Improved Method for Isolating *Phytophthora lateralis* from Soil

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ABSTRACT

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Recovery of *Phytophthora lateralis* from soil was improved by adding hymexazol to the baiting solution, then placing baits into a solid antibiotic medium. Port-Orford-cedar branchlet baits were floated over a soil sample in water amended with 25 μg/ml hymexazol. Baits were transferred after 6 days to cornmeal agar containing 20 μg/ml of pimaricin and 200 μg/ml each of vancomycin and streptomycin (CMP). Hymexazol in the baiting solution limited colonization by *Pythium* isolates. No effect on growth or sporulation of *P. lateralis* was observed. Hymexazol and the antibacterial amendments prevented faster-growing contaminants from masking or inhibiting growth of *P. lateralis* on CMP. *P. lateralis* was successfully recovered from naturally infested soil diluted 16 times with uninfested soil.

Phytophthora lateralis (Tuck. & J. A. Milb.) is the causal agent of a serious root disease of Port-Orford-cedar (*Chamaecyparis lawsoniana* (A. Murr) Parl.) (POC) in forests of southwestern Oregon (7) and northwestern California (2) and in ornamental plantings throughout the Pacific Northwest (6). Epidemiological studies have been hampered by the lack of an efficient technique to isolate this fungus from soil. Direct isolation from soil, even on selective media, has not been successful, presumably because of low numbers of propagules, but various baiting strategies using POC foliage do allow recovery. Cedar branchlets buried in infested soil are sometimes infected by the fungus (7), but the procedure is unwieldy for many purposes. A more reliable technique used stripped lateral POC branchlets cut into lengths 3–4 cm (4). These were floated on water over soil or organic matter (OM) wet-sieved from soil, then examined microscopically for sporangia after 6 days. Recovery was much greater from OM than from whole soil. Unfortunately, sporangia of *P. lateralis* cannot be distinguished from some *Pythium* or other *Phytophthora* species found in forest soils.

P. lateralis will grow from infected baits transferred to a selective medium (*unpublished*). Isolates thus obtained can be identified by morphological characteristics unless overgrown by other Oomycetes or bacteria. Hymexazol added to agar media inhibits growth of many *Pythium* species (1,5) but also slows growth of *P. lateralis* (1) and alters colony morphology of both. Our goal was to increase recovery of *P. lateralis* from forest soils while inhibiting growth of competing *Pythium* species without slowing *P. lateralis* or altering the identifying characteristics of either group. This was accomplished by adding hymexazol to the baiting solution, followed by direct plating of POC foliage, rather than by incorporating the antibiotics into the growth medium.

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MATERIALS AND METHODS

The double-cup method described by Linderman and Zeitoun (3) was used in all tests. A Styrofoam cup with its bottom replaced by a layer of cheesecloth was rested over the sample in a second, intact cup. Five POC foliage baits were floated above the cheesecloth in each cup.

The effects of hymexazol in the baiting solution on growth and sporulation were examined in three tests. Seven *Pythium* isolates suspected to be different species and three isolates of *P. lateralis* were recovered by baiting from forest soils. In the first two tests, isolates were grown 4–6 days in pea broth (8) at 20 C. A single colony was then placed in the bottom of each double cup, and sporulation was induced by flooding with soil extract water (SEW, equal amounts by volume of soil and water, mixed, left overnight and then filtered) to induce sporulation. In all tests, three replicates of each treatment (isolate × concentration) were examined.

In test 1, hymexazol was added to the SEW at 0.0, 0.1, 1.0, 10.0, or 25.0 μg/ml. After 6 days at room temperature, baits from each cup were transferred to a single petri plate containing half-strength (10 g/L) cornmeal agar with 20 μg/ml pimaricin and 200 μg/ml vancomycin (Vancocin) and streptomycin (CMP). Pimaricin was added before autoclaving; vancomycin and streptomycin were added after the agar had cooled to about 45 C. The numbers of *Pythium* and *Phytophthora* colonies (referred to as the number of infections) and their diameters growing from baits in each plate were counted and measured after 24, 48, and 72 hr at 20 C.

Test 2 was similar, except hymexazol was added to CMP instead of the SEW. The diameters of *Pythium* and *Phytophthora* colonies growing from baits were measured after 24, 48, and 72 hr.

