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Ethanol Attracts Scolytid Beetles to *Phytophthora ramorum* Cankers on Coast Live Oak

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Abstract Ethanol in sapwood was analyzed along vertical transects, through small spot cankers and larger basal cankers, of *Phytophthora ramorum*-infected stems of *Quercus agrifolia* at three sites in California. Trees with large basal cankers, known to attract scolytid beetles, had a 4.3 times higher ethanol level than trees with spot cankers that attract fewer beetles. Ethanol concentrations inside cankers, where scolytid beetles preferentially attack, varied by about four orders of magnitude among samples, with a median level of 16.0 μg.g^{-1} fresh mass. This concentration was 4.3 and 15.5 times greater, respectively, than the concentrations at 1 cm or 15–30 cm outside the canker boundaries. In the laboratory, we demonstrated that ethanol escaped through the bark of a *Q. garryana* log just 3 days after it was added to the sapwood. At the three study sites, traps baited with ethanol captured more *Xyleborinus saxesenii*, *Pseudopityophthorus pubipennis,* and *Monarthrum dentiger* (all Coleoptera: Curculionidae: Scolytinae) than traps baited with ethanol plus (−)-α-pinene, or ethanol plus 4-allylanisole (4AA). Logs of *Q. agrifolia* with a 50 % ethanol solution added to the sapwood were placed at the study sites, with or without additional bark treatments above the ethanol. The number of scolytid beetle gallery holes above the ethanol-infused sapwood was 4.4 times greater than that on the opposite side of the log where no ethanol was added. Attachment of ultra-high release (−)-α-pinene pouches to the bark surface above the 50 % ethanol solution reduced scolytid attacks to a density of 19.1 % that of logs without this treatment. We conclude that ethanol in *P. ramorum* cankers functions as a primary host attractant for scolytid beetles and is an important link in colonization of these cankers and accelerated mortality of *Q. agrifolia*. The results of this research shed light on the chemical ecology behind the focused scolytid attacks on *P. ramorum*-infected coast live oaks, and lay the groundwork for future efforts to prolong the survival of individual trees of this keystone species.

Keywords *Quercus agrifolia* · Ambrosia beetles · Bark beetles · Deterrents · (−)-α-Pinene · 4-Allyanisole · Ethanol · *Phytophthora ramorum* · Sudden oak death

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Introduction

Phytophthora ramorum Werres, De Cock & Man in't Veld, the causal agent of sudden oak death, is an invasive oomycete pathogen that has killed thousands of oak and tanoak trees in California (Meentemeyer et al., 2004; Grünwald et al., 2012). Millions of susceptible trees are at risk in California, Oregon, and the southeastern U.S. (Meentemeyer et al., 2004; Kelly et al., 2006). Successful infection of coast live oak (*Quercus agrifolia* Nee) stems by *P. ramorum* results in formation of a canker initially characterized at the bark surface by the release of a dark red-brown or black-colored necrotic exudate (Garbelotto et al., 2001; Rizzo et al., 2002; McPerson et al., 2005; Brown and Brasier, 2007). Appearance of the "bleeding" exudate from bark has been observed in some trees as early as 3 weeks after artificial inoculations (Rizzo et al., 2002), although periods of 14–70 weeks are probably more typical (Rizzo et al., 2002; McPherson et al., 2008). Canker formation usually is followed by focused attacks of bark and ambrosia beetles (Coleoptera: Curculionidae:Scolytinae) and subsequent growth of black fruiting bodies from the sapwood-rotting ascomycete, *Annulohypoxylon thouarsianum* (Lév.) Y.-M. Ju, J.D. Rogers & H.-M. Hsieh (Garbelotto et al., 2001; Rizzo et al., 2002; McPherson et al., 2008; Hsieh et al., 2005).

Scolytid beetles are attracted to *P. ramorum* cankers on *Q. agrifolia*, as shown by their attacks, almost always targeting within canker margins (Rizzo et al., 2002). In Marin Co., CA, *Monarthrum scutellare* (LeConte), *Xyleborinus saxesenii* (Ratzeburg), and *Pseudopityophthorus pubipennis* (LeConte) are the most common scolytids trapped on the bark surface of *Q. agrifolia* near *P. ramorum* cankers created by wound inoculations (McPherson et al., 2008). These beetles typically are nonaggressive, restricting their attacks and colonization to severely stressed, dying, or recently dead trees, and freshly broken branches or stumps (Furniss and Carolin, 1977). Thus, their initial preference for disease-stressed tissue within the canker boundary is consistent with their colonization of dying host materials. While scolytid attacks prior to the onset of bleeding have been noted (McPherson et al., 2008), the appearance of this exudate does not necessarily result in immediate attacks; McPerson et al. (2005) found 42–58 % of naturally infected symptomatic trees to be free of attack over a four-year period. When trees were artificially inoculated with *P. ramorum,* about 50 % of those with bleeding cankers remained free from beetle attacks 17 months post-infection (McPherson et al., 2008).

Once beetle attacks occur on *P. ramorum*-infected *Q. agrifolia*, the probability of survival of the tree decreases from 8 to 12 years to 3 years or less, depending on the site (McPherson et al., 2010). Ambrosia beetle galleries compromise a tree's protective outer bark and penetrate deep into the sapwood (Švihra and Kelly, 2004; McPerson et al., 2005; Swiecki et al., 2006), allowing access to decay fungi (Garbelotto et al., 2001; Swiecki et al., 2006; McPherson et al., 2008), which may be vectored by beetles moving back and forth to discard frass and boring dust at the gallery entrance. Environmental conditions in galleries appear ideal for fungal growth and wood decay, which, alone or in combination with the ambrosia beetle tunnels weakens the stems and branches and makes them prone to breakage in live or dead trees (Švihra and Kelly, 2004; McPerson et al., 2005, 2008; Swiecki et al., 2006). The growth of *Annulohypoxylon*, in combination with beetle attacks, can contribute to stem fracturing, but the presence of both organisms does not further decrease a tree's probability of survival compared to those attacked only by beetles (McPerson et al., 2005, 2010; Swiecki et al., 2006).

Ethanol recently was reported as a host attractant for *M. scutellare* (Noseworthy et al., 2012); *Xyleborinus saxesenii* is also often captured in traps baited only with ethanol (Klimetzek et al., 1986; Oliver and Mannion, 2001; Coyle et al., 2005; Miller and Rabaglia, 2009; Ranger et al., 2011). Ethanol has not been shown to attract P. *pubipennis*, but it does attract other congeners (Dunn and Potter, 1991; Coyle et al., 2005). Elevated ethanol concentrations often occur in tissues of hardwoods and conifers when they are subjected to stresses of various types (Kimmerer and Kozlowski, 1982), including pathogen infection (Gara et al., 1993; Kelsey and Joseph, 1998). Therefore, we hypothesized that ethanol functions as a host attractant that causes scolytids to select and colonize cankers caused by *P. ramorum*. Once an attack begins, ethanol also may synergistically enhance attraction to pheromones released by the beetles, volatiles from the host tree, or both (Pitman et al., 1975; Borden et al., 1980), which would further strengthen the attraction of beetles to the canker.

