Evaluation of a Strain of *Myrothecium roridum* as a Potential Biocontrol Agent Against *Phytophthora cinnamomi*

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ABSTRACT

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Potential antagonists of *Phytophthora cinnamomi* were evaluated from among 36 fungi and 110 bacteria isolated from the rhizosphere of avocado roots growing in a soil suppressive to *Phytophthora* where *P. cinnamomi* had been present for 40–50 yr. Strain TW of *Myrothecium roridum* proved to be the most active antagonist in controlling *P. cinnamomi* in repeated greenhouse pot tests with highly susceptible seedlings of *Persea indica* inoculated with *P. cinnamomi*. *M. roridum* was grown on a wheat-bran medium and introduced into a peat-perlite mixture at 2.5% (w/v) 2 wk before inoculation with *P. cinnamomi*. In a UC-mixture with *P. indica* inoculated with zoospores of *P. cinnamomi*, *M. roridum* suppressed root infection by 50–94% compared with uninoculated controls. In the same experiments there was no significant difference in the level of control achieved by either *M. roridum* or the fungicide potassium phosphonate (2.5 mg/pot). In three naturally infested field soils, root infection ranged from 12 to 54% in the presence of *M. roridum*, compared with 58 to 93% for controls over the same 4-wk period. On a selective medium containing carbendazim, a fungicide-resistant mutant of strain TW, TWm14, was isolated consistently from the root tips of *P. indica* growing in infested soil 4 wk after transfer, demonstrating the apparent rhizosphere competence of this strain in all three soils.

Root rot of avocado (*Persea americana* Mill.), caused by *Phytophthora cinnamomi* Rands, is the major disease affecting avocado in California (12). Only two fungicides (metalaxyl and fosetyl-Al) are available that are only partially effective in suppressing the pathogen under California conditions (13). Total dependence on such site-specific fungicides is not recommended, because, aside from their high cost, fungicide-resistant strains of *P. cinnamomi* may occur through their excessive use (13). An urgent need exists for an effective, broad-based, integrated approach to the management of this severe disease (12). Conceivably, such an approach might combine the use of resistant rootstocks, fungicides, an improvement in existing cultural practices, and development of fungicide-compatible methods of biological control.

Few attempts have been made to develop biological methods to control Phytophthora root rot of avocados in the field (41). There is a report from Queensland, Australia, of the existence of two groves with soils suppressive to *P. cinnamomi* (9). In this case, the continuous incorporation of large quantities of organic material and calcareous amendments partially restored some of the biological properties of the native rainforest soil, which naturally supports a microflora and microfauna that suppresses *P. cinnamomi* (4,9,14,36). Recently, we have detected several soils suppressive to *P. cinnamomi* in California in old avocado groves having deep alluvial soils. Occurrence of *P. cinnamomi* has been recorded in these groves for 40–50°yr, but there has been no significant decline in tree vigor despite the presence of *P. cinnamomi*. The majority of avocado groves with similar alluvial soils and in the same vicinity have declined or have been lost to Phytophthora root rot.

Bacteria and fungi isolated from avocado feeder roots growing in a soil suppressive to *P. cinnamomi* were introduced into various soil mixtures and natural soils infested with the pathogen to test their biocontrol potential on of *P. indica* L., a relative of avocado. Our study describes screening bioassays and greenhouse tests conducted with bacteria and fungi, particularly with a strain TW of *Myrothecium roridum* Tode ex Fr., which was most active in suppressing *P. cinnamomi*.

MATERIALS AND METHODS

Soils. All organisms used in these experiments originally were isolated from the feeder root zones of avocado trees in an avocado grove in Santa Barbara County, CA. *P. cinnamomi* had been present in this grove since the early 1940s, yet no severe root rot symptoms, nor subsequent tree decline, had been observed. The top 10 cm of this soil was a sandy loam with the following characteristics: pH 6.8, 1.8 mmhos conductivity, 3.6% organic matter, 29 μg/g of phosphorus, 512 μg/g of potassium. For the bioassay, five artificial mixes or soils were used: UC mix No. 4, pH 5.8, consisting of 50% fine sand, 50% peat moss plus 2.2 kg of dolomite, 1.5 kg of superphosphate, 148 g of KNO_3, 148 g of K_2SO_4 m^{-3} (3); a peat-perlite mixture (60:40, v/v), pH 5.8; and three field soils infested with *P. cinnamomi* from Goleta, Camarillo, and Santa Paula, CA, respectively. Characteristics of the Goleta soil were: a sandy clay loam, pH 7.2, 1.3 mmhos conductivity, 1.8% organic matter, 29 μg/g of phosphorus, 174 μg/g of potassium, 921 μg/g of nitrogen. The Camarillo soil was a sandy clay loam having a pH of 6.0, 3.1 mmhos conductivity, 1.3% organic matter, 21 μg/g of phosphorus, 160 μg/g of potassium, and 780 μg/g of nitrogen. The Santa Paula soil was a loam with a pH of 7.4, 1.7 mmhos conductivity, 10.7% organic matter, 8.8 μg/g of phosphorus, 248 μg/g of potassium, and 6,675 μg/g of nitrogen.