In test 3, actively growing colonies of each isolate were transferred from CMP to CMP amended with 0, 0.1, 1.0, 10.0, or 25.0 μg/ml hymexazol. Diameters of *Pythium* and *Phytophthora* colonies were measured after 5 and 10 days, respectively.

Suppression of *Pythium* species while baiting for *P. lateralis* was tested with an infested forest soil and two infested ornamental soils. The OM fraction was separated as described previously (4), lightly chopped, and 5 g was placed in each of 32 cups. Eight cups containing five POC foliage baits were set up for 6 days for each of five treatments: 1) baits were floated on distilled water containing 25 μg/ml hymexazol, then placed on CMP; 2) baits were floated on unamended distilled water, but 25 μg/ml hymexazol was added to the CMP; 3) hymexazol (25 μg/ml) was added to both the baiting solution and the CMP; 4) no hymexazol was added to either solution or CMP; and 5) the same as in 4, except petri plates were used instead of Styrofoam cups and *P. lateralis* infections were determined by microscopic examination of baits for sporangia production (the method of Ostrofsky et al) (4) before plating on CMP for comparisons. Baits from each cup were then placed into one petri plate. The number of CMP plates yielding *Phytophthora* and *Pythium* colonies was determined for each treatment and soil. The test was then repeated.

Sensitivity of the isolation technique was tested by baiting 62.5 g of OM from each of three naturally infested soils. A 12.5-g portion was removed and mixed with 50 g of sterilized OM at each of four dilution steps. The resulting 50-g portions (undiluted, 1:4, 1:16, 1:32, and 1:128) were divided among 10 cups, and five POC foliage baits were floated in distilled water containing 25 μg/ml hymexazol.

This procedure was repeated for each soil.

RESULTS

Recovery of *P. lateralis* through direct plating of baits was much more sensitive than by microscopic examination. Only baits from three cups (6%) were positive by the latter method (treatment 5) compared with 12 (25%) when identical baits were plated directly on agar. Although the number of positive plates yielding *P. lateralis* differed among soils, there were no significant differences ($P = 0.05$) between the four baiting treatments in total number of *P. lateralis* recoveries (Table 1). The total number of *Pythium* colonies obtained in each treatment, however, varied greatly. A significantly greater number ($P = 0.05$) of plates (60% or 30/48) in treatment 4 had baits with one or more *Pythium* infections, compared with treatment 1 (6% or 3/48), and none in treatments 2 and 3. Repeating treatments 1 and 4 using three new infested soils gave similar results. Hymexazol at 25 μg/ml in the baiting solution did not disrupt colony morphology, thus facilitating the identification of both genera on CMP. Increased *Phytophthora* recovery was observed when baits were pressed into the agar rather than when laid on the agar surface.

Growth of *P. lateralis* on agar from baits or from agar blocks was inhibited by hymexazol (Table 2). Neither infections of baits nor subsequent growth from the baits on unamended media were inhibited by hymexazol in the baiting solution.

Hymexazol at 25 μg/ml added to the baiting solution gave the greatest reduction in *Pythium* infections of baits floated over colonies of the fungi (Table 2). The number of *Pythium* colonies growing from each bait transferred to CMP averaged 6.7, 7.4, 7.2, 4.2, and 2.8 after 24 hr when baits were floated on 0, 0.1, 1.0, 10.0, and 25 μg/ml hymexazol, respectively. *P. lateralis* infections using this method averaged 0.5, 1.0, 4.5, 1.9, and 1.5 at the same concentrations. Counts after 72 hr indicated that infections by three of seven *Pythium* isolates had not been significantly reduced ($P = 0.05$) by hymexazol at 25 μg/ml (Table 2).

Growth reduction of *Pythium* but not *P. lateralis* isolates occurred after plating baits on CMP. Hymexazol added at 25 μg/ml to CMP significantly reduced ($P = 0.05$) growth of six of seven *Pythium* isolates during direct-transfer and baiting tests. Colonies were small and atypical compared with those on unamended CMP.

