0.3, and 0.06% at 8 days after inoculation with 3,500, 350, and 35 zoospores per ml, respectively. For each treatment, the amount of *P. cinnamomi* DNA rose in a somewhat linear fashion during the assay period. The proportional variation between duplicate plants was highest in those inoculated at the lowest concentration of zoospores (35/ml), possibly due to the stochastic nature of root colonization at low inoculum densities. This was particularly true for the 2-day sample, in which *P. cinnamomi* averaged only 0.005% of the total DNA present.

To compare the DNA values with macroscopic symptoms of infection, the plants used in the assay were inspected prior to harvesting DNA. Root lesions consistent with *P. cinnamomi* infection were evident in the 350 and 3,500/ml samples after 2 days but not

in the 35/ml sample, in which lesions were apparent only at 8 days. Wilting was observed only after 8 days on plants treated with 3,500 zoospores per ml.

When the assay was repeated on a different day with a separate lot of avocado seedlings, similar trends were evident in the data, although quantitative variation was observed. For example, in the experiment shown in Figure 6, the relative level of *P. cinnamomi* DNA at 2, 5, and 8 days after infection with 3,500 zoospores per ml equaled $0.87 \pm 0.33\%$, $2.40 \pm 0.22\%$, and $5.15 \pm 0.65\%$, respectively, with error expressed as the range between duplicate plants. Values in a repeat experiment at the same time points equaled $0.21 \pm 0.05\%$, $1.12 \pm 0.30\%$, and $2.22 \pm 0.42\%$, respectively. The quantitative variation between experiments likely was attributable to differences in the zoospore preparations or plants, because the degree of variation was greater between experiments than between plants infected on the same day.

DISCUSSION

The ability to detect and measure pathogens in infected tissue is essential for accurate studies of plant-microbe interactions and for assessing strategies for controlling disease. Using a DNA probe specific to a highly repetitive sequence of *P. cinnamomi*, we have developed such an assay and demonstrated its effectiveness at quantitating levels of the pathogen in avocado roots as a function of time and inoculum density. The sequence was specific for *P. cinnamomi*, because it showed insignificant cross-reactivity to DNA from other organisms, including closely related species of *Phytophthora*. Therefore, the probe also may be useful for species identification.

DNA-based assays for identifying phytopathogenic fungi have been described for several species, although few tests have been used in planta (12,13,14,18,19,25,35). None has been used to precisely measure levels of the pathogen in plant tissue. Such a DNA-based assay for measuring *P. cinnamomi* in avocado was developed in this study as an alternative to existing methods, which suffer from several limitations. For example, assays of lesion size (10,30) can be inaccurate because they are not species-specific, mycelia can extend beyond the visible lesion (6), and such tests generally measure longitudinal growth along roots but not lateral growth. Assays involving the plating of root sections on semiselective media for *Phytophthora* (1,6,8) are similarly only semiquantitative and not species-specific. Fungal growth in planta theoretically can be measured by microscopic analysis, but in practice, this is difficult to apply to more than a few samples. In contrast, DNA methods do not suffer from these limitations and may be more accurate at measuring viable propagules than assays involving the scoring of visible lesions, because a DNA assay would preferentially detect living pathogen biomass as opposed to dead mycelia in old lesions.

Relatively few experiments involving the quantitative assessment of fungal growth within plants have been reported, in comparison to many such studies performed on artificial media. Studies of the growth of *Colletotrichum lagenarium* and *Cladosporium fulvum* in plants were described, based on measurement of levels of chitin (33) or pathogen-specific enzymes (22). In these studies, growth was generally linear during early-to-midphase infections, except for an initial lag phase of several days. In our experiments with avocado, the level of *P. cinnamomi* within roots also increased in an approximately linear manner over the infection time course. No obvious lag phase was observed. Colonization also increased in proportion to the density of zoospores in the inoculum (35, 350, and 3,500/ml), indicating that infection sites had not been saturated under these conditions. In contrast, a decrease in the efficiency of infection was observed at >200 zoospores per ml in a study of *P. cinnamomi* on pine (8), in which infection was scored by plating root sections on agar media to test for the presence of viable fungal propagules. Unlike our DNA-based assay, this method may have underestimated the actual number of infections because plating assays cannot distinguish between singly and multiply infected root sections.