Isolation of bioassay organisms. Soil was collected from three avocado trees chosen for their exceptional vigor in the grove that exhibited suppressive-soil properties. Four samples were collected and bulked from the feeder root zone of each tree. One gram of roots was randomly removed from each bulked sample, added to 99 ml of sterile distilled water, shaken for 10 min, and a dilution series prepared. One-milliliter samples were plated on King's B medium (23), and the resultant bacterial colonies transferred to yeast-dextrose agar (1% yeast extract, 2% $CaCO_3$, 2% dextrose, 2% agar). Bacteria were stored either freeze dried,

or in 0.85% sterile saline solution at 5 C. Bacteria (110 isolates) from the rhizosphere were screened in randomly chosen groups of three for their ability to suppress Phytophthora root rot. Fungi were isolated by plating 1-ml samples on a modified Martin medium (31). They were stored on potato-dextrose agar (PDA) slants at room temperature or by cryopreservation under liquid nitrogen. Thirty-six fungi isolated from the rhizosphere of avocado roots were included in the final screening tests.

Plants. Blue lupin (*Lupinus angustifolius* L.), was used as a host to test the bacteria. Seedlings were grown in 4-cm-diameter plastic containers in UC mix No. 4 before inoculation with bacteria. Six-week-old seedlings of *P. indica* previously grown in sand were used for screening bacteria and fungi. All plants were grown in the greenhouse with natural light and a temperature range between 18 and 29 C.

Preparation of inoculum of *P. cinnamomi*. For zoospore inoculum, the fungus was grown on cleared V8C agar (10% V-8 juice, 1% $CaCO_3$, clarified by centrifugation, 2% agar) for 3–4 days. Ten 5-mm-diameter agar/mycelium disks were placed in a 9-cm-diameter plastic petri dish and flooded with a V8C broth diluted 1:4. *P. cinnamomi* was incubated for 24 hr at 24 C. After removing the broth and washing the mycelium mats three times with distilled water, 10 ml of a 1% nonsterile soil extract, previously filtered through Whatman No. 1 paper, was added to the plates. The fungus was incubated in the dark for 5–6 days at 24 C for sporangial formation. Next, the soil extract was replaced with fresh extract and the fungus chilled at 5 C for 1 hr to release zoospores. Zoospores were counted with the aid of a hemacytometer and diluted with nonsterile soil extract to the desired concentration.

Another inoculation method involved millet seeds (300 g), which were autoclaved for 1 hr and infested with blended mycelium from two 5-day-old PDA colonies of the fungus suspended in 200 ml of sterile distilled water. After 10 days of incubation at 24 C, the millet inoculum was blended twice (1 min at high speed) in a blender and mixed into UC mix No. 4 (2% v/v). The soil mixture was maintained at ~12% soil moisture at ~24 C. After 5–7 days, the population density of *P. cinnamomi* in the mix was determined by the dilution-plate technique on cornmeal-PARPH medium selective for *Phytophthora* (22). This medium consisted of 1.8% Difco cornmeal agar (Difco Laboratories, Detroit, MI) with (per milliliter) 133 μg of pentachloronitrobenzene, 125 μg of ampicillin, 10 μg of rifampicin, 10 μg of pimaricin, and 50 μg of hymexazol. The infested potting mix then was diluted with noninfested mix to give about 15–30 colony-forming units (cfu) of *P. cinnamomi* per gram of dry mix.

Finally, naturally infested field soils conducive to Phytophthora from three avocado groves with severe root rot in southern California were collected from underneath diseased avocado trees, sifted through a 10-mm-mesh sieve, and used directly as an inoculum source.

Bioassay with bacteria. Lupins were inoculated with 5 ml of a bacterial suspension ($>1 \times 10^8$/ml in water), 5–8 days after planting. After an additional 7 days, plants were inoculated with a zoospore suspension (1×10^4/ml) of *P. cinnamomi* as described above. Percent root infection and soil population density of *P. cinnamomi* were determined 4 wk after inoculation with *P. cinnamomi*. Bacterial preparations also were tested on *P. indica* by drenching soil with a mixture of three bacteria produced in 2.5% Luria base broth (Difco).