If scolytid beetle attacks to *P. ramorum* cankers were minimized, the life span of diseased *Q. agrifolia* might be extended by 5–8 years or more (McPherson et al., 2010). Spraying *P. ramorum*-infected *Q. agrifolia* with insecticide prior to the principal flight periods of various scolytids prevents the first beetle attack by about 2 months, reduces the number of attacks per stem, and decreases mortality of the treated oak (McPherson et al., 2008). It also can slow subsequent wood decay rates (Švihra and Kelly, 2004). Keeping infected coast live oak alive longer will not jeopardize the health of nearby trees, as there is no evidence that *P. ramorum* can sporulate on *Q. agrifolia* (Davidson et al., 2005). Extending the life span of *P. ramorum*-infected *Q. agrifolia* trees would help prolong their ecological function as an important food source, especially acorns, and as a critical habitat for numerous species of vertebrates and arthropods (Tempel et al., 2006; Monahan and Koenig, 2006; Winslow and Tietje, 2006). Slowing oak mortality also would reduce the rate of surface fuel accumulation to fuel wildfires (Valachovic et al., 2011).

Although insecticides can help manage beetle attacks on *P. ramorum* cankers (Švihra and Kelly, 2004; McPherson et al., 2008), more environmentally friendly or less costly alternatives might also work equally well or better. For example, while the attraction of many scolytid species to ethanol plus α-pinene, is additively or synergistically increased compared to traps baited with either compound alone (Borden et al., 1980; Schroeder and Lindelöw, 1989; Byers, 1992; Miller and Rabaglia, 2009; Ranger et al., 2011), there are some beetle species, including *X. saxesenii,* that are deterred by this combination (Miller and Rabaglia, 2009; Ranger et al., 2011). 4-Allylanisole (4AA) is another conifer oleoresin component that deters attraction of some scolytid beetles to traps baited with ethanol plus a (1:1) α-:β-pinene mixture (Joseph et al., 2001). To our knowledge, no studies have yet tested the efficacy of these semiochemicals at preventing scolytid attacks to *P. ramorum*-infected trees.

The objectives of this study were: 1) determine whether the stem tissues of *Q. agrifolia* infected with *P. ramorum* contain higher ethanol concentrations than healthy tissues; and, 2) if this were confirmed, demonstrate that the scolytid species in California oak woodlands that typically attack *P. ramorum* cankers are attracted to ethanol; 3) determine whether the combined release of (−)-α-pinene or 4AA with ethanol can substantially reduce attraction of these species over that to ethanol alone; and 4) evaluate whether the application of an antitranspirant on the bark surface of logs containing ethanol blocks the release of ethanol sufficiently to inhibit attraction of scolytid beetles.

Methods and Materials

Measuring Ethanol Concentrations in Diseased and Healthy Q. agrifolia Fourteen pairs of putatively diseased and healthy *Q. agrifolia* were chosen for sampling at three sites in California where *P. ramorum* had been previously confirmed (Table 1). Selection criteria for diseased trees included one or more bleeding canker on the stem (with or without beetle attacks), no evidence of *Annulohypoxylon* fruiting bodies on the bark, and a healthy tree of comparable diameter within 30 m or less.

Sapwood cores (2.0×0.5 cm) were sampled in September 2010 from two types of cankers: small spot cankers surrounded on all sides by apparently healthy tissue (N=9), and larger basal cankers that extended from the forest floor to the upper canker boundary, with no healthy tissue to sample below (N=6). One tree had both types. A vertical strip of surface bark that ran through the spot cankers was removed to define the upper and lower margins, as was a strip from the upper boundary of basal cankers, extending 30 cm or more below. Canker size was estimated by measuring the vertical length from top to bottom edges of spot cankers at the transect position and from top edge to forest floor for basal cankers. Sapwood cores were extracted with an increment borer at nine points along spot canker transects: three inside (center and 1 cm from the top and bottom margins) and six outside (1, 15, and 30 cm above and below the edges) a canker. Basal cankers were sampled at six points along a transect: three inside (1, 15, and 30 cm below the top edge) and three outside (1, 15, and 30 cm above the top border of) the canker. Each healthy tree

Table 1 The locations of our *Phytophthora ramorum*-infested study sites in California, number of trees sampled for ethanol, and various environmental parameters associated with beetle traps at each site

Parameters[a]	Fairfield Osborne	Mt. Burdell	Rush Creek
Location (Lat. and Long.)	38°20′28.27″N	38°7′58.72″N	38°7′42.25″N
	122°35′43.34″W	122°36′28.77″W	122°32′53.79″W
	Sonoma County	Marin County	Marin County
No. tree pairs sampled for ethanol analysis (September 2010 sample date)	3 (6)	9 (7–8)	2 (8)
Trap opening distance above forest floor (cm)	90.0 (±13.3)	84.1 (±24.4)	94.1 (±20.4)
Trap elevation ranges (m)	485–549	183–202	12–55
Trap aspect (degrees)	232.2 (±16.9)[a]	87.5 (±32.0)	100.0 (±43.7)
Slope at traps (degrees)	16.3 (±9.4)	15.5 (±9.8)	12.6 (±8.6)
Trap distance from nearest tree (m)	2.8 (±1.7)	1.9 (±0.8)	2.8 (±1.7)
Tree species ≥ 15 cm nearest to trap (frequency)			
Quercus agrifolia	0.519	0.777	0.926
Umbellularia californica (Hook & Arn.) Nutt.	0.444	0.148	0.074
Acer macrophyllum Pursh	0.037	–	–
Q. kelloggii Newberry	–	0.074	–
Most abundant tree species around traps (frequency)			
Q. agrifolia	0.333	0.481	0.481
U. californica	0.444	0.519	0.481
Q. kelloggii	0.037	–	–
Pseudotsuga menziesii (Mirb.) Franco	0.037	–	–
U. californica,Q. agrifolia	0.037	–	–
U. californica,P. menziesii	0.037	–	–
Q. agrifolia,P. menziesii	0.074	–	–
Q. agrifolia, Olea europaea L.	–	–	0.037

Oakmapper (www.oakmapper.org/oaks/index) shows that *P. ramorum* had been confirmed at Fairfield Osborn in 2000 and Mt. Burdell in 2004, while symptomatic trees were observed at Rush Creek in 2001 and confirmed in litter and soil samples in 2006 (Manter et al., 2007)

[a] Values are means (±SD), except for elevation ranges and tree species frequencies, for 27 traps at each site

was sampled at the same distances from the forest floor as its paired diseased tree.

