



Ethanol and acetone from Douglas-fir roots stressed by *Phellinus sulphurascens* infection: Implications for detecting diseased trees and for beetle host selection



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ABSTRACT

Phellinus sulphurascens (previously the Douglas-fir form of *Phellinus weirii*) is an important native pathogen causing laminated root rot in forests of western North America. Visual crown symptoms, or attacks by bark or ambrosia beetles appear only during advanced stages of the disease with extensive infection in the lower bole. Ethanol synthesis is one of many physiological responses in tree tissues stressed by pathogens. Ethanol, acetone and other volatiles from root tissues of healthy and diseased trees were analyzed using headspace gas chromatography. Xylem and phloem from 20 diseased trees at two western Oregon sites contained higher concentrations of ethanol, acetone, or other headspace volatiles than 20 healthy trees on one or more dates in September, November, or the following May. Root cross-sections from eight diseased trees were sampled along perpendicular transects and found to contain extremely variable ethanol concentrations, with highest xylem quantities in a 0–2 cm zone outside the infection boundary and lowest amounts inside the infection. Acetone concentrations were the opposite. Logistic regression models were built and tested to determine which volatiles could predict diseased trees. A model using xylem ethanol concentrations as the only parameter was selected and validated with measurements from 80 trees on the edges of *P. sulphurascens* infection centers at two different western Oregon sites. This model successfully predicted trees with laminated root rot (78% overall correct classification and 68% for known diseased trees), but worked best for those with infections observed in both root cores and the root collar (100% correct). Early detection of *P. sulphurascens* infected trees remains a challenge. Our ethanol analysis method is useful for research, but provides limited benefits for identifying individual *P. sulphurascens* hazard trees, or for extensive ground surveys in the forest. Whether ethanol is released to the atmosphere in sufficient quantities to confirm infection before the late appearance of crown symptoms, or bark beetles remains unknown. If it is, then development of sensors capable of tree side detection requiring minimal tissue sampling would be useful in managing this disease. We also propose a mechanism for how ethanol with host monoterpenes could play a central role in pioneering bark beetle primary host selection of trees infected with this pathogen.

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1. Introduction

When trees are subjected to stress from various biotic or abiotic agents one of their many physiological responses may be ethanol synthesis if the stressed cells experience impaired aerobic respiration as shown when tissues are deprived of oxygen (Kelsey et al., 1998a; Joseph and Kelsey, 2004). This allows the

cells to survive for brief periods until their O₂ supply is restored, and if not restored they die. Ethanol accumulation is dependent on the duration and rates of synthesis (Kelsey et al., 2011), its subsequent dissipation by diffusion as shown by adding it to tissues (Kelsey et al., 2013), movement in the transpiration stream (Kreuzwieser et al., 1999; Cojocariu et al., 2004), metabolism in non-stressed cells to other cellular components (MacDonald and Kimmerer, 1993), direct release to the atmosphere (MacDonald and Kimmerer, 1993; Kreuzwieser et al., 1999; Rottenberger et al., 2008; Ranger et al., 2013), or conversion to acetaldehyde that is released to the atmosphere (Cojocariu et al., 2004).

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Pathogens may be the most common biotic stress agents to induce ethanol synthesis in trees. For example, boles of lodgepole pine, *Pinus contorta* Douglas ex Loudon, with active decay fungi emit 2.4 times more ethanol to the atmosphere than those without the fungi (Gara et al., 1993). Elevated ethanol concentrations occur in stem tissues near the root collar of Douglas-fir, *Pseudotsuga menziesii* (Mirb.) Franco., infected with *Leptographium wageneri* var. *pseudotsugae* Harrington & Cobb, the cause of black stain root disease (Kelsey and Joseph, 1998). This was also observed in stem tissues of ponderosa pine, *Pinus ponderosa* Lawson & C. Lawson, infected with *L. wageneri* var. *ponderosum* (T.C. Harr. & F.W. Cobb) T.C. Harr. & F.W. Cobb, or its combination with *Heterobasidion irregular* Garbelotto & Otrosina, the cause of *Heterobasidion* root disease of pine (Kelsey et al., 1998b, 2006).