Bioassay with fungi. Seedlings of *P. indica*, grown in sand for 6 wk, were transferred to 5-cm-diameter plastic pots containing UC mix No. 4 or peat-perlite mixture, into which the potential fungal antagonists had been incorporated. Fungi were grown on wheat bran for 5–7 days at 24 C in the dark. Fifty milliliters of fungal spore suspension (1×10^6 spores/ml), or 20 3-mm-diameter, agar mycelium disks from the margins of fungal colonies, were added to 500-ml Erlenmeyer flasks containing 50 g of autoclaved (1 hr) bran and incubated at 24 C. After 5–7 days, the colonized bran was mixed either with UC mix No. 4 or peat-perlite mixture at different ratios (w/v) ranging from 1 to 50 g/L. Controls consisted of UC mix No. 4 or the planting mixes plus uninoculated wheat bran. Potassium phosphonate, a fungicide control, was prepared by mixing equimolar amounts of phosphorous acid and potassium hydroxide and titrating with the latter to a final pH of 6.8. Five or 10 ml of a 500 μg/ml solution of potassium phosphonate were applied as a soil drench to each plant 1 wk before inoculation. *Trichoderma* spp. (isolates 72, 86, 104) and a strain of *T. hamatum* grown on wheat bran, which possessed no strong antagonistic effects against *P. cinnamomi*, also were used as additional controls in different experiments. Two weeks after transferring seedlings of *P. indica* into the soil/bran mixtures, plants were inoculated with 5 ml of a zoospore suspension of *P. cinnamomi* (1×10^4 spores/ml, unless stated otherwise), which was placed 2–3 cm from opposite sides of the stem base at a depth of 3–4 cm.

Four to six weeks after inoculation with *P. cinnamomi*, experiments were terminated and the root system washed free of soil. Feeder roots were cut into 1-cm sections and 20 randomly selected pieces were plated on cornmeal-PARPH agar. After 36–48 hr at 24 C, infected root pieces were counted and results expressed as percent root infection.

Population density of *P. cinnamomi* in artificially and naturally infested soil. Soil samples were collected from three to five randomly chosen plants per treatment. With 10 g for artificially infested and 50 g for naturally infested soils, samples were suspended in 100 ml of 0.1% agar in Styrofoam cups and mixed on a rotary shaker at 200 rpm for 30 min. One milliliter of the soil-agar slurry was placed on each of five plates of cornmeal-PARPH agar. After 48 hr, the soil was washed off the plates and colonies of *P. cinnamomi*, recognizable by their distinctive growth with clusters of hyphal swellings, were counted. Population densities were expressed as colony-forming units per gram of soil.

Techniques of incorporating antagonistic fungi in soil. Besides incorporating fungi grown on wheat bran in soil, alternative methods also were tested. In one alternate method, fungi were grown on potato-dextrose broth (PDB, 100 ml per 250-ml Erlenmeyer flask) for 5–10 days, or until a thick mycelium was formed, and broth was separated from the mycelium by suction filtration. Mycelium was suspended in 50 ml of water and blended twice for 1 min in a blender. Ten milliliters of the mycelial suspension were applied to the soil of each 10-wk-old plant and watered 2 wk before inoculation with *P. cinnamomi*. In another method, 25 ml of filter-sterilized PDB used as a growth medium was applied to soil. Finally, 10-ml conidial suspensions of fungi (2×10^6/ml and 2×10^7/ml) were mixed into soil.

Production of carbendazim-resistant strains of *M. roridum*. Chemical mutagenesis was performed to obtain carbendazim-resistant strains. Conidial suspensions ($\sim 1 \times 10^7$ conidia/ml) were incubated in an aqueous solution of the chemical mutagen *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (75 μg/ml) for 20 min. After centrifuging at high speed for 2 min, sedimented conidia were washed three times with sterile distilled water. One-milliliter samples of the conidial suspension were placed on petri dishes and overlayed with a selective PDA medium containing (per milliliter) 100 μg of carbendazim, plus 125 μg of ampicillin, 10 μg of rifampicin, and 125 μg of chloramphenicol. Fifty-eight fungicide-resistant mutants were recovered and, after repeated subculture, were determined to be phenotypically stable with respect to level of fungicide resistance, colony morphology, and antibiosis in vitro against *P. cinnamomi*. One of these mutants, strain TWm14, was selected for use in further experiments because of its fast growth and prolific conidial production on a carbendazim-selective PDA medium.

Population density of *M. roridum* in soil. Ten-gram soil samples containing *M. roridum*, strain TWm14, were suspended in 100 ml of H_2O in Styrofoam cups and placed on a rotary shaker at 200 rpm for 30 min. Rhizosphere population density was determined by vigorously washing whole roots of *P. indica* in 20 ml of H_2O. One-milliliter samples of these suspensions (three replicates) were used for dilution series on carbendazim-selective PDA medium. After 4–5 days, colonies of *M. roridum* were counted. Population densities were expressed as colony-forming units per gram of soil.