Each core was placed immediately into a pre-weighed 20 ml headspace vial, which was sealed with a septum and screw cap (Agilent Technologies, Santa Clara, CA, USA), and then frozen on dry ice. At day's end, the vials were thawed and heated to 100 °C for 60 min to kill the pathogen and stop enzyme activity. Vials were reweighed to determine fresh tissue mass and stored at ambient temperature until analyzed. Ethanol concentrations were measured by static headspace gas chromatography using a PerkinElmer Turbomatrix 110 headspace autosampler connected to a PerkinElmer Autosystem XL gas chromatograph (GC) fitted with a DBWAX column (30 m×0.32 mm i.d., 0.25 μm film; J&W Scientific-Agilent) and flame ionization detector. The GC injector and detector were set at 200 °C and 250 °C, respectively. The column oven was held at 50 °C for 2. 4 min, and then increased to 120 °C at 45 °C.min^{-1} with no hold. The oven then was returned to 50 °C at 45 °C. min^{-1}, at which it remained for a total run time of 5.96 min. Helium was the carrier gas, with an initial flow of 2.0 ml. min^{-1} for 3.0 min, and then increased to 4.0 ml.min^{-1} with a 2.0 min hold. Temperature settings on the headspace autosampler were 110 °C for the transfer line, 90 °C for the needle, and 70 °C for the vial oven. Vials were heated 40 min with a 4.0 min pre-injection pressurization, 0.04 min injection, and 0.1 min needle withdrawal time. The autosampler helium carrier pressure was 122.7 kPa.

Sapwood ethanol concentrations were determined by the external standard method, using a four-level curve (vials with 5 μl of aqueous ethanol solution, prepared with deionized water and 100 % ethanol, Pharmco-Aaper, Shelbyville, KY, USA) analyzed with each set of samples. Concentrations were normalized to μg of ethanol.g^{-1} fresh mass, rather than dry mass, as this more accurately represents ethanol levels in water throughout the tissue.

All statistical analyses in this study were conducted with SAS ver. 9.2 (2008). Diseased tree sapwood ethanol concentrations from the three sites were analyzed as a completely random split-plot design. Canker type (spot or basal) was the whole plot, and the three within-tree sampling positions the split plot: 1) 15–30 cm outside the canker, averaged above and below for spot cankers; 2) 1 cm outside the canker, averaged above and below for spot cankers; and 3) all inside canker values averaged. Values were natural log (μg ethanol+0.001) transformed to assure normality of residuals and homogeneous variances, and analyzed using a mixed effects ANOVA (SAS PROC MIXED) to model both whole and subplot error, with site analyzed as a fixed effect. Because the data were unbalanced, the Kenward-Roger method was used for calculating degrees of freedom (Littel et al., 2006). Least squares means were compared by the protected (model α=0.05) Fisher's Least Significant Difference test, with P values≤0.05 considered significant. The means and 95 % confidence intervals (CIs) were back-transformed to medians for presentation. The ethanol measured in paired healthy trees was not included in the statistical analysis. Mean spot and basal canker sizes were compared by ANOVA.

Confirming the Presence of P. ramorum in Q. agrifolia Cankers Immediately after collecting cores from cankered trees in September 2010, a sample of phloem at the canker boundary was excised and sealed in a plastic bag. In the laboratory, small sections of this tissue were embedded in petri plates of PARP (cornmeal agar with pimaricin, ampicillin, rifampicin, and pentachloronitrobenzene), a *Phytophthora*-selective media (Erwin and Ribeiro, 1996). The plates were stored in the dark at 18 °C and examined under a microscope from days 4–21 for growth of *P. ramorum,* identified by the characteristic chlamydospore morphology. Cankered trees were all resampled again in June 2011 and processed as described above.

Quantifying Ethanol Release from Q. garrayana Dougl. ex Hook Sapwood An Oregon white oak log (65×35 cm, l×w), with no visual signs of decay, was cut 23 February 2011 from a tree blown over in January 2011 near Corvallis, OR (44°34′47.93″N, 123°20′39.95″ W; ele. 228 m), and stored outside until 9 March. In the laboratory, cut ends were sealed with two coats of the antitranspirant Moisturin (undiluted; GSI Horticultural, Bend, OR, USA) to reduce water loss. With the cut surface upright, 5 holes (1.0× 30 cm) were drilled into the sapwood with centers 1.5 cm inside the sapwood-cambium boundary and 2.5 cm apart around the circumference. A wire-wrapped absorbent rayon wick (250×3.2 mm, folded lengthwise) was inserted into each hole, followed by 10 ml of a 50 % aqueous ethanol solution and a rubber plug.

Ethanol vaporizing through the bark was trapped in 20 ml headspace autosampler vials (13 mm opening, Agilent Technologies) containing 0.1000 to 0.1005 g of activated charcoal (20–60 mesh; Sigma-Aldrich, St. Louis, MO, USA), held against the vial bottom with a 13 mm diam. nylon mesh cloth. These traps were attached by pieces of closed cell plank foam (4×4×2 cm, Uline, Pleasant Prairie, WI, USA), with a center hole cut with a no. 6 cork borer, glued to the bark surface above the ethanol infused sapwood. Two traps were attached over natural fissures, where the bark is thinner, and the third was attached over a thicker bark plate. Traps remained in place for 2 hr, 3, 6, 8, 10, 13, 15, 17, and 29 d after adding ethanol to the sapwood. They were then sealed with a PTFE-lined silicone septum. After 5 min, 40 μl of *n*-butanol were added to help desorb ethanol.

Trapped ethanol was analyzed by a static headspace method, using the same instruments and settings described for the sapwood analysis, except that the autosampler needle temperature was 102 °C, and the vial oven 100 °C (for 30 min). Ethanol standards were prepared by adding 5 μl of an aqueous standard solution to vials containing charcoal and sealing immediately. The vials were shaken and allowed to sit 5 min before adding 40 μl of *n*-butanol. Duplicate vials of a three-level standard curve were analyzed with day 6 samples, and duplicate vials of the mid-level standard were analyzed on all other days to validate instrument calibration. The coefficient of variation for the mid-level ethanol standard across all analysis dates was 1.9 %.

Evaluating Beetle Primary Attraction to Ethanol and Efficacy of Two Semiochemical Repellents The three lures tested were ethanol, ethanol plus (−)-α-pinene, and ethanol plus 4AA, set up as a blocked design with each of the three sites from September 2010 considered a block (Table 1). Within sites, Japanese beetle traps (Trece Inc. Adair, OK, USA) were positioned 30 m apart along a linear transect. To ensure even treatment distribution, the three lure types were randomly assigned within each consecutive group of three traps, and replicated nine times per site. The traps were attached to metal rods with their funnel base 84–94 cm above the forest floor (Table 1), where basal cankers are generally located on oak stems.

