Antimicrobial Activity of Extracts and Select Compounds in the Heartwood of Seven Western Conifers Toward *Phytophthora ramorum* [1]

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Abstract

Previously, we demonstrated that wood chips, essential oil, and four individual compounds from Alaska yellow-cedar (*Chamaecyparis nootkatensis* [D. Don] Spach) heartwood strongly inhibit the germination of *Phytophthora ramorum* (Werres, de Cock, Man int Veld) zoospores or sporangia, and reduce hyphal growth in culture (Manter and others 2006). Essential oils from heartwood of incense cedar (*Calocedrus decurrens* [Torr.] Florin), western redcedar (*Thuja plicata* Donn ex D. Don), Port-Orford-cedar (*Chamaecyparis lawsoniana* [A. Murr.] Parl.), and western juniper (*Juniperus occidentalis* Hook.*)* were also tested and found to be equally active, but not investigated further until now. The objectives of this study were to: 1) test the procedure described by Kuhajek and others (2003) as a bioassay technique for *P. ramorum*; 2) use this bioassay with other individual compounds from yellow-cedar heartwood that were not previously tested for bioactivity toward *P. ramorum*; and 3) bioassay extracts and compounds from the heartwood of the other conifers with antimicrobial essential oils and compare their *P. ramorum* activity to the yellow-cedar constituents.

Bulk heartwood samples were gathered from one tree of the five species above, plus Douglas-fir (*Pseudotsuga menziesii* [Mirb.] Franco) and redwood (*Sequoia sempervirens* [D. Don] Endl.). Air dried chips were ground (20 mesh), extracted with ethyl acetate (triplicate subsamples), filtered and bioassayed. In addition, 14 compounds known to occur in the heartwood of these seven conifers were selected for testing individually (hinokitiol, thymoquinone, nootkatin, nootkatol, carvacrol, valencene-11, 12-diol, α-terpineol, valencene-13-ol, taxifolin, (+)-β-cedrene, (-)-thujopsene, (+)-cedrol, δ-cadinene, methylcarvacrol). They were all commercially available, or previously isolated in our laboratory, and soluble in ethyl acetate.

Zoospores were obtained from one *P. ramorum* isolate (A1 Oregon source) after growing on clarified V8 agar (6.6 percent) for two weeks. One isolate was considered adequate based on previous results (Manter and others 2006). Bioassays of *P. ramorum* growth were conducted in a 96-well microtiter format by measuring optical density (OD, 650 nm) at 0, 16, 24, 48 and 72 h using a microplate reader (Kuhajek and others 2003). Antimicrobial activity of all test compounds was analyzed by adding zoospore solution (50 µl, 10^4 zoospores ml^{-1}) and V8

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broth (100 µl, 6.6 percent) amended with an extract, or individual test compound to each of six replicate wells. Heartwood extracts were tested at eight concentrations from 0 - 10000 ppm (v/v). Individual compounds were tested at eight concentrations from 0 - 1000 ppm (v/v). All experimental plates also included six replicate sterility control wells (100 µl of V8 broth; 50 µl dH_2O), and any plates with growth in these wells were discarded. All experimental plates were replicated three times and their average OD values used for growth analysis. For each concentration of extract, or compound bioassayed, a sigmoidal curve was fitted to the OD growth values to obtain an estimate of $time_{1/2}$, or time (h) it takes to reach the OD mid-point between its minimum and maximum value. Antimicrobial activity of each test compound was then determined by fitting a sigmoidal regression curve to the $time_{1/2}$ vs. $\log_{10}$ concentration (ppm) plot and calculating the EC_{50} from this curve.

After completing the bioassays, chemical constituents in heartwood extracts of incense cedar, western red cedar, Port-Orford-cedar, and western juniper were identified by gas chromatography-mass spectrometry (GC-MS). Constituents in yellow-cedar extracts were identified recently by GC-MS and were recognizable from their relative retention times. Concentrations of compounds in the extracts were determined by adding an internal standard and analyzing them by GC with an FID detector.

To determine whether chips of red cedar heartwood can impact *P. ramorum* under natural conditions we conducted a field test on the Rush Creek Open Space Preserve, at Novato, California. Bulk samples of red cedar (same tree sampled for lab bioassays) and redwood (commercial boards) chips were prepared in January 2006, and enclosed (140 g dry wt equivalent) in nylon mesh bags (approx. 20 x 20 cm). Redwood was selected as a control because it was inactive toward *P. ramorum* in culture bioassays (see below). On March 1, 2006 ten California bay laurel (*Umbellularia californica* [Hook. & Arn.] Nutt.), trees with leaves symptomatic of *P. ramorum* infection were selected to serve as blocks in the preserve. Two plots (1 x 1 m) were located beneath each tree where the canopy had the most symptomatic leaves. Six bags of red cedar chips were randomly positioned within one plot and six bags of redwood in the other plot. Bags were placed on top of the litter and secured with long wire pins in the soil. After about two months (May 2) the litter and soil (1.4 cm dia. x 3.0 cm deep) beneath three randomly chosen bags were sampled on each plot. Sampling was repeated for the remaining three chip bags after four months (June 29). Total genomic DNA was extracted from each litter or soil sample using plant or soil DNA kits and 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 using the species-specific nested protocol developed by Hayden and others (2006). The amount of *P. ramorum* DNA present per unit dry weight of soil or litter was calculated from measured Ct values (BioRad, iCycler) and an external standard curve generated from serially diluted *P. ramorum* genomic DNA isolated from pure cultures.