Ethanol can also accumulate within the boundaries of cankers caused by *Phytophthora ramorum* Werres, De Cock & Man in't Veld, the microbe responsible for sudden oak death, on the stems of coast live oak, *Quercus agrifolia* Nee, whereas concentrations in tissues outside the cankers and in adjacent healthy trees remain low (Kelsey et al., 2013). In the absence of any abiotic stressors, especially flooding as it induces synthesis in the roots and transport into the bole (Kreuzwieser et al., 1999; Cojocariu et al., 2004; Ranger et al., 2013), elevated ethanol concentrations in a tree can be a strong indicator of infection. However, it is important to note that low ethanol concentrations do not explicitly confirm the tree is disease free because of the many interacting factors influencing the rates of synthesis and subsequent dissipation. If ethanol escapes from stressed trees into the atmosphere in sufficient quantities and duration it can function, usually in combination with other volatiles released from the tree, as a signal that attracts various bark or ambrosia beetles to land and attack (Kelsey and Joseph, 2001, 2003; Kelsey et al., 2013, 2014; Ranger et al., 2013).

Other compounds occur with ethanol in the headspace analysis of tree tissues and their concentration changes associated with pathogen infections may also function as biomarkers for detecting disease. Acetaldehyde, acetone, methanol, and propanol were all more strongly correlated than ethanol with the severity of black stain and *Heterobasidion* root diseases in ponderosa pine (Kelsey et al., 1998b). Acetaldehyde was selected as the single best predictor of black stain disease, followed by acetone. In a related study, 284 ponderosa pine trees were randomly selected in stands where crown symptoms were unreliable for identifying diseased trees (Kelsey et al., 2006). In this case, sapwood acetone concentrations were selected as the best chemical headspace markers for predicting root disease. We suspected ethanol, or one of the other headspace compounds might also serve as a useful marker for earlier and more accurate detection of trees with laminated root rot caused by *Phellinus sulphurascens* Pilát (previously the Douglas-fir form of *P. weirii*).

P. sulphurascens infects various conifer hosts, with Douglas-fir, true firs and mountain hemlock being most susceptible (Thies and Sturrock, 1995). It spreads across root contacts between healthy and diseased trees or stumps, with ectotrophic mycelium transfers considered more important than endotrophic transfers (Bloomberg and Reynolds, 1982). Because of root-to-root spread, multiple trees often die near one another creating gaps in the forest canopy with standing dead and fallen trees. These gaps are easily recognized signatures of this pathogen that slowly expand outward in an uneven radial pattern at a rate of less than 50 cm per year (Nelson and Hartman, 1975; McCauley and Cook, 1980). However, some diseased trees also occur sporadically throughout a stand, unassociated with gaps. The infection mechanism for these trees is yet unknown and their detection is challenging (Thies and Nelson, 1997).

Distinct visual symptoms often do not appear in the crowns of infected trees (Wallis and Reynolds, 1965; Bloomberg and Wallis, 1979; Wallis and Bloomberg, 1981; Thies, 1983), and they are typically not attacked by bark beetles (Buckland et al., 1954; Lane and Goheen, 1979; Goheen and Hansen, 1993), until the infections reach advanced stages in the bole. Reduced annual height growth can be an early crown symptom for *P. sulphurascens* (Bloomberg and Wallis, 1979), but this change may be gradual, or variable, and difficult to recognize when an infected tree is among healthy ones whose height growth may also be impacted by competition, water deficits, or other stresses. Trees with healthy appearing crowns and no evidence of beetle colonization may be wind-thrown during storms exposing the broken roots weakened by decay. These trees are hazardous when growing in campgrounds, along road right-of-ways, or near homes. Their detection and removal is critical. Because of its combined economic and ecological importance in Washington forests, *P. sulphurascens* was recently chosen over other root pathogens as best suited for focused research directed toward improving applied management options (Cook et al., 2013). Their report cites methods for detecting infected stands and conducting ground surveys, but emphasizes the importance for further research to improve early detection, identification accuracy, and cost-effectiveness.

Objectives for the study we report here were to: (1) determine whether roots of Douglas-fir stressed by *P. sulphurascens* infections synthesize and accumulate higher ethanol concentrations than roots from uninfected trees as observed for other pathogens; (2) determine if there are differences in the quantities of acetaldehyde, acetone, methanol, or propanol detected during headspace analysis of ethanol between roots of infected and uninfected trees; and (3) attempt to develop a predictive model using these compounds to help identify trees infected with *P. sulphurascens* before the disease reaches an advanced stage.