The ethanol (120 g ultrahigh release pouch) and (−)-α-pinene (75 % purity, 170 g ultrahigh release pouch) lures were both obtained from Synergy Semiochemical Corp., Burnaby, BC, Canada, and the 4AA (98 %) was from Sigma-Aldrich. The pouch field-release rates range from 0.5 to 1.0 g.d^{-1} at a mean daily temperature of 20–30 °C for ethanol, or 7–13 °C for (−)-α-pinene (data from Synergy Semiochemical Corp.). The 4AA (5 ml) was released from a Nalgene narrow mouth bottle (8 ml capacity) with a rayon cloth wick (7.5×1.0 cm) extending 2.0 cm outside the cap. The 4AA release rates from three vials at Mt. Burdell and Fairfield Osborne Preserve and two at Rush Creek averaged 247.1 (±8.5 SE) mg.d^{-1}, from 18 to 25 August 2011, at a mean daily temperature of 17–21 °C (from the Petaluma Airport, 10–15 km away).

Lures were attached to the outside edge of trap funnels on 19 April at the Fairfield Osborn Preserve and 20 April 2011 at Mt. Burdell and Rush Creek. If two lures were present, they were attached on the same side of the trap next to one another. Diluted ethylene glycol (50 % aqueous) was added to the catch cups to preserve beetles and minimize losses to predators (Miller and Duerr, 2008). Trap contents were collected and antifreeze refreshed periodically, until 12 November at the Fairfield Osborne Preserve and 13 November 2011 at the other two sites, for a total of 207 trap days at each site. The (−)-α-pinene pouches were replaced once on 18 August, and the 4AA bottles were refilled at two-weekly intervals, with their wicks replaced on 25 August. Ethanol pouches were not refreshed, but still contained liquid when the experiment ended. After each trap collection, the insects were recovered from the antifreeze by suction filtration and stored in 95 % ethanol for cleaning, sorting, and counting at a later date.

The total number of beetles caught over the entire trapping period was summed for each species and for total scolytids. Treatment effects were analyzed with generalized linear models (PROC GENMOD), using the log link function and keeping block as a fixed effect. All responses were initially modeled assuming a Poisson distribution of count data using the deviance scale dispersion parameter to adjust for over dispersion caused by excess zeroes. When over dispersion was present, the same model was fit using a negative binomial distribution. The deviance and dispersion parameters were checked to assure model fit. All means comparisons were protected by conducting tests only when the ANOVA model indicated a difference ($\alpha = 0.05$). The least squares means and 95 % confidence intervals were back-transformed for presentation. Counts of *Gnathotrichus pilosus* (LeConte) and the *Ips* spp. were not analyzed because of low numbers.

Assessing Beetle Attacks to Q. agrifolia Logs with Ethanol-Infused Sapwood, With or Without Semiochemical Repellants on the Bark Ten, apparently healthy, *Q. agrifolia* trees were cut by commercial arborists between March 22 and May 13, 2011 in Marin, Napa, and Sonoma Counties. Six logs were collected from each tree on the same day, or no more than 10 d after cutting (60 total, mean l×w of 67.1×24.8 cm), and the ends covered with two coats of Anchorseal® (U.C Coatings Corporation, Buffalo, NY, USA) to slow water loss. Any damaged bark (minor saw cuts, cracks, etc.) was sealed with an acrylic latex caulk with silicone (DAP Products Inc., Baltimore, MD, USA), and the logs stored outside under cover to protect them from sunlight and rainfall.

A field experiment was set up as a randomized complete block design with 10 blocks, each with 6 logs from an individual tree, allowing six treatments per block. Five blocks of logs, spaced≥10 m apart, were positioned and prepared for treatment at Mt. Burdell and Rush Creek on 24 and 25 May 2011, respectively. Logs within blocks were linearly separated ≥ 10 m apart under the canopy of adjacent trees, with some direct sun for varying periods of the day. Five holes were drilled vertically into the sapwood on the top cut surface, as described above for *Q. garryana*, and plugged; mean bark thickness was 16.6 and 22.0 mm at the thinnest and thickest points covering the sapwood. The outer bark surface above the sapwood holes was brushed clean and a rectangular area (32.0 cm down from the top and about 17 cm wide, extending 2.0 cm beyond first and last hole) marked for subsequent treatment.

Log treatments within blocks were randomly assigned and applied at both sites on 26 May. There were six treatment combinations of ethanol (E), (−)-α-pinene (P), or the antitranspirant Moisturin (M). Ethanol was added to each sapwood hole by inserting a cloth wick (as above for *Q. garryana*) soaked with a 50 % aqueous solution (diluted 100 %, KOPTEC, King of Prussia, PA, USA). The hole then was sealed with a rubber stopper. Bark treatments were (−)-α-pinene spray (SP, 98 %, Sigma-Aldrich), ultrahigh release pouch (PP, 75 % purity, 170 g, the same used for beetle traps), or Moisturin as a 50 % aqueous spray solution. The six treatments were: 1) E, 2) E+M, 3) E+PP, 4) E+PP+M, 5) E+PS, and 6) E+PS+M. The PS was applied to saturation over the marked treatment area with a hand held spray bottle, then reapplied when the bark no longer appeared wet (10–20 min.). The M also was sprayed to saturation, allowed to dry until tacky (about 30 min), and sprayed again. In the E+PS+M treatment, M was applied last, covering the PS. The PP ultrahigh release (−)-α-pinene pouches were stapled to the bottom edge of the treatment areas. To avoid ethanol depletion, five ml of a 50 % solution were added to each hole on 2, 9, 16, 30 June and 7, 14 July 2011.

The experiment was terminated 21 July 2011, 8 weeks after initiation. The number of scolytid beetle attacks, or gallery entrance holes, was counted as a group in the treatment area because of their similar appearance. Gallery holes for *Scobicia declivis* (Coleoptera: Bostrichidae) had a larger diameter and were counted separately. Observations of the E logs indicated higher numbers of attacks within or near the marked ethanol-infused sapwood compared to other log positions. To confirm this difference, a second area of bark, comparable in size to the E-infused zone, was marked directly across on the opposite side of the log and the gallery holes counted.

The influence of M, PP, or SP treatments on beetle attacks was modeled as for the beetle trapping data using generalized linear models (PROC GENMOD) with block as a fixed effect. The best-fitting model was the negative binomial distribution; however, there was still evidence of overdispersion (deviance =1.57) that was corrected with the deviance scale option to build more conservative confidence intervals. Contrast statements were used to compare the counts of gallery entrance holes for logs with, against those without, treatments of M, SP, or PP. Another contrast also was used to compare the number of gallery holes for logs treated with PP vs. SP. For logs treated with E only, the number of beetle gallery holes in the area above the ethanol-infused sapwood was compared with the number in a similar area on the opposite side of the log without E in the underlying sapwood, using a paired *t*-test with the TTEST procedure.