In vitro experiments. Fungal isolates were tested in vitro for antibiosis against *P. cinnamomi*. Cultures of *P. cinnamomi* were grown on PDA in the presence of potential antagonists. Disks (3 mm diameter) from the margin of 5-day-old fungal colonies were placed 30 mm from 5-day-old colonies of *P. cinnamomi* and effects on its growth monitored daily with a final reading after 5 days.

Cellulolytic activity of *M. roridum*. To test qualitatively the cellulolytic abilities of *M. roridum*, the wild type and mutant strain TWm14 were grown in a basic salts medium at pH 7.0 containing (g/L): KH_2PO_4, 1.0; K_2HPO_4, 1.0; NH_4NO_3, 1.0; $CaCl_2$, 0.02; $MgSO_4 \cdot 7\ H_2O$, 0.2; $FeCl_3$ (saturated solution), 2 droplets; cellulose (Sigmacell Type 100, Sigma, St. Louis, MO), 4.0. Spore suspensions (1×10^6 spores/ml) from 7-day-old cultures grown on PDA were transferred to 100 ml of salts medium in 250-ml Erlenmeyer flasks. Controls consisted of medium without fungus, a medium without cellulose but with the fungus, and *Trichoderma* sp., isolate 104, grown on the cellulose medium. Cultures were incubated at room temperature for 4 wk and visually checked periodically for fungal growth and cellulose residue.

Effect of culture filtrates on growth and sporulation of *P. cinnamomi*. *M. roridum* was grown for 5–7 days at 24 C in a basic salts medium (pH 6.4) composed of (g/L): K_2HPO_4, 0.2; NH_4NO_3, 0.3; $CaSO_4$, 0.2; $MgSO_4 \cdot 7H_2O$, 0.2; sucrose, 2; yeast extract, 4; Difco casamino acids, 2. A conidial suspension from a 7-day-old culture of *M. roridum* was transferred to 100 ml of medium in 250-ml Erlenmeyer flasks to give a final spore concentration of 1×10^4/ml. Flasks were shaken on a rotary shaker at 130 rpm for 5 days. Broth was separated from mycelium by filtration through Whatman No. 1 paper, then filter-sterilized with a 0.2-μm Nalgene filter (Nalgene, Rochester, NY). Dilution series of the sterilized growth medium were prepared to test the antifungal activity of the filtrate against *P. cinnamomi*; salts medium or H_2O were added to control plates. One milliliter of each dilution was placed in a petri plate and amended with 9 ml of molten PDA. When solidified, agar disks with *P. cinnamomi* were placed in the center of each petri plate. Plates were incubated at 24 C and the growth monitored daily.

The effect of the culture filtrates on zoospore germination also was tested. Three sterile cellophane squares (10 mm^2) were placed on the surface of PDA in 9-cm-diameter plastic petri dishes, 5–10 μl of a zoospore suspension (5×10^5/ml) of *P. cinnamomi* was spotted in the center of each square, and the droplet was let dry completely before incubating at 24 C for 10–13 hr. Liquid growth medium of another strain of *M. roridum* (IMI 142375) was used for comparison. The cellophane squares were removed from the agar and placed on a micro-slide, and droplets of 2% lactophenol-cotton blue were used to stop fungal growth. Percentage of germinated spores was determined by counting 100 spores on each of three cellophane squares.

Experimental design and statistic analysis. All greenhouse experiments were performed at least twice, with 12–15 replicates using completely randomized block designs. In vitro experiments were repeated twice with three replicates. Data were statistically analyzed by one-way ANOVA, and Duncan's multiple range tests or an LSD were used to separate means. Where applicable, regression analysis was performed with a MSTAT computer program. Percent values were transformed to arcsins before analysis.

RESULTS

Evaluation of microorganisms. Because zoospores of *P. cinnamomi* are considered one of the primary infectious agents, different levels of zoospore inoculum were tested. Zoospore inoculum directly applied in the vicinity of the root ball of *P. indica* built up a high population density of *P. cinnamomi*, able to kill most of the control plants during the first week after inoculation. Initially, four different fungal isolates (two *Trichoderma* spp., *T. hamatum*, and *M. roridum*) demonstrated evidence of some disease suppression. However, none of the bacteria tested in the bioassay caused a significant reduction of root rot.

The fungi were tested repeatedly with different inoculum levels, methods, and soil regimes to determine the consistency of initial results. Only strain TW of *M. roridum* consistently and significantly ($P = 0.05$) suppressed root infection at three inoculum levels of *P. cinnamomi* (Table 1). Besides UC mix No. 4, a peat-perlite mixture, routinely used in nurseries as a rooting medium for woody cuttings such as avocado, was tested as a medium for incorporation of bran-grown *M. roridum*. Again, seedlings of *P. indica* inoculated with zoospores of *P. cinnamomi* exhibited very little disease in all experiments, even where high levels of inoculum (5×10^4 zoospores/ml, 10 ml/plant) were used (data not shown).