The Kuhajek and others (2003) bioassay procedure was easy to use, allowed many samples to be quickly analyzed, and required small amounts of media and test materials. Disadvantages include the inability to directly test heartwood activity because of interference with light transmittance. Also, it does not distinguish inhibition or disruption of zoospore or sporangia germination from inhibition of hyphal growth. Nevertheless, this photometric method is useful for detecting compounds with antimicrobial activity toward *P. ramorum*.

The heartwood extracts were grouped into three levels of antimicrobial activity: 1) strong (EC_{50} 500-700 ppm) – for incense cedar and western redcedar; 2) moderate (EC_{50} 1500-2100 ppm) – yellow-cedar, western juniper, and Port-Orford-cedar, and 3) weak or no activity (EC_{50} > 10000 ppm) – Douglas-fir and redwood. Since the chemical composition of conifer heartwood can be extremely variable among trees, our ranking of species activities above applies only to the samples used in this study. Confirmation of these species differences will require testing a larger random sample. Four categories of activity were used for the

individual compounds tested: 1) strong (EC_{50} 1-10 ppm) – hinokitiol, thymoquinone; 2) moderate – (EC_{50} 11-100 ppm) nootkatin, nootkatol, carvacrol, valencene-11, 12-diol; 3) weak (EC_{50} 101-1000 ppm) – α-terpineol, valencene-13-ol, taxifolin, β-cedrene, and thujopsene; and 4) no activity (EC_{50} > 1000 ppm) – cedrol, δ-cadinene, and methylcarvacrol. All compounds with strong or moderate antimicrobial activity contained a free hydroxy group, except for thymoquinone. The importance of this functionality is demonstrated by the large difference in activity between carvacrol (EC_{50} 59.8 ppm) and methylcarvacrol (> 1,000 ppm), with a methyl group blocking the hydroxyl.

Major components in the heartwood extracts were distinct for each species, except for a few select compounds. Activities of the heartwood extracts tended to parallel the activities of individual compounds they contained. For example, extracts of incense cedar and western redcedar expressed the strongest antimicrobial activity and they each contained one of the individual compounds with strong activity; thymoquinone in incense cedar and hinokitiol in western redcedar. Yellow-cedar extracts were moderately active and contained numerous compounds that individually were moderate inhibitors (nootkatin, nootkatol, carvacrol, valencene-11, 12-diol, and valencene-13-ol). Western juniper and Port-Orford-cedar extracts also were moderately antimicrobial, but their individual compounds we tested had only weak, or no activity. The chemical constituents in Douglas-fir and redwood extracts were not analyzed further by GC because they lacked activity.

On May 1, two months after placing chips on the forest floor there was no difference between the western redcedar and redwood plots in the amount of *P. ramorum* DNA in litter. However, during the two months 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. At the end of June the redwood control plots had 4.3 times more *P. ramorum* DNA than the western redcedar plots. Soils from redwood and redcedar plots had the same quantities of DNA in May and June. Increases in *P. ramorum* biomass, and DNA, during the spring rains is thought to occur from either spores washing from the surface of infected leaves by rainfall (Davidson and others 2005), or newly germinated chlamydospores in the litter, which give rise to new sporangia and zoospores and a new infection cycle. Redcedar chips were effective in reducing the inoculum potential and future development of new infections.

An integrated management program may be able to utilize bioactive conifer heartwoods in some instances to help limit the proliferation of *P. ramorum* spores in litter and the potential for new infections via splash dispersal (Davidson and others 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 2006, Davidson and others 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 distribution from these areas (Manter and others 2006). Our results here suggest heartwood chips from western redcedar, or incense cedar are as active, and possibly more active toward *P. ramorum* than those of yellow-cedar. Both of these species are abundant and readily attainable in large volumes from western forests. Alternatively, some of the most active individual heartwood compounds might be developed into useful products. It may be worthwhile examining the efficacy of foliar spray applications for some of the most active compounds such as hinokitiol and thymoquinone.

Key words: Photometric bioassay, conifer heartwood, antimicrobial activity.

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