2. Methods and materials

2.1. Study sites and tree selection

Four sites were utilized in this study; sites 1 and 2 were used to gather data for model building, while sites 3 and 4 were used to gather data for testing the model. Trees selected for model development were from two Douglas-fir stands near Philomath, Oregon. Site 1 was at 44.475461°, –123.430978° (202 m elev.) and site 2 at 44.545358°, –123.497525° (268 m elev.). At each site 10 tentatively diseased trees were selected and tagged as encountered when they fit the selection criteria of (1) being located on the perimeter of a *P. sulphurascens* infection center; (2) being a dominant or co-dominant tree; (3) having *P. sulphurascens* ectotrophic mycelium present on the bark surface of one or more partially excavated roots; and (4) having no evidence of bark or ambrosia beetle attacks. Visible crown symptoms for these trees were variable with some suggesting advanced infections. Rot or the characteristic stain from infection (Thies and Sturrock, 1995) was detected in at least one root from 13 of the 20 trees considered diseased. Ten putatively healthy trees were also selected and tagged as encountered when they fit the selection criteria of (1) being located well beyond root contact with trees on the infection center perimeter; (2) being a dominant or co-dominant tree; (3) having no ectotrophic mycelium on the bark surface of one or more partially excavated roots; and (4) having no evidence of bark or ambrosia beetle attacks. No rot or stain was observed in their sample cores on any dates. As described later, the phloem and xylem from root cores of these trees were analyzed for headspace volatile concentrations and the values were used to build a model for predicting whether trees at the sites below were likely infected with this pathogen.

An additional 40 trees were sampled from each of two stands near Forest Grove, Oregon for validation of the model developed from two sites above. Site 3 was located at 45.450608°, –123.345275° (611 m elev.) and site 4 at 45.767447°, –123.353411° (375 m elev.). These trees were selected and tagged as encountered from around the periphery of *P. sulphurascens* infection centers marked for harvest, without knowing if they were diseased or healthy. Four main roots entering the root collar near the soil surface was the only selection criterion.

2.2. Root sampling for headspace volatile analysis and water contents

At sites 1, 2, the root cores were collected from healthy and diseased trees in September, November and the following May to detect any seasonal changes in the volatile concentrations. Four main lateral roots were partially exposed from the soil and tagged. The following day (27 September) a tissue core was extracted with an increment borer (5 mm i.d.) from the top-right side (facing the bole) of each root at a distance of 5–30 cm below the point where they entered the surface litter layer or soil. The phloem and first 1.0 cm of xylem were separated, sealed in glass vials (15 × 45 mm o.d.) then immediately frozen with dry ice until stored in a –36 °C freezer. Root holes were plugged with corks to minimize further contamination and improve visibility for future sampling. At site 1, one additional sample on diseased trees only was collected from the lower bole above each sampled root at a distance of 5–20 cm above the soil surface. Roots were resampled on the top left-side at the same distance from the bole on 28 or 29 November and finally about 3–5 cm down the root from previous sample holes on 23 May to evaluate seasonal changes in ethanol concentrations.

Trees at sites 3–4 were sampled one year later, 16 and 23 May for site 3, and 5 June for site 4. Four roots per tree were partially excavated and one increment core (0.5 × 6 cm xylem depth) extracted from the top of each at 5–30 cm distal to their point of entry into the soil litter layer, or soil and processed as above. Xylem depth was increased to 6 cm rather than 1 cm at sites 1, 2. This would contain the same tissue as a 1 cm core, but for larger roots might improve the likelihood of reaching tissue near an infection with higher ethanol concentrations, as shown by the root transect experiment described below. Any staining in the xylem core was noted as an infection. These trees were then harvested, their tagged stumps relocated and the presence of any brown stain on the cut surface recorded.