Effect of incorporating different forms of the antagonist *M. roridum*, strain TW, into soil. Wheat bran was the only delivery system used that significantly suppressed root rot in *P. indica*. Liquid culture filtrates, conidial suspensions, or blended mycelium of *M. roridum* did not suppress *P. cinnamomi* (data not shown).

Effect of bran inoculum concentration. *M. roridum* was effective in the range of 1.5–2.5% (w/v) bran inoculum added to UC mix No. 4 (Table 2). Below 1.0% (w/v) bran, results were inconsistent. A temporary retardation of the growth of transplanted seedlings of *P. indica* sometimes was noted with 3.5–5.0% bran inoculum of *M. roridum*. This undesirable effect was overcome by a slight change in transplanting technique, which involved placing the root ball of *P. indica* on a 2- to 5-mm-thick layer of UC mix No. 4 placed on top of the bran/soil mixture.

TABLE 1. Effect of different fungal isolates grown on wheat bran on the percentage of root infection of *Persea indica* inoculated with different zoospore concentrations of *Phytophthora cinnamomi*

	Percent root infection		
	Zoospore/ml		
Treatments[x]	1×10^3[y]	0.7×10^4	4×10^4
Control	73 ab[z]	78 a	91 a
Trichoderma sp. (isolate 86)	47 b	86 a	61 b
Trichoderma sp. (isolate 72)	78 a	67 a	43 b
Trichoderma hamatum		89 a	67 ab
Myrothecium roridum (strain TW)	16 c	5 b	46 b
Potassium phosphonate	9 c		16 b

[x] Fungi grown on wheat bran for 5 days at 24 C and added to UC mix No. 4 at 2.5% bran (w/v). No bran was used in the control; potassium phosphonate was used at the rate of 500 μg/ml (pH 6.2, 5 ml/pot).
[y] Zoospore suspensions of *P. cinnamomi* applied at 5 ml/plant.
[z] Means within columns followed by the same letter are not significantly different according to Duncan's multiple range test ($P = 0.05$).

TABLE 2. Effect of different fungal isolates grown on wheat bran and added at three rates in UC mix No. 4 on the percent root infection of seedlings of *Persea indica* 4 wk after inoculation with a zoospore suspension of *Phytophthora cinnamomi*

	Percent root infection		
	g of bran/fungus mixture per liter UC mix No. 4		
Treatments[y]	25 (2.5%, w/v)	15 (1.5%, w/v)	5 (0.5%, w/v)
Control	77.9 a[z]	72.9 ab	47.7 b
Trichoderma sp. (isolate 86)	67.4 a	77.5 a	52.1 ab
Trichoderma sp. (isolate 72)	86.0 a	46.7 b	80.8 a
Myrothecium roridum (strain TW)	4.7 b	15.7 c	67.6 ab
Potassium phosphonate		12.1 c	9.4 c

[y] Fungi grown on wheat bran for 5 days at 24 C and added to UC mix No. 4. No bran was used in the control; potassium phosphonate was used at the rate of 500 μg/ml (pH 6.2, 10 ml/pot). All treatments were inoculated with zoospore suspension of *P. cinnamomi* (5 ml, 1×10^4/ml).
[z] Means within columns followed by the same letter are not significantly different according to Duncan's multiple range test ($P = 0.05$).

Two *Trichoderma* spp. tested as controls did not significantly inhibit disease development.

Artificially and naturally infested soils. In the UC mix, an artificially high inoculum level of 15–30 cfu/g of soil of *P. cinnamomi* was used, whereas 3–6 cfu/g were present in three naturally infested field soils. In the UC mix and field soils, soil amendment with both strains of *M. roridum* significantly suppressed root rot of *P. indica*, compared with a nonamended control (Table 3). A *Trichoderma* sp., isolate 104, isolated from the same avocado soil as the strain of *M. roridum*, had no suppressive effect. Soil samples were analyzed for *P. cinnamomi* at the conclusion of the experiments. Population density of the pathogen was significantly lower in treatments with *M. roridum* (Fig. 1).

Comparison of wild type and mutants of *M. roridum*. Carbendazim-resistant mutants of *M. roridum* were tested on PDA for changes in growth rate, colony morphology, and in vitro antibiosis of *P. cinnamomi*. No differences in these characteristics could be detected among 58 mutants studied (data not shown). Three randomly selected mutants (TWm14, TWm15, and TWm30) were tested further for their biological control capabilities in comparison with the wild type. The standard bioassay method with 2.5% bran-peat perlite mixture (w/v) and inoculation with zoospores was used as described earlier. No significant differences were found among these three resistant mutants and the wild-type TW in controlling *P. cinnamomi* in the artificially infested UC mix (data not shown).