Other laboratories also have developed DNA-based methods for identifying and detecting *Phytophthora* species, but these either have not been used for quantitation or failed to detect the pathogen in direct in planta assays. Repetitive probes specific for either *P. parasitica* or *P. citrophthora* were obtained by Goodwin et al. (12,13,14) that could detect 1 ng of DNA in dot blots, but attempts to use the *P. citrophthora* probe to detect pathogen DNA in greenhouse-infected citrus roots were unsuccessful (14). A probe for *P. cinnamomi* was identified by Dubrowolski and O'Brien (7) that was capable of detecting 200 ng of purified fungal DNA in a dot-blot assay, but this probe was not tested on infected material. In contrast to these hybridization probes, our *P. cinnamomi* probe quantitatively detected 5 to 50 pg of DNA and was useful for in planta studies. A qualitative PCR assay was developed for detecting *P. fragariae* var. *rubi* in roots of raspberry (29); in theory, such a test could be performed quantitatively with suitable controls (27).

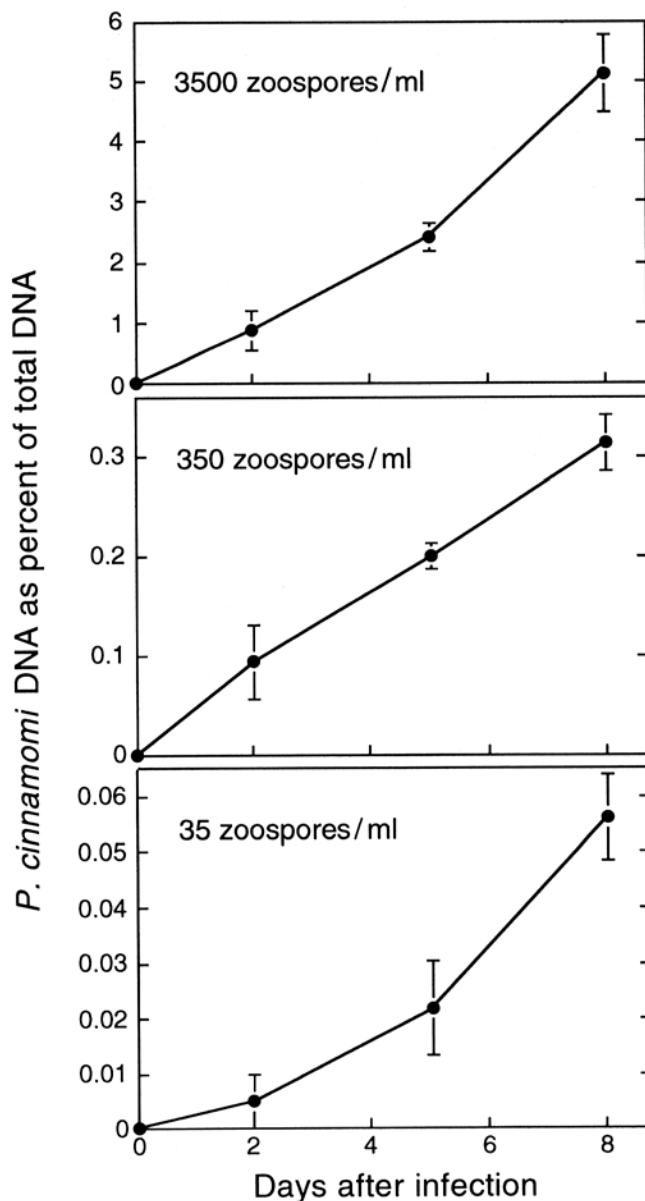


Fig. 6. Relative amount of *Phytophthora cinnamomi* DNA in avocado roots. Data from the experiment shown in Figure 5 were used to calculate the amount of *P. cinnamomi* DNA as a percentage of total DNA extracted from roots inoculated with suspensions of 3,500, 350, or 35 zoospores per ml. Band intensities were determined by densitometry, and the amount of *P. cinnamomi* and avocado DNA in the experimental samples was determined by comparison to the standard curves. Error bars reflect the range of values obtained from duplicate plants.

Several technical factors may be responsible for the greater sensitivity and utility of our assay relative to previous hybridization tests, including differences in hybridization procedures and use of probes enriched for the repetitive sequence (i.e., synthesized from a 460-bp subfragment of the 4.2-kb insert of pPC19). It also is likely that the copy number of our cloned sequence is particularly high. Based on several assumptions, the abundance of the sequence cloned in pPC19 was estimated at 12,000 copies per haploid genome. If the element spans 630 bp, as suggested by the *Hae*III digest shown in Figure 1, then it would account for >5% of the genome. Although repetitive sequences in Oomycetes have been poorly characterized, they have been shown to account for more than half of the total DNA in other members of the Peronosporales (9,20). The abundance of the *P. cinnamomi* sequence is in line with that of repeated sequences in mammals, such as the *Alu* element, which accounts for about 9% of the human genome (28). An additional factor that enabled the detection of low levels of *P. cinnamomi* within avocado was the isolation of suitable root DNA by a CTAB protocol. Other methods gave unacceptable levels of nonspecific hybridization, possibly due to humic acids in soil clinging to the roots (31). Root tissue also is notorious for containing other compounds that interfere with hybridization and that likely have interfered with other attempts to detect pathogens in roots with DNA probes (25).