Results

Ethanol Concentrations in Diseased and Healthy Q. agrifolia The quantity of ethanol in sapwood from *Q. agrifolia* trees infected with *P. ramorum* was dependent on type of canker (Fig. 1; $F_{1,\ 13.4}$=4.92; P=0.044) and location of the sapwood relative to the canker boundary (Fig. 2; $F_{2,\ 25.7}$=13.28; P<0.001), but not their interaction ($F_{2,\ 25.7}$=0.58; P=0.568). Sapwood from trees with large basal cankers had 4.3 times more ethanol than trees with spot cankers (Fig. 1; $t_{1,\ 13.4}$=2.22; P=0.044, 95 % CI=1.04–17.80 times). Ethanol concentrations inside cankers ranged from 0.3 to 1036.1 μg.g^{-1} fresh mass, with a median level 4.3 times greater than sapwood 1 cm outside the canker boundary ($t_{2,\ 25.3}$=2.85; P=0.009, 95 % CI=1.5–12.5 times), and 15.5 times greater than sapwood 15–30 cm outside the canker boundary ($t_{2,\ 25.9}$=5.13; P<0.001, 95 % CI=5.2–46.7 times). The median concentration 1 cm outside the canker boundary was 3.6 times greater than that of tissues sampled 15–30 cm from the canker (Fig. 2; $t_{2,\ 25.9}$=2.39; P=0.024, 95 % CI=1.2–10.8 times). Ethanol concentrations in sapwood from the healthy control trees ranged from 0 to 0.65 μg.g^{-1} fresh mass across all sample points from all trees, with a mean of 0.27 (±0.02 SE) μg.g^{-1} fresh mass. The mean vertical length of spot cankers at the transect position (33.4 cm, ±30.7, 95 % CI) was smaller than the vertical length of basal cankers (109.5±37.7, 95 % CI; $F_{1,\ 13}$=11.42; P=0.005). The average breast height diameter of the diseased trees was 46.7 cm (±8.5, 95 % CI), while that of healthy trees was 43.9 cm (±8.2, 95 % CI).

Confirming the Presence of P. ramorum in Cankers The presence of *P. ramorum* was confirmed in phloem samples from three of the diseased trees (all from Mt. Burdell), one with a spot canker and the other two with basal cankers. Resampling cankers in June 2011 was unsuccessful in further isolating *P. ramorum*. Standard isolation procedures may not be the best technique for detecting *P. ramorum* in *Q. agrifolia* phloem (Hayden et al., 2004). However, the presence of *P. ramorum* had been previously established at each site (Table 1), and there was mortality and stem fracturing of other trees in these areas, consistent with symptoms described for

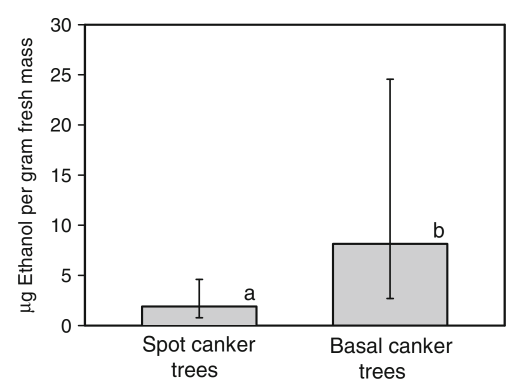

Fig. 1 Median (± 95 % CI) sapwood ethanol concentrations in trees with large basal cankers (N=6) and small spot cankers (N=9). Medians are different at P=0.044

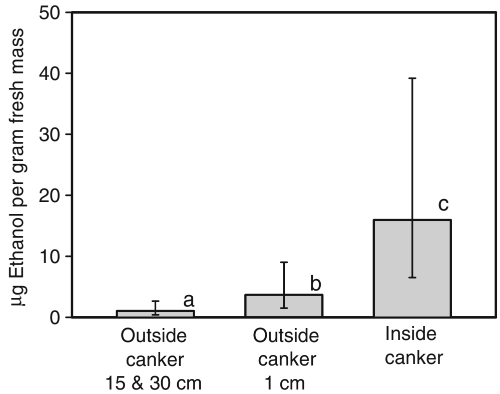

Fig. 2 Median (± 95 % CI) sapwood ethanol concentrations inside and outside cankers of all types. Medians with different letters are different at $P \leq 0.05$

this pathogen (Garbelotto et al., 2001; Rizzo et al., 2002; McPherson et al., 2008).

Ethanol Movement from Q. garryana Sapwood to the Atmosphere It took 3 days for ethanol to move radially from sapwood to the atmosphere under laboratory conditions, with the fastest release rates over fissures where the bark was thinnest (Fig. 3). The release rates increased until about day 15, when they ranged from 0.14 to 0.80 mg.d^{-1}, depending on bark thickness, and remained near this level through to day 29.

Scolytid Beetle Responses to Trap Lures A total of 1333 scolytid beetles were captured in the funnel traps at the three study sites over the entire trapping period (207 trap days). *Xyleborinus saxesenii*, an ambrosia beetle, was the most common scolytid trapped, followed by *Pseudopityophthorus pubipennis*, a bark beetle species, and *Monarthrum scutellare* and *M. dentiger*, both ambrosia beetles (Table 2). Small numbers of *Xyleborus xylographus* (Say), and *Gnathotrichus*

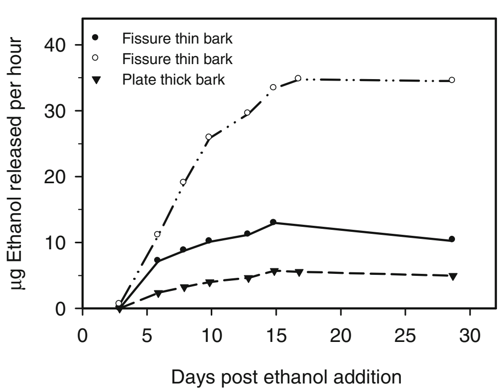

Fig. 3 Hourly ethanol release rates from a *Quercus garryana* log in the laboratory after infusing the sapwood with a 50 % ethanol solution. Three charcoal traps were used to measure ethanol release rates: two were positioned over fissures where the bark was thinner and one over a thick bark plate

pilosus (LeConte), both ambrosia beetles, and one *Ips* bark beetle also were trapped, but not analyzed statistically. All counts, with the exception of *M. dentiger,* were modeled using a negative binomial distribution due to over dispersion of data. The Poisson distribution model was used in the analysis of *M. dentiger*, because this species had relatively low trap counts and no evidence of over dispersion. Differences in beetle numbers among sites (Table 2) were detected for total scolytids, *X. saxesenii*, and *P. pubipennis* (all $P \leq 0.026$), but not for *M. scutellare* or *M. dentiger* (both $P \geq 0.070$), with no further comparisons tested.

Traps baited with ethanol captured greater numbers of total scolytid beetles, *X. saxesenii*, and *M. dentiger* than traps in which ethanol was combined with (−)-α-pinene or 4AA (Fig. 4, see Table 3 for *P* values). Traps baited with ethanol also captured more *P. pubipennis* than those with ethanol plus (−)-α-pinene, but not for traps with ethanol plus 4AA. Traps baited with ethanol plus (−)-α-pinene tended to capture fewer numbers of scolytid beetles than did those with ethanol plus 4AA, but none of the differences were significant (Fig. 4, *P* values in Table 3).

