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Antimicrobial Activity of Extractable Conifer Heartwood Compounds Toward *Phytophthora ramorum*

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Abstract Ethyl acetate extracts from heartwood of seven western conifer trees and individual volatile compounds in the extracts were tested for antimicrobial activity against *Phytophthora ramorum*. Extracts from incense and western redcedar exhibited the strongest activity, followed by yellow-cedar, western juniper, and Port-Orford-cedar with moderate activity, and no activity for Douglas-fir and redwood extracts. Chemical composition of the extracts varied both qualitatively and quantitatively among the species with a total of 37 compounds identified by mass spectrometry. Of the 13 individual heartwood compounds bioassayed, three showed strong activity with a Log_{10} EC_{50} less than or equal to 1.0 ppm (hinokitiol, thymoquinone, and nootkatin), three expressed moderate activity ranging from 1.0–2.0 ppm (nootkatol, carvacrol, and valencene-11,12-diol), four compounds had weak activity at 2.0–3.0 ppm [α-terpineol, valencene-13-ol, (+)-β-cedrene, (−)-thujopsene], and three had no activity [(+)-cedrol, δ-cadinene, and methyl carvacrol]. All of the most active compounds contained a free hydroxyl group, except thymoquinone. The importance of a free hydroxyl was demonstrated by the tremendous difference in activity between carvacrol (Log_{10} EC_{50} 1.81±0.08 ppm) and methyl carvacrol (Log_{10} EC_{50} >3.0 ppm). A field trial in California, showed that heartwood chips from redcedar placed on the forest floor for 4 months under *Umbellularia californica* (California bay laurel) with symptoms of *P. ramorum* leaf blight significantly limited the accumulation of *P. ramorum* DNA in the litter layer, compared with heartwood chips from redwood.

Keywords Sudden oak death · Fungicide · Tropolone · Monoterpene · Sesquiterpene

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Introduction

The heartwood of trees has long been known to produce and accumulate biologically active compounds from a variety of chemical classes (Rennerfelt and Nacht 1955; Rudman 1962, 1963; Scheffer and Cowling 1966) that protect them from microbial decay. Alaska yellow-cedar (*Callitropsis nootkatensis*; previously *Chamaecyparis nootkatensis*; Little et al. 2004; Little 2007) heartwood is an excellent example. Even after these trees die, defensive heartwood chemicals continue to protect standing snags for up to a century in the coastal forests of southeast Alaska and western Canada (Hennon et al. 1990, 2005, 2007a). Changes in snag heartwood chemistry are minimal until after the protective bark sloughs off, and the sapwood is lost from decay and weathering, about 26 years after death (Kelsey et al. 2005). Direct exposure of the snag heartwood to abiotic environmental conditions results in a continuous decline in the concentration of protective chemicals (Kelsey et al. 2005) and eventually a reduction in decay resistance, about 80 years after death (Degroot et al. 2000; Hennon et al. 2007b).

The presence of these strong antimicrobial compounds in yellow-cedar heartwood and its greater resistance than Port-Orford-cedar (*Chamaecyparis lawsoniana*) to the root disease, *Phytophthora lateralis* (McWilliams 2000), were the basis for our previous testing of its heartwood and select components against *P. ramorum* (Manter et al. 2006). *P. ramorum* is an exotic pathogen (Ivors et al. 2006) that causes the stem canker disease known as sudden oak death, which has killed thousands of tanoak (*Lithocarpus densiflorus*), coast live oak (*Quercus agrifolia*), California black oak (*Q. kelloggii*), and Shreve's oak (*Quercus parvula* var. *shrevei*) in coastal counties of California and one county in southwest Oregon since 1995 (Rizzo et al. 2002a, b; Garbelotto and Rizzo 2005; Goheen et al. 2006a). In Europe and the UK, this pathogen is primarily in nurseries or seminatural managed gardens (Werres and De Merlier 2003). It can also cause nonlethal shoot blight or leaf blight in various hosts (Goheen et al. 2006a). The list of proven and suspected hosts has grown to over 50 genera of predominately trees and shrubs (updated list at http://www.aphis.usda.gov/ppq/pramorum). The potential ecological and economic impacts from *P. ramorum* could be extensive depending on its level of direct or indirect influence on nurseries or the composition, structure, diversity, and function of native forests (Rizzo et al. 2005; Goheen et al. 2006a). Management of this pathogen focuses on slowing its spread by preventing introductions and establishment into new areas (Garbelotto and Rizzo 2005; Rizzo et al. 2005). As spores are the key dispersal mechanism (Davidson et al. 2005), control measures that target spore survival may contribute to successful management actions.