The assay developed here is appropriate for studies in which sensitive and accurate methods are required for assessing the degree of host colonization by *P. cinnamomi*. In terms of time and effort, when the representative sampling of large amounts of root tissue is required, the DNA assay is no more demanding than the traditional method of plating root sections on selective media. Our experience is that low-frequency infections of avocado by *P. cinnamomi*, such as those obtained with 35 zoospores per ml of inoculum, are difficult to detect by the plating approach unless a large number of root sections is analyzed. Using the DNA assay, low-level infections can be detected by pooling the equivalent of 100 or more root sections into a single sample. High-level infections also can be quantified more accurately. On the other hand, the assay does require expertise in technologies such as DNA extraction and hybridization. Although the assay presented here relies on the use of radioactive detection procedures, similar sensitivities might be achieved by nonisotopic methods according to claims made by vendors of several such systems.

LITERATURE CITED

- Benson, D. M. 1991. Detection of *Phytophthora cinnamomi* in azalea with commercial serological assay kits. *Plant Dis.* 75:478-482.
- Cahill, D. M., and Hardham, A. R. 1994. A dipstick immunoassay for the specific detection of *Phytophthora cinnamomi* in soils. *Phytopathology* 84:1284-1292.
- Cantino, E. C., and Lovett, J. S. 1960. Respiration of *Blastocladiella* during bicarbonate induced morphogenesis in synchronous culture. *Physiol. Plant.* 13:35-40.
- Caten, C. E., and Jinks, J. L. 1968. Spontaneous variability of single isolates of *Phytophthora infestans*. I. Cultural variation. *Can. J. Bot.* 46:329-348.
- Coffey, M. D. 1987. *Phytophthora* root rot of avocado: An integrated approach to control in California. *Plant Dis.* 71:1046-1052.
- Davison, E. M., Stukely, M. J. C., Crane, C. E., and Tay, F. C. S. 1994. Invasion of phloem and xylem of woody stems on roots of *Eucalyptus marginata* and *Pinus radiata* by *Phytophthora cinnamomi*. *Phytopathology* 84:335-340.
- Dobrowolski, M. P., and O'Brien, P. A. 1993. Use of RAPD-PCR to isolate a species specific DNA probe for *Phytophthora cinnamomi*. *FEMS Microbiol. Lett.* 113:43-48.
- Fraedrich, S. W., Tainter, F. H., and Miller, A. E. 1989. Zoospore inoculum density of *Phytophthora cinnamomi* and the infection of lateral root tips of shortleaf and loblolly pine. *Phytopathology* 79:1109-1113.
- Francis, D. M., Hulbert, S. H., and Michelmore, R. W. 1990. Genome size and complexity of the obligate fungal pathogen, *Bremia lactucae*. *Exp. Mycol.* 14:299-309.
- Gabor, B. K., and Coffey, M. D. 1990. Quantitative analysis of the resistance to *Phytophthora cinnamomi* in five avocado rootstocks under greenhouse conditions. *Plant Dis.* 74:882-885.
- Gees, R., and Coffey, M. D. 1989. Evaluation of a strain of *Myrothecium rostratum* as a potential biocontrol agent against *Phytophthora cinnamomi*. *Phytopathology* 79:1079-1084.
- Goodwin, P. H., English, J. T., Neher, D. A., Duniway, J. M., and Kirkpatrick, B. C. 1990. Detection of *Phytophthora parasitica* from soil and host tissue with a species-specific DNA probe. *Phytopathology* 80:277-281.
- Goodwin, P. H., Kirkpatrick, B. C., and Duniway, J. M. 1989. Cloned DNA probes for the identification of *Phytophthora parasitica*. *Phytopathology* 79:716-721.
- Goodwin, P. H., Kirkpatrick, B. C., and Duniway, J. M. 1990. Identification of *Phytophthora citrophora* with cloned DNA probes. *Appl. Environ. Microbiol.* 56:669-674.
- Hoagland, D. R., and Arnon, D. I. 1950. The water-culture method for growing plants without soil. *Calif. Agric. Exp. Stn. Circ.* 347.