In addition to the targeted scolytid beetles, we also captured large numbers of *Scobicia declivis* in traps with ethanol, an occurrence consistent with previous observations (Burke et al., 1922). Although *S. declivis* comprised 5.7 % of the beetle catch on *P. ramorum*-inoculated trees (McPherson et al., 2008), the role of this species in colonization and mortality of *Q. agrifolia* appears to be minor relative to that of scolytid beetles, and will be reported elsewhere. Additionally, two click beetles (Coleoptera: Elateridae), *Limonius ornatulus* (LeConte) and *Dolerosomus debilis* (LeConte), and the fire-colored beetles (Coleopotera: Pyrochroidae) *Pedilus* spp. were captured in high numbers in traps with ethanol plus 4AA.

Table 2 Trap catch totals of scolytid bark and ambrosia beetles at three study sites in California, 2011

Scolytid species	Sites			Totals	% of total
	Fairfield Osborne	Mt. Burdell	Rush Creek		
Gnathotrichus pilosus	0	4	4	8	0.6
Ips spp.[a]	1	0	0	1	0.1
Monarthrum dentiger	10	12	4	26	2.0
Monarthrum scutellare	81	96	38	215	16.1
Pseudopityophthorus pubipennis[a]	74	161	125	360	27.0
Xyleborinus saxesenii[b]	489	131	88	708	53.1
Xyleborus xylographus	11	2	2	15	1.1
Total scolytid beetles	666	406	261	1333	100.0

[a] Bark beetle

[b] Exotic species

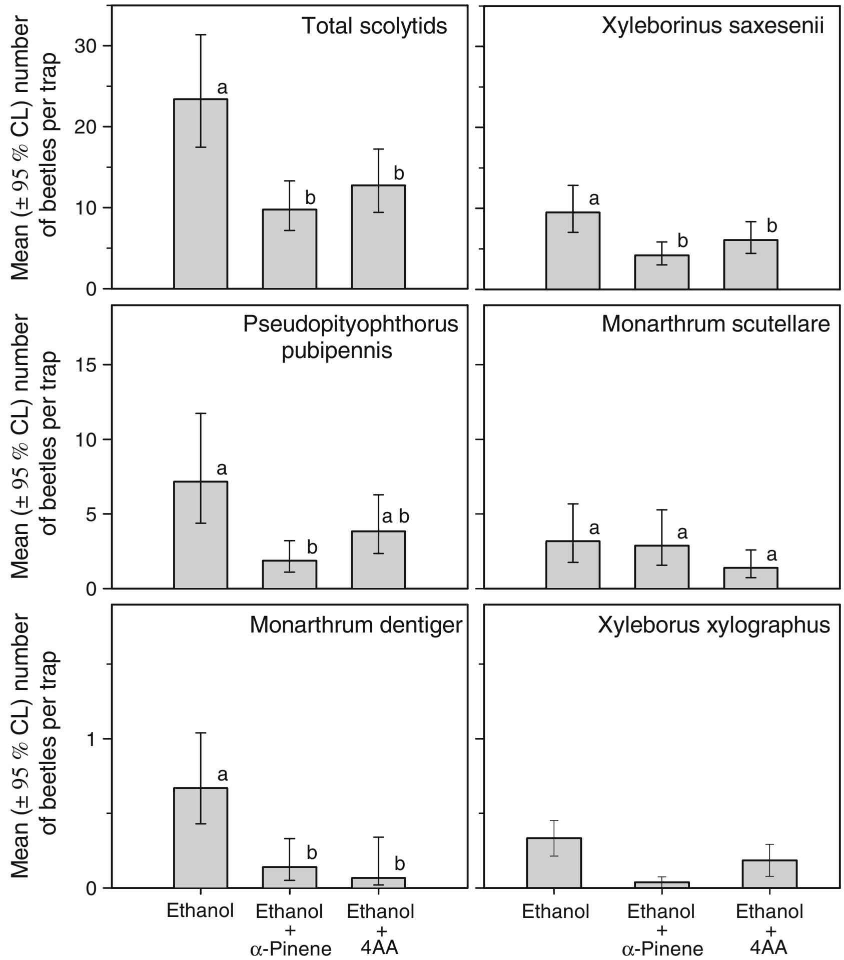

Fig. 4 Mean (± 95 % CI) total trap catches for three lure types (N=27) across three study sites in California. The traps were deployed from April to November 2011, for a total of 207 d. Counts of *Xyleborus xylographus* (± SE) were not statistically analyzed because of low numbers. Means with different letters are different at P values listed in Table 3 for each species

Because these species are not associated with *P. ramorum* cankers, these results also will be reported elsewhere.

Beetle Responses to Oak Logs Containing Sapwood Ethanol and Semiochemical Repellants on the Overlaying Bark Logs treated with only ethanol had 4.4 (95 % CI 1.8–7.0) times more scolytid beetle gallery holes in bark covering ethanol-infused sapwood than did bark on the opposite side of the log, where the sapwood had no added ethanol (Fig. 5, t_9=2.96; P=0.016).

Table 3 P values for comparisons of means in Fig. 4, showing numbers of beetles caught in traps with three trap lures across three California study sites

Species	Trap lures compared		
	EtOH vs α-Pinene	EtOH vs 4AA	α-Pinene vs 4AA
Monarthrum scutellare[a]	–	–	–
Monarthrum dentiger	0.001	< 0.001	0.384
Pseudopityophthorus pubipennis	0.001	0.084	0.052
Xyleborinus saxesenii	< 0.001	0.047	0.112
Total scolytids	< 0.001	0.005	0.232

[a] Model treatment P=0.143

The scolytid beetle attacks for the complete group of bark-treated *Q. agrifolia* logs (Fig. 6) did not show a site by treatment interaction (χ^2_5=5.20; P=0.393), but there were treatment differences (χ^2_5=17.90; P=0.003). Attachment of a single pouch of (−)-α-pinene (PP) to the bark at the base of the ethanol-treated area reduced the mean scolytid beetle attacks to 19.1 % (95 % CI=6.97–52.52 %) that of the attacks on all other treatments without a pouch (χ^2_1=10.30; P= 0.001). Mean scolytid beetle attacks on PP-treated logs were 14.2 % (95 % CI=4.98–40.26 %) that of attacks on logs treated with the (−)-α-pinene spray (SP, χ^2_1=13.45; P< 0.001). One-time application of Moisturin (M) to the bark covering the ethanol-treated sapwood had no impact, with these logs having 68.4 % (95 % CI=29.10–160.86 %, χ^2_1=0.76; P=0.384) of the attacks that occurred on logs without this treatment. Similarly, the (−)-α-pinene spray was not effective at deterring beetles, with these logs having 135.1 %, (95 % CI=48.90–373.03 %, χ^2_1=0.34; P=0.562) of the attacks observed on logs without this treatment.