Previously, we demonstrated that wood chips, essential oil, and four individual compounds (nootkatin, carvacrol, nootkatone, and valencene) in yellow-cedar heartwood were fungitoxic to *P. ramorum* zoospores and sporangia and fungistatic against hyphae in culture assays (Manter et al. 2006). Microscopic observations detected rupturing of the zoospore outer membrane, and disruption, or release of the cellular contents from developing sporangia before zoospore formation. In addition, heartwood essential oils from incense cedar (*Calocedrus decurrens*), western redcedar (*Thuja plicata*), Port-Orford-cedar (*Chamaecyparis lawsoniana*), and western juniper (*Juniperus occidentalis*) were tested. All four essential oils killed zoospores and inhibited hyphal growth just as strongly as the oil from yellow-cedar, but their heartwood constituents were not further investigated until now. The main objectives of this study were to test the procedure described by Kuhajek et al. (2003) as a potential bioassay technique for antimicrobial activity against *P. ramorum* and to test the antimicrobial activity of individual compounds (most of them commercially available) present in the heartwood of the other conifer

species with antimicrobial activity against *P. ramorum*. In addition, we conducted a field experiment to determine if heartwood chips can limit the proliferation of *P. ramorum in situ*.

Methods and Materials

Heartwood Extracts Tested An initial study was conducted to identify heartwood extracts with antimicrobial activity against *P. ramorum*. We obtained bulk samples of heartwood from single trees of seven conifer species at the locations listed in Table 1. After air drying, each sample was mechanically chipped and then ground in a Wiley mill to pass a screen with 4 or 6 mm openings and finally through a 20 mesh screen. Extracts were prepared in triplicate by weighing 1.0 g of ground heartwood into a glass vial followed by 10.0 ml of ethyl acetate (99.9%, Fisher Scientific). Sealed extracts were kept at room temperature for 24 h with periodic swirling and then filtered through paper (Whatman No. 1 qualitative) into a storage vial.

Compound Identification and Quantification in Heartwood Extracts Volatile constituents in the extracts from incense cedar, western redcedar, Port-Orford-cedar, and western juniper were identified by gas chromatography–mass spectrometry (GC–MS) using a Hewlett Packard 5890 Series II gas chromatograph, with a 5970 mass selective detector and J&W Scientific DB-5 column (30 m×0.25 mm×0.25 μm film thickness). Helium was the carrier gas, with a 1.0 ml min^{-1} flow through the column at 100°C and a 1:10 split. Injector and detector temperatures were 250°C. The oven program started a 100°C for 1.0 min, then increased at 5°C min^{-1} to 150°C, followed by an increase of 3°C min^{-1} to 220°C, with no final hold. Instrument conditions for the analysis of western redcedar extracts were modified to those used by Taylor (2004). The injector and detector were set at 275°C, with an initial column temperature of 100°C and no hold, then increased at 5°C min^{-1} to 130°C, followed by an increase of 30°C min^{-1} to 250°C with a 7.0 min hold. Helium flow through the column was 2.0 ml min^{-1} at 100°C with a 1:10 split. Extracts of western redcedar, western juniper, and Port-Orford-cedar were concentrated by gently evaporating 50–75% of the solvent with a steam of compressed air. Two microliters of these concentrates, or the original incense cedar extract, was injected. Compounds were tentatively identified by comparison of their spectra and relative retention times with those in the Wiley and Adams

Table 1 The antimicrobial activity of ethyl acetate extracts from the heartwood of seven western conifer trees toward *Phytophthora ramorum*

Species	Log_{10} EC_{50} (ppm)[a]	Tree\heartwood Source
Strong inhibitors		
Incense cedar	2.74 (0.08)a	Warm Springs, OR
Western red cedar	2.78 (0.06)a	Alsea, OR
Moderate inhibitors		
Yellow-cedar	3.18 (0.08)b	Prince of Wales Island, AK
Western juniper	3.27 (0.11)b	Warm Springs, OR
Port-Orford-cedar	3.29 (0.09)b	East of Orick, CA
Weak or no activity		
Douglas-fir	>4.00	Warm Springs, OR
Redwood	>4.00	Fortuna, CA

[a] Mean (standard error) from three replicate trials. Means with different letters are significantly different ($P<0.05$).

(2007) libraries and authentic samples of the compounds tested for antimicrobial activity (Table 2). Constituents in yellow-cedar heartwood extracts were recently identified by GC–MS (Kelsey et al. 2005) and were recognized by their relative retention times.

Compound concentrations in the extracts were determined on the same GC with an flame ionization detector (FID) detector and Phenomenex ZB-5 column (30 m×0.25 mm× 0.25 μm film thickness), which is equivalent to the DB-5. The instrument operating conditions were the same as above including the modified settings for western redcedar. Before injection, an internal standard solution containing known quantities of limonene or fenchone in ethyl acetate (Fisher Scientific 99.9%) was added to each extract (1:4, standard/extract measured with analytical syringes). Limonene was the standard for yellow-cedar and fenchone the standard for western juniper, Port-Orford, incense, and western redcedar. Two microliters of each extract/standard mixture was injected. Mean compound concentrations are reported from the three technical replicates of each heartwood extract, except western juniper that had just two replicates.