Recovery of a carbendazim-resistant mutant of *M. roridum* from soil. Two weeks after bran was mixed with potting mix, samples from the root ball had a high population density ($>1 \times 10^8$ cfu/g) of carbendazim-resistant mutant TWm14 (data not shown). A second assay of TWm14 after 6 wk showed that the initial population density had dropped, but still was $>1 \times 10^5$ cfu/g in the rhizosphere. The overall percent root infection of *P. indica* caused by *P. cinnamomi* in the TWm14 treatment was 49% compared with 81% for the control (Table 3). In addition, the population density of *P. cinnamomi* dropped from initially 33 to 12 cfu/g in the same treatment. There was no decrease in pathogen population in the controls (Table 3). TWm14 also was recoverable after 6 wk from most root pieces plated on carbendazim-selective medium.

Growth of *M. roridum* on cellulose. Both wild-type and mutant strains of *M. roridum* grew readily in the presence of cellulose powder as the only carbon source. After 4 wk of incubation, *M. roridum* formed a dense layer of mycelium and no free cellulose was left in the flask, whereas the *Trichoderma* sp. used as a control formed a less dense mycelium, and free cellulose remained in the bottom of the flasks. No growth occurred in the control without cellulose.

In vitro antagonism of *M. roridum* against *P. cinnamomi*. *M. roridum*, strain TW, strongly inhibited the growth of *P. cinnamomi* in vitro. Once both fungi had established on the new medium, growth of *P. cinnamomi* was almost totally inhibited, the hyphae forming thick clusters of hyphal swellings. However, *M. roritum* was a slow grower compared with *P. cinnamomi* and needed a longer period of time to establish on PDA.

Effect of liquid growth medium from *M. roridum* on *P. cinnamomi*. All dilutions of sterile culture filtrates from two strains of *M. roridum* grown on a basic salts medium for 6 days, when

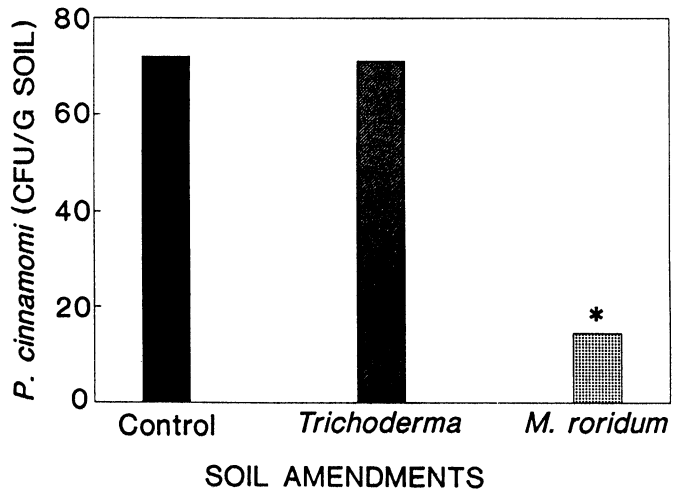

Fig. 1. Effect of *Myrothecium roridum* (strain TW) and *Trichoderma* sp. (isolate 104) applied to UC mix No. 4 on the population density of *Phytophthora cinnamomi* 6 wk after inoculation of *Persea indica*. Zoospore suspensions ($1\text{–}5 \times 10^3$/ml 5 ml/plant) of *P. cinnamomi* served as inoculum. Data from three experiments were pooled (three to five replicates per test); * = significantly different than the control according to an LSD at $P = 0.05$.

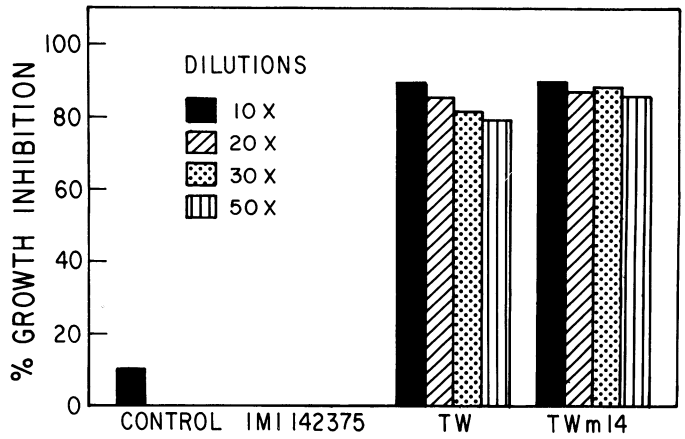

Fig. 2. Mycelial growth of *Phytophthora cinnamomi* at different dilutions of sterilized liquid growth medium of *Myrothecium roridum* strain TW, carbendazim-resistant strain TWm14, or strain IMI 142375 added to potato-dextrose agar after 96 hr. Water was added as a control.