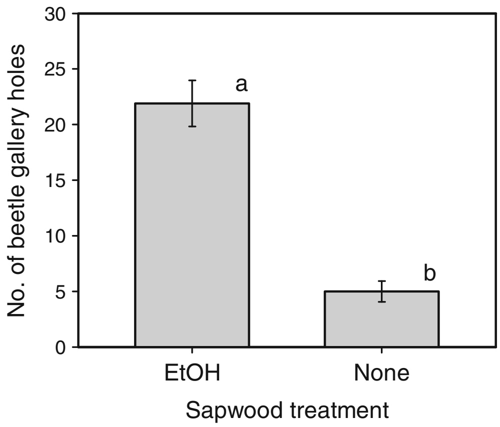

Fig. 5 Mean (± SE) numbers of scolytid beetle gallery entrance holes in *Quercus agrifolia* logs (N=10) within a marked area over the ethanol-infused sapwood, and from a comparable area on the opposite side of the log with no added ethanol in the underlying sapwood. There was no treatment applied to the bark surface. These means are different at P=0.016

Discussion

Ethanol is synthesized, and accumulates, within the boundaries of *P. ramorum* cankers on *Q. agrifolia* stems. Based on several lines of evidence, we believe that this ethanol functions as a primary host attractant for scolytid beetles to attack diseased trees. First, insect traps baited with ethanol caught the greatest number of beetles out of all the attractants we tested. The suite of beetle species captured in these traps was similar to that reported by McPherson et al. (2008) as landing on *Q. agrifolia* trees with *P. ramorum* cankers created by artificial inoculations. Of the total scolytids captured, our traps caught 53.1 % *X. saxesenii,* 27.0 % *P. pubipennis,* and 16.1 % *M. scutellare*, compared to the 29.0 %, 27.3 %, and 31.1 % of these species, respectively, reported in McPherson et al. (2008). Ethanol attraction of *X. saxesenii* has been observed in Europe and many locations in the U.S. (Klimetzek et al., 1986; Oliver and Mannion, 2001; Coyle et al., 2005; Miller and Rabaglia, 2009; Ranger et al., 2011). Ethanol recently was reported as a host attractant for *M. scutellare* (Noseworthy et al., 2012). Our results may be the first report of ethanol as an attractant for *M. dentiger* and *P. pubipennis,* although other species in the genus *Pseudopityophthorus* respond to this compound (Dunn and Potter, 1991; Oliver and Mannion, 2001; Coyle et al., 2005).

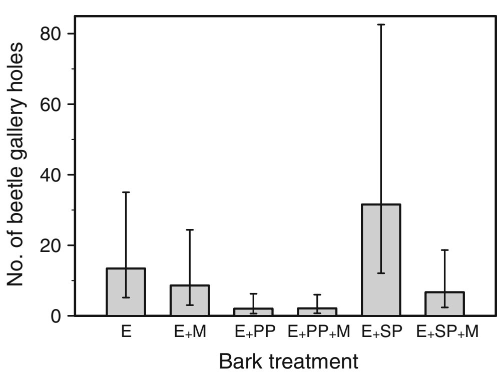

Fig. 6 Mean (± 95 % CI) numbers of scolytid beetle gallery entrance holes in *Quercus agrifolia* logs (N=10) counted within a marked area over ethanol-infused sapwood (E) with or without additional treatments on the bark surface. Bark treatments: M = Moisturin antitranspirant; PP = ultra-high release pouch containing (−)-α-pinene; SP = spray application of (−)-α-pinene. See the Results section for P values of the means comparisons for logs treated with vs. without M, PP, SP, or logs treated with PP vs. SP

Our second line of evidence of attraction to ethanol is demonstrated by the E-treated *Q. agrifolia* logs having 4.4 times more scolytid entrance holes above the ethanol-infused sapwood than did the opposite side of the log, where no ethanol was added. Part of this response, however, may be a result of a synergism of ethanol with the beetles' pheromones after initial attack (Pitman et al., 1975; Borden et al., 1980). McPherson et al. (2008) reported that once beetles attack a *P. ramorum* canker on an inoculated tree, their presence becomes the overriding factor in further attacks.

The third line of evidence for attraction to ethanol is that scolytid beetles typically restrict attacks within the canker borders (Rizzo et al., 2002), where sapwood ethanol concentrations are much higher than in adjacent uninfected sapwood from the same tree or from nearby healthy trees. Our final line of evidence is that large basal cankers contained higher ethanol concentrations than did the smaller spot cankers, consistent with the observation by McPherson et al. (2008) that trees with larger cankers attracted more beetles prior to the first attacks than did trees with smaller cankers. Beetles had attacked five of the six basal cankers we sampled, whereas only one of the nine spot cankers was attacked.

While ethanol is released from *P. ramorum* cankers, the two *Monarthrum* species and *P. pubipennis* all restrict attacks to oak species, suggesting that other tree components may function as kairomones or pheromone synergists in host recognition and selection, as demonstrated for *Dendroctonus* species (Pureswaran and Borden, 2005). Further, the dead and dying woody substrates that these beetles typically colonize often synthesize and accumulate ethanol (Kelsey, 1994; Kelsey and Joseph, 1999). What role, if any, ethanol plays in their selection of these materials remains to be determined.

The scolytid beetles' attraction to ethanol was deterred by simultaneous release of (−)-α-pinene or 4AA. These two compounds provided comparable levels of repellency for all beetle species. Similar repellant activity by ethanol and (−)-α-pinene has been reported previously for *X. saxesenii* (Miller and Rabaglia, 2009; Ranger et al., 2011), but our study, to our knowledge, is the first report for *P. pubipennis*.

Attaching ultrahigh release pouches of (−)-α-pinene to *Q. agrifolia* logs with ethanol-infused sapwood reduced attraction of scolytid beetles, whereas spray application of (−)-α-pinene to logs did not. Whether the ultrahigh release rate (−)-α-pinene pouches would be as effective in deterring attraction to natural cankers was not tested, but seems likely given the (probably) much lower ethanol release rates from natural cankers (compared to infused logs) and the importance of release rates for ethanol and (−)-α-pinene in determining the responses of beetles (Klimetzek et al., 1986; Schroeder and Lindelöw, 1989). The presence of 4AA reduces attraction of various conifer scolytid beetles to a mixture of ethanol plus (1:1) α-:β-pinene (Joseph et al., 2001), but to our knowledge, this is the first time that this compound has been reported as a deterrent for any of the hardwood scolytid beetles discussed here. Treatment of the bark surface with Moisturin, alone or in combination with (−)-α-pinene, was not effective in deterring attraction of scolytid beetles to logs releasing ethanol, perhaps because the amount released was too high for effective suppression by Moisturin.

McPerson et al. (2005, 2008) reported that bleeding cankers remain free from beetle attacks for many months, suggesting that the bleeding exudate is not an attractant for beetles. Absence of attack would result if ethanol were not being synthesized and/or released, if beetle population densities were extremely low, or a combination of these factors. When beetle densities are low, most individuals are attracted to trees with cankers releasing the greatest amounts of ethanol and, subsequently, to the pheromones and kairomones released following initial attack. In this situation, cankers without ethanol, or with very low amounts, would remain attack free. Given the highly variable ethanol concentrations measured (0.3–036.1 mg.g^{-1} fresh mass), it is likely that some cankers do not release sufficient ethanol to attract beetles.