Individual Compounds Bioassayed Thirteen different volatile compounds were identified from the GC–MS analysis of the conifer heartwood extracts and were selected for further testing. Selected compounds were limited to those that were soluble in ethyl acetate and commercially available or previously isolated in our laboratory (Table 2). Compound purity was determined by transferring 1–2 mg to 1.0 ml of ethyl acetate and analyzing 2μl by GC–FID as described above for compound quantitation. Preliminary studies showed that ethyl

Table 2 The antimicrobial activity, purity as tested, and source for individual compounds tested in *Phytophthora ramorum* bioassays and found in the heartwood extracts of one or more western conifer species

Compound	Log_{10} EC_{50} (ppm)[a]	Purity (%)	Class[b]	Source[c]
Strong inhibitors				
Hinokitiol	0.30 (0.06)a	99.7	T	TCI America
Thymoquinone	0.78 (0.08)b	99.9	M	Acros
Nootkatin	1.00 (0.09)b	99.6	T	Yellow-cedar isolation
Moderate inhibitors				
Nootkatol	1.63 (0.09)c	66.6	S	Lab synthesis
Carvacrol	1.81 (0.08)c	99.4	M	TCI America
Valencene-11,12-diol	1.82 (0.08)c	90.2	S	Yellow-cedar isolation
Weak inhibitors				
α-Terpineol	2.36 (0.07)d	84.1	M	TCI America
Valencene-13-ol	2.38 (0.11)d	55.2	S	Yellow-cedar isolation
(+)-β-Cedrene	2.75 (0.10)e	96.7	S	Fluka (Sigma -Aldrich)
(−)-Thujopsene	2.92 (0.10)e	96.9	S	Fluka (Sigma -Aldrich)
No activity				
(+)-Cedrol	>3.00	99.7	S	Fluka (Sigma -Aldrich)
δ-Cadinene	>3.00	85.5	S	Florida Chemical
Methyl carvacrol	>3.00	98.5	M	Fluka (Sigma -Aldrich)

[a] Mean (standard error) from three replicate trials. Means with different letters are significantly different ($P<0.05$).

[b] Compound class: *T* tropolone; *M* monoterpene; *S* sesquiterpene

[c] For details of isolation and identification of yellow-cedar compounds, see Xioung (2000) and Khasawneh (2003). Nootkatol synthesized from sodium borohydride reduction of nootkatone

acetate exhibited no antimicrobial activity against *P. ramorum* at concentrations less than or equal to 1% (data not shown).

Bioassays Antimicrobial activity of heartwood extracts or individual compounds was measured with the photometric assay developed by Kuhajek et al. (2003) for bioassays with *Phytophthora* spp., but not tested with *P. ramorum*. Zoospores from one isolate of *P. ramorum* (A1 mating type, obtained from an infected rhododendron plant at a commercial nursery in Clackamas, Oregon) were obtained by flooding 2-week-old cultures grown on clarified V8 agar plates (66.7 ml of V8 juice and 2 g $CaCO_3$, filtered through cheesecloth, plus 15 g of agar [Bacto-agar, Difco Laboratories, Detroit, MI, USA] per liter of distilled water) with 10 ml H_2O and incubating at 4°C for 4 h. Zoospore solutions were quantified by direct counting with a hemocytometer (×40 magnification) and diluted to appropriate concentrations with dH_2O. One isolate was considered adequate as six *P. ramorum* isolates (A1 and A2 mating types) responded similarly in previous bioassays (Manter et al. 2006). Growth of *P. ramorum* was evaluated in a 96-well microtiter format by measuring optical density at 650 nm (OD_{650}) at 0, 16, 24, 48, and 72 h with a microplate reader (Vmax Kinetic Microplate Reader, Molecular Devices, Menlo Park, CA, USA). Between measurements, plates were covered and incubated at room temperature with gentle shaking at 80 rpm. All OD_{650} values were corrected for background absorbance at time zero. The effect of starting zoospore concentrations on growth curves was analyzed by adding 50µl of a zoospore solution (0, 10, 100, 1,000, 2,500, 5,000, 7,500, or 10,000 zoospores ml^{-1}) and 100µl of clarified V8 broth to each of six replicate wells. Antimicrobial activity of all test compounds was analyzed by adding 50µl of a zoospore solution (10^4 zoospores ml^{-1}) and 100µl of V8 broth amended with an individual test compound to each of six replicate wells. All heartwood extracts were tested at 0, 10, 50, 100, 500, 1,000, 5,000, and 10,000 ppm (*v*/*v*), and all individual compounds were tested at 0, 1, 5, 10, 50, 100, 500, and 1,000 ppm (*v*/*v*). Additional concentrations at 25 ppm were included for hinokitiol, nootkatin, nootkatol, carvacrol, and valencene-11,12-diol and at 250 ppm for α-terpineol and valencene-13-ol. Each experimental plate also included six replicate sterility control wells (100µl of V8 broth and 50µl dH_2O). Plates with growth in the sterility control wells were discarded from the analysis. Each compound and concentration combination was replicated ×3, and a Log_{10} EC_{50} was determined for each trial as described below.