2.3. Headspace and water content analysis

Headspace volatiles in the samples from sites 1, 2 and root transect were processed and analyzed with the gas chromatography (GC) instruments and settings previously described in detail (Kelsey and Joseph, 1998). Briefly, the samples were thawed on ice, weighed into headspace vials, and then sealed with a septum and crimp cap (PerkinElmer, Akron, OH, USA). Vials were held on ice until a full set was processed then placed in a 102 °C for 30 min to deactivate enzymes in the tissue. The GC was a Hewlett Packard 5890 with flame ionization detector and J&W DB-Wax column (30 m × 0.32 mm i.d., 0.25 µm film thickness) using helium as the carrier gas. The GC injector was set at 50 or 60 °C and the detector at 250 °C. The HS40 settings were 90, 100 and 60 °C for the sample heating block, needle, and transfer line temperatures, and 1.0, 0.04, and 0.4 or 0.1 min for the vial pressurization, injection, and needle withdrawal times, respectively. The column oven was held isothermally at 50 °C for 4.5 min for sites 1, 2, and the root transect samples. For samples from sites 3, 4 the column oven was held at 50 °C for 1.0 min, then increased to 80 °C at 20 °C per min. and held for 1.5 min to minimize monoterpene carryover

between samples. Duplicate vials, each containing 5 µL of a mixed acetaldehyde and ethanol standard were included with each sample set for quantification by the external standard method using a linear response curve from zero for both compounds. All samples were analyzed twice by the multiple headspace extraction technique for calculating concentrations of acetaldehyde and ethanol (Kolb et al., 1984). Because acetone, methanol and propanol are generated during heating (Kelsey et al., 1998b) their concentrations were determined by static headspace calculations with values from the first analysis only and those of the ethanol standards using a relative response factor of 1. Ethanol concentrations, except for root transect samples, were calculated as µg g^{–1} fresh mass because of its strong association with water by hydrogen bonding. Concentrations of the other compounds were reported in the same units for consistency. Tissue water content was measured on these samples after analysis by removing the caps and heating at 102 °C for 16 h, then cooling in a sealed container with desiccant for 30 min before taking a final weight.

2.4. Root transect sampling and analysis

This experiment was setup to examine concentrations of headspace volatiles in *P. sulphurascens* infected root tissues and compare them with quantities in the surrounding healthy tissues. One root on each of eight diseased Douglas-fir stumps from trees harvested the previous 30–45 days near site 1 was collected between 7 and 16 December. Diseased trees were identified by the characteristic brown *P. sulphurascens* stain visible on the stump surface and selected as encountered. One root beneath the stained stump surface was excavated, scribed on top for future reference, then severed 15–232 cm from the bole, and again at 1 m or less distal from the first cut. Each root segment (dia 6–31 cm; mean 12 cm) was placed in a plastic bag to minimize water loss and returned immediately to a 5 °C coldroom for storage and processing. Each root was first cut perpendicular to length near the proximal end and the xylem examined for a distinct zone of rot or stain surrounded by apparently healthy tissue. If this infection pattern was observed a disk (about 10 mm thick) was removed, but if there was decay with loss of tissue strength and integrity, or no stain, then a new cut was made further down the root. When disks matched the selection criteria, the stained-infected area was outlined with a marker for future reference. A transect (0.5 cm wide) was marked across the cut surface from root top to bottom, and another left to right with both passing through the disk center. Segments 10 mm long were marked and numbered along each transect starting at the center and progressing outward through the phloem. Xylem segments adjacent to the phloem were of variable length.

After marking, disk surfaces were photocopied for measuring segment distances from stain or decay. The transect segments (about 1 × 1 cm) were then cut out on a band saw and held in vials on ice. Each segment was split into smaller pieces then quickly placed into a pre-weighed headspace vial, sealed with a septum and held on ice until all segments were processed. The vials were heated for 60 min in a 102 °C oven to deactivate enzymes and prevent further ethanol synthesis. Volatiles and tissue dry mass were analyzed as described above, except the samples were heated 60 min in the HS40 autosampler because of their larger mass. Volatile concentrations are reported as µg g^{–1} dry mass, because tissue fresh mass was not measured to facilitate faster sample processing.

To verify the pathogen was *P. sulphurascens*, a second disk immediately adjacent to the one used for headspace analysis was cut and marked with the same transect for six of the eight roots processed. Each 10 × 10 mm segment of tissue was cut from the transect, noted for presence of stain, then split into three pieces and each placed separately on 1.5% malt extract agar slants in

culture tubes. The tubes were incubated for 21 days at 20 °C then examined for the presence of *P. sulphurascens* setal hyphae in developing colonies. Three samples of decayed tissue from one root cross section were also cultured.