TABLE 3. Effect of wild-type strain TW and carbendazim-resistant strain TWm14 of *Myrothecium roridum*, grown on wheat bran, on percent root infection of seedlings of *Persea indica* and on the population density of *Phytophthora cinnamomi* in artificially infested UC mix No. 4 and three naturally infested field soils

Treatments[x]	Percent root infection				Soil population densities of *P. cinnamomi*
	UC mix	Field soils			
	No. 4	Goleta	Camarillo	Santa Paula	cfu/g[y]
Control	81.3 a[y]	92.5 a	57.5 a	70.5 a	46
Trichoderma sp. (isolate 104)	81.7 a	94.0 a	66.5 a	61.0 ab	32
Myrothecium roridum (TW)	40.5 b	54.0 b	11.5 b	30.5 b	na
M. roridum (TWm14)	48.7 b	32.5 b	38.0 ab	27.0 b	12

[x] All plants were grown in UC mix No. 4 amended with wheat bran inoculum (2.5%, w/v) for 2 wk before transplanting into field soils naturally infested with *P. cinnamomi* (Goleta and Camarillo, sandy clay loams; Santa Paula, loam). No wheat bran was used in the control treatment.

[y] Colony-forming units (cfu)/g soil of *P. cinnamomi* for UC mix No. 4 after 4 wk of incubation; na = not assessed.

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

added to PDA, strongly inhibited mycelial growth of *P. cinnamomi* (Fig. 2). A 10-fold dilution of the liquid growth medium inhibited the growth of the fungus for at least 60 hr; subsequent growth was very slow and the mycelium was distorted. In the control, *P. cinnamomi* reached the edge of plates after 72 hr. A fourfold dilution caused complete inhibition of the pathogen over a period of 8 days. The pathogen, however, resumed normal growth when transferred to nonamended PDA.

Inhibition of zoospore germination. A 20-fold dilution of growth medium of TW and TWm14 completely inhibited zoospore germination of *P. cinnamomi*, and a 100-fold dilution prevented germination of cysts by 26 and 59%, respectively (Fig. 3). There was, however, no significant difference between the wild-type and the mutant strain in their activity. In comparison, all dilutions of growth medium of IMI 142375 had no activity against zoospores of *P. cinnamomi* (Fig. 3).

DISCUSSION

Through introduction of single antagonistic organisms, repeated attempts have been made to enhance suppressive effects of soils against specific plant pathogens (17,18,33,34). Our results focus on the ability of a strain of *M. roridum*, originally isolated from soil suppressive to *Phytophthora* in southern California, to suppress *P. cinnamomi* in tests with susceptible seedlings of *P. indica*. The potential of *M. roridum* as a biological control agent to suppress *P. cinnamomi* in greenhouse potting mixes and in naturally infested field soils was clearly demonstrated. Interestingly, the isolate of *M. roridum* was recovered from the top 10 cm of soil in this particular avocado grove. Similarly, the top layer of decomposing leaf litter was the most suppressive to *Phytophthora* found in the Ashburner grove at Mt. Tamborine in Queensland according to Cook and Baker (14). Although it is unlikely that *M. roridum* is the only cause of suppression in this particular avocado grove, its presence and proven activity against *P. cinnamomi* in various bioassays with a highly susceptible host strongly suggest its potential role in a more complex soil ecosystem.

Soils suppressive to *Phytophthora* have been described from several parts of the world; however, the nature of such suppression is still largely unresolved (14,37). Generally, no single physical, chemical, or biological property of a soil has been identified as a primary determinant in soils suppressive to *Phytophthora*. No single organism has been isolated that could completely reproduce the suppression (10). Additionally, the suppressive phenomenon was not transferable to new soils (14), as is the case with some soils suppressive to *Fusarium* (37).

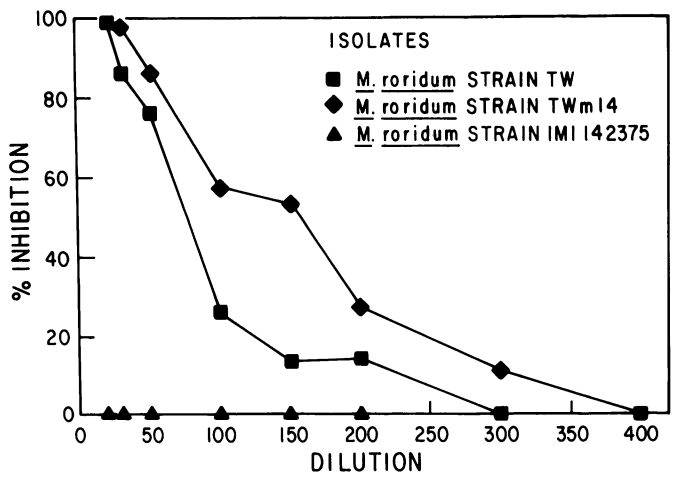

Fig. 3. Inhibition of zoospore germination of *Phytophthora cinnamomi* by filtrates of sterilized liquid growth medium of strain TW, carbendazim-resistant strain TWm14, and strain IMI 142375 of *Myrothecium roridum* grown for 5 days and added at different dilutions to potato-dextrose agar. Each point represents the mean of three replicates.