Heartwood Field Trial To determine whether chips of redcedar heartwood can limit *P. ramorum* buildup in soil and litter under natural conditions, we conducted a field test on the Rush Creek Open Space Preserve, at Novato, California (38°7.22′N, 122°33.70′W), in the spring and early summer of 2006. A fresh bulk sample of redcedar heartwood was collected in January 2006 from the same tree sampled for the laboratory bioassays reported in Table 1. The wood was cut into pieces, air dried in the laboratory, and chipped (1–3 cm long, 0.5–3 cm wide, 0.1–0.5 cm thick). Redwood was selected as a control because it was inactive in the laboratory bioassays. Kiln-dried and planed redcedar boards were obtained from a commercial source in Eugene, Oregon in January 2006 and chipped in our laboratory. Water content was measured on six subsamples of each species by oven drying them for 16 h at 102°C and cooling to room temperature in a desiccator box before reweighing. The dry weight equivalent of 140 g of fresh chips was enclosed in a nylon mesh bag (approx. 20×20 cm, 2.5×3.5 mm hole) sealed on the edges with cloth tape and staples.

On March 1, 2006, ten *Umbellularia californica*, California bay laurel, trees with leaves symptomatic of *P. ramorum* infection (Goheen et al. 2006a) were selected to serve as

blocks on the northwest side of Pinheiro ridge in the Rush Creek Preserve. Two plots (1× 1 m) were located beneath each tree where the canopy had the most symptomatic leaves. Six bags of redcedar chips were randomly positioned on one plot (selected from a 5×5 grid of 20×20 cm subplots) and six bags of redwood on the other plot. The bags were placed on top of the litter and secured with long wire pins on each corner penetrating into the soil to minimize movement by passing animals. On two sample dates (2 months posttreatment: May 2, 2006; 4 months posttreatment: June 29, 2006), three randomly chosen subplots were sampled from each plot. Bags were lifted carefully, and the litter beneath the center of the bag was gathered. We wore plastic gloves that were changed after each sample to minimize cross contamination. The litter depth varied tremendously; in some instances, it was only one or two leaves deep, and on other sites, it was 1–2 cm or greater. Although leaves of *U. californica* were usually present, leaves from other tree species nearby or grass blades were also common and were included in the sample that was sealed in a glass scintillation vial. Bits and pieces of surface soil may have adhered to leaves in direct contact with the soil. A plug of soil beneath the litter was taken to a depth of 3.0 cm with a #8 cork borer (1.4-cm diameter) and sealed in a glass scintillation vial. The borer was cleaned between samples with a 70% isopropyl alcohol swab and dried with a fresh tissue to reduce cross contamination.

Analysis of Phytophthora ramorum Proliferation by Quantitative Polymerase Chain Reaction Soil and litter samples from the Rush Creek Preserve were shipped back to the laboratory on ice and stored at −20°C until DNA extraction. Total genomic DNA was extracted from each sample by using Ultraclean Soil or Plant DNA kits (MoBio, Carlsbad, CA) per the manufacturer's protocol. DNA from the litter samples was further purified by phenol/chloroform/isoamyl alcohol (25:24:1) extraction and ethanol precipitation. Amplification of *P. ramorum* DNA was performed by using the species-specific nested protocol developed by Hayden et al. (2006). The amount of *P. ramorum* DNA present in each sample was calculated from measured cycle threshold values (BioRad, iCycle, Hercules, CA, USA) and an external standard curve generated from serially diluted *P. ramorum* genomic DNA isolated from pure cultures. All reported values of *P. ramorum* DNA are per unit dry weight of soil or litter extracted. The three subsamples (bags) from each plot were averaged, and treatment differences were analyzed by analysis of variance (ANOVA) as described below.

Data Analysis Kuhajek et al. (2003) suggested that bioactivity can be determined from the ratio of treatment and control OD values at a particular time point. However, unless a linear relationship between OD and initial zoospore concentration exists at the chosen time point, this technique will lead to an inaccurate estimate of activity. In our study, we did not observe a linear relationship at any of the selected time points (data not shown); furthermore, activity estimates will vary depending upon the chosen time point, if the slope of the OD vs. initial zoospore concentration regression line changes. To minimize these problems, the following analysis procedures were used. For each concentration of extract or compound bioassayed, a sigmoidal curve was fitted to the corrected OD_{650} growth curves (e.g., Fig. 1a) to obtain an estimate of $time_{1/2}$ or time (h) it takes to reach the midpoint between minimum and maximum OD_{650} values. This calculated parameter ($time_{1/2}$) was linearly related to initial zoospore concentration (Fig. 1b) and has the advantage that it is independent of the specific time points included in the analysis. Antimicrobial activity of each compound was then determined by fitting a sigmoidal curve to the $time_{1/2}$ vs. Log_{10} concentration (ppm) of each test compound and calculating the Log_{10} EC_{50} from this curve.