2.5. Pre-dawn water potentials at sites 1, 2

Pre-dawn water potential was measured on all trees to determine whether the pathogen infection caused greater water stress in diseased trees than healthy trees. The first measurements were taken at the end of summer on 28 and 29 of September and again on 30 November and 1 December, after the fall rain had begun. A branch tip was detached from the crown and a secondary lateral removed for measurements with a Scholander pressure chamber (PMS Instruments, Albany, OR, USA). Heights of the sampled branches were measured and used to adjust for gravitational effects on water potentials.

2.6. Diameter and radial growth measurements

Tree diameters at breast height (DBH) were measured at all sites with a diameter tape at 1.4 m above the forest floor. The last five-year radial growth at sites 1, 2 was measured to the nearest mm on one increment core per tree at BH on the uphill side of the bole. At sites 3, 4, five-year radial growth was measured to the nearest mm at two random positions on each stump surface and averaged, except for one stump damaged during harvest.

2.7. Statistical analysis

Although each compound was analyzed in the phloem and xylem of four roots per tree on each sampling date, only the maximum concentration among the four roots was assigned to each tissue on each date for analysis. A tree was categorized as diseased regardless of the number of infected roots, and this helped to minimize any dilution effect that might occur from averaging low concentrations in three healthy roots with a high concentration in one diseased root. The maximum concentration for all compounds did not necessarily occur in the same root within a sampling date, nor did the maximum value for each compound always occur in the same root across dates. Alternatively, the water content in each tissue was averaged across the four roots of a tree on each sample date to help mitigate the influence of a single low value among the four roots, since water can be influenced by various factors other than pathogen stress.

All analyses were done with the linear mixed procedure in SAS 9.4 (SAS, 2012). Assumptions of normality and constant variance of the residuals was checked for all models using quantile–quantile plots of the residuals and residual vs fitted value plots, respectively. If assumptions were not met, the response was natural log transformed and assumptions were checked again to ensure they were met. Estimates and confidence intervals for any transformed responses were back-transformed and inference made to this median response. The Kenward–Roger method was used for calculating degrees of freedom (Littell et al., 2006). For each analysis of repeated measures data we fit models with compound symmetry (CS), unstructured (UN1–3), Toeplitz (TOEP1–3), auto-regressive (AR1) and spatial power (SP) covariance structures and chose a final one based on the suitability with our repeated sampling and the lowest AICc values. Comparisons among group levels were calculated for each model using protected Fisher's least significant difference to guard against error from multiple comparisons. All statistical testing was done using an alpha of 0.05.

For diseased trees at site 1, the highest ethanol concentrations per tree from the four bole samples were compared with the highest quantity among the four roots using a paired *t*-test for the

phloem and xylem separately after transforming the data to natural logarithms. The relationship between bole and root ethanol concentrations in each tissue were evaluated by Pearson correlation coefficients using all four bole and root samples per tree.

Tissue mean water contents and maximum volatile concentrations for trees at sites 1, 2 were compared between the healthy and diseased trees by a randomized block (site) repeated measures (month) analysis for each tissue separately with the UN(1) covariance structure used in all repeated measures analyses. Main effect of tree disease condition was fixed and site and tree were random effects. At sites 3, 4 the mean root water content and maximum ethanol and acetone concentrations were analyzed separately for phloem and xylem with tree condition (diseased or healthy) as a fixed effect and site as a random effect.

Transect sampling of eight diseased roots was used to examine volatile concentrations inside and outside the infection and determine how proximity to the infection boundary influenced concentrations. For each tissue, transect segments were assigned to one of five categories based on their distance from the nearest xylem infection boundary; (1) segment partially or entirely stained, (2) 0–2 cm, (3) 2–4 cm, (4) 4–6 cm and (5) 6+ cm from the nearest stain border (not included in the analysis because of low numbers). A mean compound concentration was determined from the pool of cores in each category and these analyzed separately for the xylem and phloem in a randomized complete block (root) repeated measures (distance) design with distance a fixed main effect and root a random effect. The SP and TOEP (1) covariance structures were used for repeated measures analysis of the phloem and xylem volatile concentrations, respectively.

Tree water potentials at sites 1, 2 were compared using a randomized complete block (site), repeated measures (month) analysis using UN(1) covariance structure, with tree disease condition and sampling month as fixed main effects, and site as a random effect. Tree diameter and five-year radial growth were each combined across sites 1–4 for the diseased and healthy trees, then compared using a randomized complete block (site) analysis, with tree disease condition a fixed main effect, and site a random effect.