Other workers have shown that wheat bran is an effective and economic food base for introducing specific biological control agents into soils (18,25,26,32). This technique was successfully applied in the present study. In contrast, drenching the soil with conidial suspensions or mycelial preparations from liquid cultures, even when mixed directly into infested soil, did not suppress Phytophthora root rot.

M. roridum together with another species (*M. verrucaria*) are well documented as soil microorganisms and have a cosmopolitan distribution (16,40). They are found in a wide range of soils, especially those high in organic matter, and on a number of other substrates (7,16,39,40).

Pathogens such as *P. cinnamomi*, which attack the roots of their hosts and grow internally until the plant is killed, are difficult to control once penetration of the host root has occurred. Consequently, promising biological control organisms may need to be rhizosphere-competent (1,2) to antagonize the pathogen before infection. Species of *Myrothecium*, including *M. roridum*, have strong cellulolytic activity (16). The wild-type strain TW and the carbendazim-resistant mutant TWm14 both grew well on a basic salt medium with cellulose powder as their only carbon source. The ability to colonize cellulosic material might be a significant factor in permitting these fungi to compete in the soil and colonize the rhizosphere of *P. indica* (2). Our tests with the mutant strain TWm14 of *M. roridum* have demonstrated its ability to colonize the rhizoplane of *P. indica*. Undoubtedly, other factors than cellulolytic activity are involved in rhizosphere-competence, however.

Some isolates of *M. roridum* have been reported as pathogenic on a number of hosts (11,24,38) and, generally, attack above-ground plant organs. The TW strain of *M. roridum* and its mutants were nonpathogenic on *P. indica* in our experiments. However, at high bran/fungus concentrations, the root system of *P. indica* was sometimes stunted, which might possibly have been caused by high concentrations of antibiotics produced by the fungus.

M. roridum, and other species of *Myrothecium*, are strong antibiotic producers (6,8,19,21). Some macrocyclic trichothecenes produced by these species are active against leukemia (6,20) and, consequently, have attracted considerable scientific interest. The compounds produced by *Myrothecium* also have shown high fungistatic activity in vitro against several other fungi, including various pathogens (8,27–29,35). Involvement of these antibiotic compounds in suppression of *P. cinnamomi* in soil cannot be excluded. Mutants that do not produce the antibiotics could be invaluable tools in distinguishing the effect of antibiotics in situ from other forms of antagonism (30). Our attempts at producing mutants of *M. roridum*, strain TW, that do not produce antibiotics were not successful. In fact, some mutants produced slightly more antibiotics than the wild type. However, recently we obtained an isolate of *M. roridum* (IMI 142375) that did not produce antibiotics capable of inhibiting *P. cinnamomi* in vitro. Preliminary experiments with seedlings of *P. indica* indicated that IMI 142375 does not control Phytophthora root rot to the same degree as strains TW and TWm14. This strain could provide a tool for studying the role, if any, of the trichothecene toxins in the suppression of *P. cinnamomi* in soil. Although there is no immediate answer to the question as to whether the antibiotics produced by *M. roridum* in vitro play an active role in the suppression of root rot in soil, their potential in inhibiting key stages of the fungal life cycle was demonstrated in our study.

Incorporating conidial suspensions with a high antibiotic concentration into soil, or drenching soil with liquid culture filtrates, did not suppress Phytophthora root rot. Obviously, antibiotic properties of TW and its mutant TWm14 do not fully explain the level of disease suppression demonstrated. In addition, it is unknown to what extent these antibiotics are actually produced in soil. Jarvis et al (19) found no correlation between antibiotic production of isolated *Myrothecium* spp. and the high amount of macrocyclic trichothecenes in tissue of certain species of *Baccharis*. These plants are thought to accumulate and store large amounts of these antibiotics, believed to be produced by various soil fungi. It also has been demonstrated that pH and

temperature can profoundly affect the production of these compounds in culture (6). It can be assumed that these effects, together with other properties of the soil, could influence the level of antibiotic production. Factors such as rhizosphere-competence and, perhaps, competition for nutrients and space (5), mycoparasitism (39), and induced resistance (14,15) also may be involved in biocontrol of *P. cinnamomi* by *M. roridum*.

Because Phytophthora root rot of avocado is a long-term disease problem, strategies will have to be developed that permit *M. roridum* to persist as a large, rhizosphere-competent population density on avocado feeder roots over extended periods of time. Current use of strain TW at a rate of 2.5% (w/v) bran inoculum will probably require application of 6.5–26.0 kg/hectare based on recommended avocado planting densities of 260 trees per hectare. Future research will focus on developing improved methods of application, including formulation and delivery systems.

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