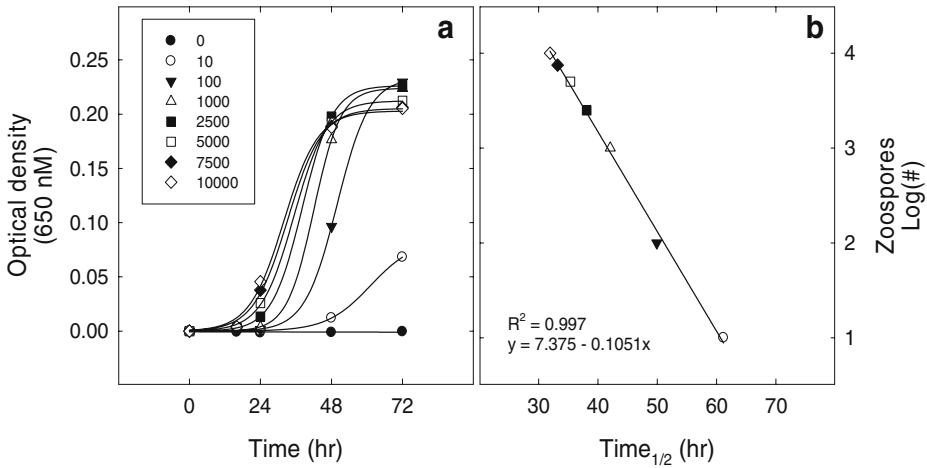

Fig. 1 Changes in optical density (OD_{650}) over time for microplate well cultures of *P. ramorum* initiated with different zoospore concentrations (number ml^{-1}; **a**) and the relationship between starting zoospore concentrations on a log_{10} scale and the well culture OD_{650} midpoints, or $time_{1/2}$ (**b**)

All regressions were performed by using SigmaPlot 9.0 (Systat Software Inc., Point Richmond, CA, USA). Significant differences between treatments (compounds) were tested by ANOVA and Fisher's least significant difference post hoc comparison ($P<0.5$) using SAS Vers 9.1 (Cary, NC).

P. ramorum DNA concentrations in the litter beneath the redcedar and redwood chips after 0, 2, or 4 months of treatment were analyzed as a randomized complete block design with ten replications by ANOVA using SAS Ver. 9.1. Data were natural log transformed to meet the assumption of homogeneous variance, and back-transformed least squares means and pooled standard errors are presented. Differences among means were determined by Fisher's protected least significant difference ($P<0.05$). The DNA concentrations in soil samples were analyzed separately using the same analysis procedure.

Results

Heartwood extracts from the trees sampled were grouped into three levels of antimicrobial activity: (1) strong—incense cedar and western redcedar; (2) moderate—yellow-cedar, western juniper, and Port-Orford-cedar, and (3) weak or no activity—Douglas-fir (*Pseudotsuga menziesii*) and redwood (*Sequoia sempervirens*) (Table 1). Individual compounds were separated into four activity categories: (1) strong (Log_{10} $EC_{50}<1.0$ ppm)—hinokitiol, thymoquinone, nootkatin; (2) moderate—(Log_{10} EC_{50} 1.0–2.0 ppm) nootkatol, carvacrol, valencene-11,12-diol; (3) weak (Log_{10} EC_{50} 2.0–3.0 ppm)—α-terpineol, valencene-13-ol, β-cedrene, and thujopsene; and (4) no activity (Log_{10} $EC_{50}>3.0$ ppm)—cedrol, δ-cadinene, methylcarvacrol (Table 2).

The volatile compounds identified in each extract are shown in Table 3 and were distinct for each tree (species), except for a few compounds. Carvacrol was detected in three trees—incense cedar, western redcedar, and yellow-cedar. α-Terpineol and δ-cadinene were both present in western redcedar and Port-Orford-cedar. Constituents in the Douglas-fir and

Table 3 Compound concentrations in heartwood extracts tested for antimicrobial activity toward *Phytophthora ramorum*

No.	Compound[a]	Incense Cedar[b]	Western Redcedar	Yellow-cedar	Western Juniper	Port-Orford-cedar
1	*Carvacrol*	189.5	4.4	77.4		
2	Heyderiol	673.0				
3	Libocedrol	186.1				
4	*p*-Methoxythymol	479.6				
5	*p*-Methoxycarvacrol	129.9				
6	*Thymoquinone*	193.3				
7	Thymohydroquinone	66.4				
8	*Hinokitiol*		12.8			
9	Myrtenol		7.0			
10	Nezukone		32.0			
11	Terpinen-4-ol		5.0			
12	α-*Terpineol*		5.9			374.1
13	Thujic acid		76.6			
14	*δ-Cadinene*			10.7		740.4
15	*Methyl carvacrol*			4.9		
16	Nookatone			74.4		
17	Nootkatene			126.0		
18	*Nootkatin*			55.0		
19	*epi*-Nootkatol			17.1		
20	*Nootkatol*			29.3		
21	Valencene			18.9		
22	*Valencene-13-ol*			28.4		
23	*Valencene-11,12-diol*			44.6		
24	α-Cedrene				62.6	
25	*Cedrol*				288.0	
26	*β-Cedrene*				14.9	
27	*Thujopsene*				15.3	
28	α-Amorphene					151.3
29	Borneol					93.9
30	α-Cadinene					57.7
31	γ-Cadinene					395.2
32	α-Cadinol					934.4
33	Fenchol					57.0
34	α-Muurolene					288.8
35	*epi*-α-Muurolol					860.6
36	α-Terpenyl acetate					65.2
37	α-Muurolol					168.4
38	Unknowns	82.1 (2)[c]	48.4 (7)		412.4 (7)	177.0 (1)
	Total	1,999.9	192.1	486.7	793.2	4,364.0