2.8. Model building with tissue measurements at sites 1, 2

Logistic regression models for predicting the probability that a tree is diseased were created from compounds measured in the root xylem and phloem from the 20 putative healthy and 20 diseased trees at sites 1, 2 using the SAS 9.4 logistic procedure. For each tissue the binary response variable was tree disease condition, with mean water content and the maximum root concentrations for acetaldehyde, acetone, ethanol, propanol, and methanol tested as explanatory variables. The tissue mean water content was an average for all four roots across the three sample dates. The maximum value for each compound was obtained by calculating an average concentration for each of the four roots using the measurements from the three sampling dates, and then using the highest average value from among the four root values to represent the tree. Models were built using the purposeful selection of covariates method as described by Hosmer et al. (2013). The fit of each model to the data was checked during this process using a receiver operating characteristic (ROC) area under the curve (AUC), and the Hosmer–Lemeshow goodness-of-fit tests.

2.9. Model validation with measurements at sites 3, 4

The model developed from sites 1, 2 was tested by comparing its predicted probability of infection to the actual infection observed in 80 independent trees at sites 3, 4; 25 known infected plus 55 presumed uninfected. Three methods were used to evaluate fit; (1) ROC AUC, (2) Hosmer–Lemeshow goodness-of-fit tests,

and (3) classification tables using a probability cut-point of 0.40 (Hosmer et al., 2013). ROC AUC assesses the models ability to discriminate between infected and uninfected trees in a range from 0.0 to 1.0, with values ≤ 0.5 indicating no discrimination, ≥ 0.7 as acceptable, ≥ 0.8 as excellent, and ≥ 0.9 as outstanding. The Hosmer–Lemeshow test partitions the trees into 10 percentile groupings from low to high probability and compares the model's expected number of infected with the actual observed infected. A Pearson chi-square test is then done on a 10×2 table of the expected and observed frequencies, with a chi-square P -value greater than 0.05 indicating good model fit. Classification tables compare the observed versus predicted outcomes by selecting a cut-point probability; in this case we chose 0.40 because it maximized the correct calls for both healthy and infected. If the estimated probability was equal to or exceeded the cut-point the tree was predicted to be infected, otherwise it was predicted to be uninfected.

Finally, the trees from sites 3, 4 were sorted into four categories based on observed infections: (1) stump surface only, (2) root core only, (3) both stump surface and root core, and (4) no stain-infection observed anywhere. Then the number of correct and incorrect model predictions were tallied for the trees in each category and converted to percentages to evaluate what infection types the model identified best.

3. Results

Results for the initial statistical model analyses are reported in tables, with graphs presented for the most relevant variable comparisons. Additional results for headspace volatiles other than ethanol and acetone, some water content information, tree diameters, and growth are presented in Appendix A.

3.1. Sites 1, 2 root ethanol and acetone concentrations

Ethanol concentrations in root phloem were dependent on tree condition only (Table 1) with $12.9 \mu\text{g g}^{-1}$ fresh mass in diseased tree roots that was 3.2 (95% CI 1.8, 5.6) times higher than in healthy trees. However, monthly values are presented in Fig. 1A for consistency with xylem results. In xylem there was a tree condition by month interaction (Table 1), nevertheless at each month the xylem ethanol in diseased trees was greater than in healthy trees (Fig. 1B). This interaction was caused by declining quantities in diseased trees at each subsequent sampling date that were all different from one another, whereas in healthy tree roots the concentrations remained the same among all months (Fig. 1B). The largest difference observed for diseased xylem over healthy xylem occurred in September roots (22.9, 95% CI 11.3, 46.4 times) and smallest difference in May roots (4.1, 95% CI 1.3, 13.5 times).

For diseased trees only at site 1 (not graphed) the mean phloem ethanol in the bole ($12.2, \pm\text{SE } 1.1 \mu\text{g g}^{-1}$ fresh mass) was lower than in the roots ($93.6, \pm\text{SE } 46.3 \mu\text{g g}^{-1}$ fresh mass), but not statistically different ($t_9 = 2.06, P = 0.070$), whereas xylem ethanol concentrations in the bole ($28.2, \pm\text{SE } 13.9 \mu\text{g g}^{-1}$ fresh mass) were statistically lower ($t_9 = 2.35, P = 0.043$) than in the roots ($76.9, \pm\text{SE } 22.2 \mu\text{g g}^{-1}$ fresh mass). Nevertheless, the maximum concentration measured in bole xylem ($144.2 \mu\text{g g}^{-1}$ fresh mass) was nearly as high as in the root xylem ($167.0 \mu\text{g g}^{-1}$ fresh mass). Also, bole xylem concentrations had a strong, positive correlation ($r = 0.729, n = 39$ one outlier removed, $P < .001$) with quantities in the root xylem, whereas there was no correlation ($P = 0.105$) between quantities in the bole and root phloem.