The high variability of sapwood ethanol concentrations observed in this study may be a result of many factors influencing ethanol accumulation, including how and when synthesis is initiated, the rates and duration of synthesis, and dissipation. Plant tissues that produce fermentation enzymes synthesize ethanol when cellular O_2 declines to hypoxic (low O_2) or anoxic (no O_2) levels (Gibbs and Greenway, 2003; Greenway and Gibbs, 2003; Vartapetian, 2006). However, synthesis can stop quickly if O_2 levels return to normal. Alternatively, damage to cellular membranes or enzymes systems, especially in mitochondria, initiates ethanol synthesis, as evident when plant tissues are injured by freezing, crushing, or heating (Kimmerer and Kozlowski, 1982; Anderson, 1994; Forney et al., 2000).

Ethanol synthesis may be initiated in *P. ramorum* cankers when O_2 supply routes are blocked and/or rapid cell respiration depletes available O_2 faster than it is supplied. Radial gas exchange with the atmosphere through intercellular spaces (Hook and Brown, 1972; Hook et al., 1972; Spicer and Holbrook, 2005), and dissolved gas in the transpirational stream (Hook et al., 1972; Mancuso and Marras, 2003; Spicer and Holbrook, 2005; Sorz and Hietz, 2006), are the two primary O_2 supply routes for tree stem tissues; *P. ramorum* infection could impact both. Radial diffusion pathways may be blocked when the necrotic exudate is synthesized and fills intercellular spaces or necrotic cavities, as oxygen diffusion in water is much lower than in air (Sorz and Hietz, 2006). Brown and Brasier (2007) commonly found clear, pale pink, or orange liquid-filled necrotic cavities or lagoons in phloem or xylem below the external bleed points associated with *P. ramorum*. Callus tissue walled off many lagoons, effectively blocking O_2 from reaching the liquid as well as any healthy tissues beneath the callus. The liguids light color is indicitive of minimal O_2, as it quickly oxidizes to red-brown on exposure to air. *Phytophthora ramorum* also can disrupt diffused transpirational O_2 in xylem by interfering with hydraulic conductivity, as observed in *Rhododendron* (Manter et al., 2007) and tanoak, in which reduced water transport is caused by induced tyloses formation and, probably, associated embolisms (Parke et al., 2007; Collins et al., 2009). Similar disruption in *Q. agrifolia* is likely, as *P. ramorum* has been isolated from discolored xylem at depths of 1–5 mm and occasionally 20–25 mm in other *Quercus* species (Brown and Brasier, 2007).

In addition to blocked O_2 supply routes, tree tissues within a canker may deplete available O_2 through enhanced respiration rates for synthesizing phenolic compounds (Ockels et al., 2007) or the necrotic exudate, used as part of the trees chemical defense against the pathogen. The respiration rates of *P. ramorum* in host tissues may also contribute to lower O_2 levels. Ethanol synthesis also could be initiated in cankers by disruption of mitochondrial membranes and enzyme systems in host cells, either during cellular degeneration associated with synthesis of the necrotic exudate (Brown and Brasier, 2007), or through excretion of elicitins by *P. ramorum* that scavenge sterols from membranes of host tissues to support growth and spore production (Manter et al., 2007; Stong et al., unpublished).

The quantities of ethanol produced will depend on the tissues involved, their nutrient status and phenological stage (Kimmerer and Stringer, 1988; Kelsey et al., 1998), temperature (Kelsey et al., 2011), and the pool of carbohydrates available for synthesis (Gibbs and Greenway, 2003; Vartapetian, 2006). These factors all interact to determine rates and amounts of ethanol produced, but peak concentrations within tissues also will depend on dissipation, a function of movement and metabolism. High sapwood ethanol levels within canker boundaries are consistent with a severe disruption of hydraulic conductivity, as observed for infected *Rhododendron* and tanoak (Manter et al., 2007; Collins et al., 2009). Because ethanol is nonionic and hydrogen bonded to water, it is not constrained by membranes and can diffuse freely between cells and tissues, moving from high to low concentrations after synthesis begins. The abundant water in sapwood functions as a diffusion sink, causing ethanol generated in adjacent vascular cambium and phloem to move inward (Kimmerer and Stringer, 1988; MacDonald and Kimmerer, 1991). Upon entering sapwood trachieds or vessels, ethanol moves upward in the transpirational stream at a faster rate than diffusion, as demonstrated in flooded roots (Cojocariu et al., 2004; Rottenberger et al., 2008). Ethanol entrained in the transpirational stream continues to dissipate by diffusion and eventually will be metabolized into organic acids, amino acids, and proteins when it enters living cells in the stem, branches, or leaves (MacDonald and Kimmerer, 1993). Additionally, a small amount of ethanol may be released to the atmosphere or first converted to acetaldehyde and then released to the atmosphere (MacDonald and Kimmerer, 1993; Kreuzwieser et al., 2000; Cojocariu et al., 2004). Healthy trees use these mechanisms during brief periods of stress to maintain low ethanol concentrations in their tissues. Thus, the impairment of hydraulic conductivity in sapwood infected with *P. ramorum* would allow ethanol to accumulate within cankers, as observed in this study.

Ethanol that accumulates in canker tissues eventually will move radially toward the outer bark, as demonstrated by the *Q. garryana* log with no hydraulic conductivity containing a solution of 50 % ethanol in its sapwood. Release of ethanol from tree stems has been demonstrated for several species under a variety of conditions (Gara et al., 1993; Ranger et al., 2010). The rate of radial movement outward of ethanol from *P. ramorum* cankers depends on bark thickness, how many healthy host cells remain to metabolize it, to what extent intercellular spaces are blocked by necrotic exudate or callus tissues, and tissue water content. Additionally, any ethanol entering *P. ramorum* cells might be metabolized as a carbon and energy source, as reported for the tree pathogen *Armillaria mellea* (Vahl) P. Kumm. (Weinhold and Garraway, 1966). Ambrosia beetle galleries penetrating deep into the sapwood of cankers could function as unobstructed pathways allowing enhanced release rates of ethanol to the atmosphere. Microbes vectored into these galleries by beetles also may metabolize ethanol as described above for *A. mellea*. All these interacting parameters are likely to contribute to the wide range of ethanol concentrations within cankers, release rates to the atmosphere, and subsequent attraction of scolytid beetles.

In summary, ethanol accumulates in *Q. agrifolia* sapwood within the boundaries of *P. ramorum* cankers, likely as a consequence of reduced hydraulic conductivity from infection. Eventually, ethanol diffuses radially and is released as an attractant for various scolytid beetle species. Ethanol, thus, serves as an important link in accelerated mortality of *P. ramorum*-infected *Q. agrifolia*.

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