[a] Compounds (μg ml^{-1}) listed alphabetically within species, except those in multiple species. All compounds were identified by comparison of their spectra and retention times with those in the Wiley and Adams (2007) libraries. Those in *italicized font* were also compared with the authentic samples tested individually for antimicrobial activity (Table 2).

[b] Identities of *p*-methoxythymol and *p*-methoxycarvacrol were assigned based on molecular weights (Zavarin and Anderson 1955) and molecular ions in their mass spectra and relative retention times for thymol and carvacrol provided by Adams (2007).

[c] Number of unknown peaks in parentheses.

redwood extracts were not analyzed because they exhibited no antimicrobial activity. Two or more of the individual compounds tested for antimicrobial activity were present in the extracts that exhibited bioactivity (Table 3).

P. ramorum DNA was present at detectable levels in all soil and litter samples on both sample dates. For both heartwood chip treatments, *P. ramorum* DNA increased over time with the greatest increases in the litter layer. On May 1, 2 months after placing the chips on the forest floor, there was no difference between treatments in the amount of *P. ramorum* DNA in the litter (Fig. 2a). Between May 1 and June 29, *P. ramorum* DNA on the redwood control plots increased 11.1 times, compared with only 2.6 times on the redcedar plots, resulting in the redwood plots having 4.3 times more *P. ramorum* DNA than the redcedar (Fig. 2a). Soils from redwood and redcedar plots had the same quantities of DNA in May and June (Fig. 2b).

Discussion

The new protocol tested here and developed initially by Kuhajek et al. (2003) is easier to use, allows more samples to be quickly analyzed, and requires much smaller amounts of media and test materials than the bioassay procedure used previously for testing the activity of yellow-cedar compounds toward *P. ramorum* (Manter et al. 2006). Disadvantages include the inability to directly test heartwood activity because of interference with light transmittance. Furthermore, it does not distinguish inhibition or disruption of zoospore or sporangia germination from inhibition of hyphal growth. Our previous results suggest that the key mode of action for many of the toxic yellow-cedar compounds is lysis of zoospore and sporangia cell membranes (Manter et al. 2006). The antibacterial activity of essential oils and individual oil constituents, including carvacrol, has been attributed to their hydrophobicity, which allows them to enter the cell or mitochondrial membranes (reviewed by Burt 2004). This disruption increases membrane permeability and leakage of the cellular contents. Hinokitiol, a redcedar component, also causes cell lysis for some Gram-negative bacteria (Trust and Coombs 1973) and can rupture cercariae of *Schistosoma mansoni* (Chisty et al. 2004). However, regardless of the mechanism for reducing growth (i.e.,

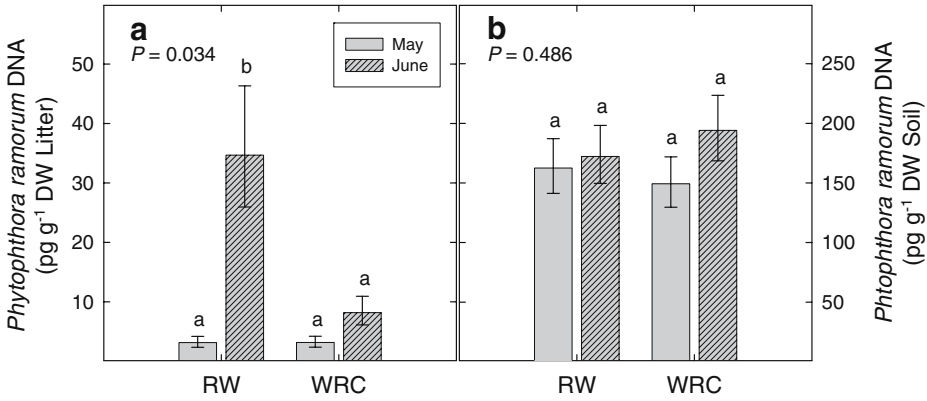

Fig. 2 Concentrations of *P. ramorum* DNA in the litter (**a**) and soil (**b**) after 2 and 4 months treatment with western redcedar (*WRC*) or redwood (*RW*) chips beneath the canopy of *U. californica* trees with leaves showing symptoms of *P. ramorum* leaf blight. *Bars* represent the median and pooled standard errors resulting from back-transformations. *Bars with different letters* are statistically significant at $P<0.05$

disruption of zoospore or sporangia membranes and/or limiting hyphal growth rates), compounds with antimicrobial activity will be detected by the photometric methodology used in this study. Nootkatin and carvacrol were tested here and with our earlier procedure (Manter et al. 2006). In both instances, nootkatin was more active than carvacrol. Previously, nootkatin had a Log_{10} EC_{50} of 0.18 and 0.84 ppm for inhibition of zoospore and sporangia germination, respectively, compared with 1.03 and 1.85 ppm for carvacrol (Manter et al. 2006). In the current assay, the Log_{10} EC_{50} for nootkatin was 1.00± 0.09 ppm, and carvacrol was 1.81±0.08 ppm.