P. lateralis was recovered consistently from each soil after two dilutions (1:16) during sensitivity testing. The mean number of CMP plates (of 10) with *P. lateralis* for each soil decreased from 6.5 in the undiluted OM to 4.2, 1.8, 0.0, and 0.0 after 1, 2, 3, and 4 dilutions, respectively.

DISCUSSION

P. lateralis was isolated more quickly and more reliably from soil by baiting organic matter with POC foliage than reported previously (4). Direct plating of POC baits, adding hymexazol to the baiting solution, and incorporating pimaricin, vancomycin, and streptomycin in the isolation medium aids in *P. lateralis* observation and prevents growth of *Pythium* species and bacteria. *P. lateralis* could be recovered by this method after a 16-fold dilution. Both the number of *Pythium* infections on baits and subsequent growth of *Pythium* on CMP is best reduced by hymexazol in the baiting solution (Table 2). In addition, baits need not be surface-sterilized before plating onto the selective medium. By this method, *P. lateralis* has been isolated throughout the year.

This technique should also aid recovery of other species of *Phytophthora* from soil. *P. cinnamomi* and *P. lateralis* are readily recovered in soils containing both pathogens and varying *Pythium* populations (P. B. Hamm and E. M. Hansen, *unpublished*). In addition, using this fungicide as an agar amendment when isolating hymexazol-tolerant *Phytophthora* species (eg, *P. cinnamomi*) (1) could increase recovery. *Pythium* species were best restricted when hymexazol was used both in the baiting solution and in the isolation medium (Table 2).

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Table 1. Recovery of *Phytophthora lateralis* and *Pythium* spp. from soils by baiting with hymexazol added to the baiting solution or the selective medium to which baits are transferred

	Hymexazol concentration (μg/ml)							
	Treatment 1		Treatment 2		Treatment 3		Treatment 4	
	Solution (25)	Agar (0)	Solution (0)	Agar (25)	Solution (25)	Agar (25)	Solution (0)	Agar (0)
Phytophthora lateralis	17[a]		21		12		12	
Pythium spp.	3		0		0		30	

[a] Plates with *P. lateralis*. Eight replicates of three soils, each repeated once (48 plates per treatment). No significant differences ($P = 0.05$) were obtained among treatments 1–4 during *P. lateralis* recovery, whereas a significant difference in *Pythium* spp. recovery occurred comparing treatment 4 with treatments 1–3.

Table 2. Effect of hymexazol in baiting solution or selective medium (CMP) on growth and sporulation of *Pythium* and *Phytophthora lateralis*

	Colonies transferred directly to CMP (growth rate mm/day)		Colonies baited[a]			
			25 μg/ml hymexazol in solution/ hymexazol in CMP:		0 μg/ml hymexazol in CMP/ hymexazol in baiting solution:	
Isolates	0[b]	25	0	25	0	25
Pythium spp.						
1	8.5	1.1*[c]	>30.0	12.4*	>30.0 (>11.0)[d]	22.7 (5.9*)
2	3.3	0.5*	11.8	7.2*	12.7 (8.5)	4.7* (0.9*)
3	3.8	0.0*	8.6	1.3*	10.7 (7.2)	2.3 (0.1*)
4	9.0	0.0*	>30.0	2.1*	>30.0 (>11.0)	22.3* (>11.0)
5	13.7	8.4*	>30.0	20.7*	>30.0 (>11.0)	20.1* (>11.0)
6	11.4	10.3	>30.0	22.0	>30.0 (>11.0)	21.3* (6.4*)
7	12.4	0.8*	>30.0	5.9*	>30.0 (>11.0)	20.3* (>11.0)
Phytophthora lateralis						
1	1.0	0.2	1.6	0.7	1.7 (6.8)	3.3 (10.0)
2	1.1	0.4	2.2	0.2*	3.3 (4.3)	3.3 (10.0*)
3	0.8	0.2	0.4	0.5	3.0 (8.6)	3.7 (8.0)

[a] Growth of largest colony (mm) 72 hr after baits placed on selective media.
[b] Hymexazol concentration (μg/ml).
[c] * = Values that are significantly different ($P = 0.05$) between 0 and 25 μg/ml hymexazol.
[d] Figures in parentheses denote the average number of infections per Port-Orford-cedar bait.

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