- Jhingan, A. K. 1992. A novel technology for DNA isolation. *Methods Mol. Cell. Biol.* 3:15-22.
- Judelson, H. S. 1993. Intermolecular ligation mediates efficient cotransformation in *Phytophthora infestans*. *Mol. & Gen. Genet.* 239:241-250.
- Lanfranco, L., Wyss, P., Marzachi, C., and Bonfante, P. 1995. Generation of RAPD-PCR primers for the identification of isolates of *Glomus mosseae*, an arbuscular mycorrhizal fungus. *Mol. Ecol.* 4:61-68.
- Lee, S. B., White, T. J., and Taylor, J. W. 1993. Detection of *Phytophthora* species by oligonucleotide hybridization to amplified ribosomal DNA spacers. *Phytopathology* 83:177-181.
- Mao, Y., and Tyler, B. M. 1991. Genome organization of *Phytophthora megasperma* f. sp. *glycinea*. *Exp. Mycol.* 15:283-291.
- Murray, M. G., and Thompson, W. F. 1980. Rapid isolation of high-molecular-weight plant DNA. *Nucleic Acids Res.* 8:4321-4325.
- Oliver, R. P., Farman, M. L., Jones, J. D. G., and Hammond-Kosack, K. E. 1993. Use of fungal transformants expressing β -glucuronidase activity to detect infection and measure hyphal biomass in infected plant tissues. *Mol. Plant-Microbe Interact.* 6:521-525.
- Oudemans, P., and Coffey, M. D. 1991. Isozyme comparison within and among worldwide sources of three morphologically distinct species of *Phytophthora*. *Mycol. Res.* 95:19-30.
- Ribeiro, O. K. 1978. A Source Book of the Genus *Phytophthora*. J. Cramer, Vaduz, Liechtenstein.
- Rollo, F., Amice, A., Francesco, F., and di Silvestro, I. 1987. Construction and characterization of a cloned probe for the detection of *Phoma tracheiplila* in plant tissues. *Appl. Microbiol. Biotechnol.* 26:352-357.
- Sambrook, J., Fritsch, E. F., and Maniatis, T. 1989. *Molecular Cloning: A Laboratory Manual*. 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
- Simon, L., Levesque, R. C., and Lalonde, M. 1992. Rapid quantitation by PCR of endomycorrhizal fungi colonizing roots. *PCR Methods Appl.* 2:76-80.
- Singer, M., and Berg, P. 1991. *Genes and Genomes*. University Science Books, Mill Valley, CA.
- Stammler, G., and Seemuller, E. 1993. Specific and sensitive detection of *Phytophthora fragariae* var. *rubi* in raspberry roots by PCR amplification. *J. Plant Dis. Prot.* 100:394-400.
- Stukely, C. E., and Crane, C. E. 1994. Genetically based resistance of *Eucalyptus marginata* to *Phytophthora cinnamomi*. *Phytopathology* 84:650-656.
- Tebbe, C. C., and Vahjen, W. 1993. Interference of humic acids and DNA extracted directly from soil in detection and transformation of recombinant DNA from bacteria and a yeast. *Appl. Environ. Microbiol.* 59:2657-2665.
- Tooley, P. W., and Therrien, C. D. 1987. Cytophotometric determination of the nuclear DNA content of 23 Mexican and non-Mexican isolates of *Phytophthora infestans*. *Exp. Mycol.* 11:19-26.
- Toppan, A., Esquerre-Tugay, A., and Touze, A. 1976. An improved approach for the accurate determination of fungal pathogens in diseased plants. *Physiol. Plant Pathol.* 9:241-251.
- Waterhouse, G. M., Newhook, F. J., and Stamps, D. J. 1983. Present criteria for classification of *Phytophthora*. Pages 130-147 in: *Phytophthora: Its Biology, Taxonomy, Ecology, and Physiology*. D. C. Erwin, S. Bartnicki-Garcia, and P. H. Tsao, eds. The American Phytopathological Society, St. Paul, MN.
- Yao, C. L., Magill, C. W., and Frederiksen, R. A. 1991. Use of an A-T-rich DNA clone for identification and detection of *Peronosclerospora sorghi*. *Appl. Environ. Microbiol.* 57:2027-2032.
- You, M. P., and Sivasithamparan, K. 1995. Changes in microbial populations of an avocado plantation mulch suppressive of *Phytophthora cinnamomi*. *Appl. Soil Ecol.* 1:33-43.
- Zentmeyer, G. A. 1980. *Phytophthora cinnamomi* and the diseases it causes. *Phytopathology Monograph* 10. The American Phytopathological Society, St. Paul, MN.