Of the seven different conifer heartwood extracts bioassayed in the initial experiment, those from western redcedar and incense cedar were more than twice as active (less than half of the EC_{50}) toward *P. ramorum* than extracts from the group including yellow-cedar, western juniper, and Port-Orford-cedar (Table 2). Douglas-fir and redwood extracts were inactive. Previously, the steam distilled essential oils from yellow-cedar, western juniper, Port-Orford-cedar, and incense cedar were shown to have nearly identical levels of activity toward *P. ramorum* zoospore germination and hyphal growth when tested at 140 ppm (Manter et al. 2006). As the chemical composition of conifer heartwood can be extremely variable among trees (DeBell et al. 1999; Kelsey et al. 2005), our ranking of species activities in Table 2 applies only to the trees used in this study. Confirmation of these species differences will require testing of a larger random sample.

All individually tested compounds with strong or moderate antimicrobial activity contained a free hydroxy group, except for thymoquinone. The importance of this functionality is demonstrated by the tremendous difference in activity between carvacrol (Log_{10} EC_{50} 1.81±0.08 ppm) and methyl carvacrol (>3.0 ppm) with a methyl group blocking the hydroxyl. The antibacterial action of carvacrol against the food pathogen, *Bacillus cereus*, is also dependant on the free hydroxyl (Ultee et al. 2002). In contrast, the cytotoxicity of thymoquinone decreases 1.7-fold when the two ketones are reduced to alcohols (Johnson et al. 1998). Hydroxylated compounds with the lowest antimicrobial activity in our bioassays included two sesquiterpenes (cedrol and valencene-13-ol) and one monoterpene (α-terpineol). Valencene-13-ol and nootkatol expressed weak activity as tested at 55 and 66.6% purity, respectively. Had they been at 95% purity, the valencene-13-ol would probably have increased to the moderate level of activity, and nootkatol might have reach the strong category. Of the three sesquiterpene hydrocarbons tested, β-cedrene and thujopsene were weakly active, and δ-cadinene had no activity toward *P. ramorum*.

All compounds in the strong and moderate categories here have been shown to be active against a range of other organisms. Hinokitiol is probably the most rigorously investigated with activity against human tumor cells and a variety of microorganisms that cause plant disease or decay, and diseases of humans (Trust and Coombs 1973; Fallik and Grinberg 1992; Inamori et al. 1993, 1999; Baya et al. 2001; Arima et al. 2003; Voda et al. 2003; Chisty et al. 2004; Yamano et al. 2005). Studies of carvacrol have demonstrated bactericidal, fungicidal, insecticidal, nematicidal, and acaricidal activities (Rennerfelt and Nacht 1955; Ahn et al. 1998; Oka et al. 2000; Voda et al. 2003; Burt 2004). Nootkatin has antimicrobial properties against wood decay fungi (Rennerfelt and Nacht 1955) and *Candida albicans*, a yeast that causes infections in animals (Johnston et al. 2001). Thymoquinone is cytotoxic for certain human tumor cells and is insecticidal toward yellow fever mosquito larvae (Johnson et al. 1998).

Conifer heartwood typically contains a complex mixture of compounds from various chemical classes and in varying concentrations (Renerfelt and Nacht 1955; Rudman 1962, 1963; Scheffer and Cowling 1966). This study focused on volatile compounds soluble in ethyl acetate and detectable by GC. Not all of the active compounds in these extracts would

be detected by this method, and there could be other active compounds remaining in the wood because of their low solubility in this solvent. This is especially relevant for redcedar because its heartwood contains many nonvolatile phenolics, such as plicatic acid, plicatin, or thujaplicatin (Barton and MacDonald 1971) with weak antimicrobial properties (Roff and Atkinson 1954). Some proportion of these compounds would be removed with ethyl acetate, as they are soluble in acetone (Barton and MacDonald 1971), but they are not detectable by GC. Thus, woody tissues of the conifers tested here could have stronger *in vivo* bioactivities than their extracts demonstrated *in vitro*. Despite our limited search of the chosen conifer metabolomes, we successfully identified a number of highly active, commercially available compounds that provide strong activity against *P. ramorum* in culture.

Results from the field trial with chips of western redcedar and redwood were consistent with *in vitro* bioassays of their heartwood extracts. After just 2 months of treatment, there was no difference in the *P. ramorum* DNA or biomass in the litter beneath the redwood or western redcedar chips. In the subsequent 2 months, however, *P. ramorum* DNA on the redwood plots increased 4.3 times the amount on the redcedar plots. A combination of rainfall, spore production, and bioactivity of the wood chips may explain these results. Warm winter or spring rains stimulate *P. ramorum* to grow and sporulate on wet leaf surfaces of suitable hosts, especially bay laurel. Once zoospores or chlamydospores are produced on the leaves, subsequent rainfall washes them to new leaves or the forest floor. Upon installation of the field plots at the beginning of March, there were relatively few leaves on the bay laurel with visible symptoms. During March and April, the site experienced heavy rainfall, 205 and 127 mm, respectively (California Climate Data Archive [http://www.calclim.dri.edu/ccda/ncacoop.html], weather station 046826 located 16 km North of the field site). At the first sampling date in early May, the number of visibly infected bay laurel leaves had increased substantially in the lower canopy. In May and June, rainfall decreased to 8 and 0 mm, but the level of bay laurel canopy infection had expanded further by the last collection date on 29 June.

In the absence of woodchips covering the forest floor, Davidson et al. (2005) observed a 2- to 4-month lag between peak rainfall and detection of *P. ramorum* in the litter and soil. The low levels of *P. ramorum* DNA in the two chip treatments of the May sample are thus not unexpected because of the time lag between rainfall and spore deposition. Additionally, the presence of a layer of woodchips, with or without antimicrobial activity, could physically trap and reduce spore movement to the litter layer below. Furthermore, spores directly contacting the western redcedar wood, or its leachate, would be exposed to antimicrobial compounds that can damage spore membranes and stop their further movement to the litter layer. It is also possible that chlamydospores present in the litter before treatment germinated and formed new sporangia and zoospores in response to the heavy March and April rainfall. Here again, those exposed to the antimicrobial compounds of western red cedar would experience lower germination and growth rates compared to those in the redwood treatment, which had no antimicrobial activity. An alternative interpretation of the field data is that western redcedar had no impact on *P. ramorum*, and the redwood treatment stimulated its growth in the litter. This seem unlikely given the strong activity of western redcedar extracts *in vitro* and the observation that direct inoculations of logs and stems of both redwood and western redcedar showed no differences in the growth of *P. ramorum* (Hansen et al. 2005).

We chose to quantify *P. ramorum* biomass in the field trial by quantitative polymerase chain reaction because of its high reproducibility and sensitivity to detect changes in *P. ramorum* biomass (Hayden et al. 2006; Schena et al. 2006). However, this technique cannot

distinguish between viable and nonviable biomass present in a sample. Although other techniques, such as leaf baiting or direct plating, will detect only viable *P. ramorum* biomass, they are at best semi-quantitative or not species specific. Furthermore, even if some nonviable *P. ramorum* was detected in our samples, it is clear that viable *P. ramorum* is present and is increasing significantly over time under the redwood, but not the redcedar chips. Finally, we suggest that the presence of any nonviable *P. ramorum* in the redcedar plots may be masking our ability to detect the full bioactivity of redcedar chips *in situ*—not only does it limit the proliferation and development of new biomass, but it also kills any *P. ramorum* already present in the system.

Management of this pathogen focuses on slowing its spread by preventing introductions and establishment into new areas (Garbelotto and Rizzo 2005; Rizzo et al. 2005). With spores being the key dispersal mechanism (Davidson et al. 2005), control measures that target spore survival may contribute to successful management actions. An integrated management program may be able to utilize bioactive conifer heartwoods in some instances to help limit the number of *P. ramorum* spores in soils and the potential for new infections via splash dispersal (Davidson et al. 2005) or long-distance transport to new areas. For example, people living, working, and recreating in areas infested with *P. ramorum* can vector spores on their shoes, bicycle tires, or other items that contact spore contaminated soils or plant tissues (Cushman and Meentemeyer 2005; Davidson et al. 2005). Previously, we proposed that chips of yellow-cedar might be spread over trails, bike paths, or parking lots used by recreationists to reduce spore movement and redistribution from these areas (Manter et al. 2006). However, this will not affect movement of viable hyphal fragments.

Alternatively, some of the most active individual heartwood compounds might be developed into useful products. There are several fungicides that can protect against *P. ramorum* infection when applied in advance of inoculation (Heungens et al. 2006; Linderman and Davis 2006; Tjosvold et al. 2006; Turner et al. 2006), but their protection against foliar infection varies with host species, age of leaves, and source of the fungal isolate (Goheen et al. 2006b). Post inoculation applications tend to be less effective, and none of the fungicides is capable of eradicating the pathogen from infected plants (Heungens et al. 2006; Linderman and Davies 2006; Tjosvold et al. 2006). In addition, there are concerns for rapid development of fungicide resistance (Turner et al. 2006). Thus, it may be worthwhile to test additional hydroxylated compounds from conifer species that have not yet been bioassayed and to examine the efficacy of foliar spray applications of the most active compounds such as hinokitiol and thymoquinone.

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