Acetone concentrations in root phloem and xylem (Fig. 2) were dependent on tree condition, but not month or their interaction (Table 1). The $9.1 \mu\text{g g}^{-1}$ fresh mass of acetone in the phloem of

Table 1

Sites 1, 2 statistical analysis results for ethanol and acetone contents in root phloem and xylem from diseased and healthy trees.

Volatile	Effect ^a	Phloem			Xylem		
		d.f.	F	P ^b	d.f.	F	P ^b
Ethanol	Tc	1, 35.3	17.20	<0.001	1, 46.7	41.33	<0.001
	M	2, 49.1	2.02	0.143	2, 46.3	6.96	0.002
	Tc $\times$ M	2, 49.1	0.67	0.515	2, 46.3	6.20	0.004
Acetone	Tc	1, 37.0	21.53	<0.001	1, 33.8	24.91	<0.001
	M	2, 42.1	0.31	0.733	2, 38.4	0.64	0.534
	Tc $\times$ M	2, 42.1	0.06	0.945	2, 38.4	2.55	0.092

^a Tc = tree condition (diseased, healthy), M = month.

^b Bold P values are statistically significant.

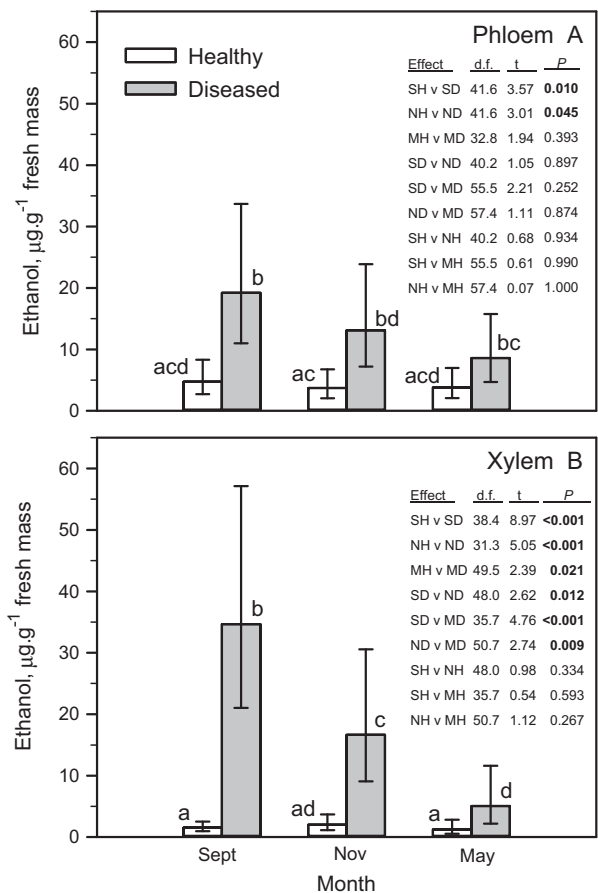


Fig. 1. Sites 1, 2 backtransformed mean ethanol concentrations (95% CI) in Douglas-fir root phloem (A), and xylem (B). See Table 1 for model P values; with P values listed in graphs for the most biologically relevant comparisons, S = Sept, N = Nov, M = May; H = healthy, D = diseased. Bold P values are statistically significant.

diseased roots was 3.9 (95% CI 2.1–7.0) times higher than in roots of healthy trees (Fig. 2A). Root xylem from diseased trees contained $10.5 \mu\text{g g}^{-1}$ fresh mass acetone that was 3.3 (95% CI 2.0, 5.4) times greater than in healthy trees (Fig. 2A).

3.2. Sites 1, 2 root transect sampling for ethanol, acetone, and pathogen identification

P. sulphurascens was confirmed in culture from five of the six diseased roots sampled, but only from the visually stained tissue. The fungus was cultured also from three samples of xylem with advanced decay removed from one root.

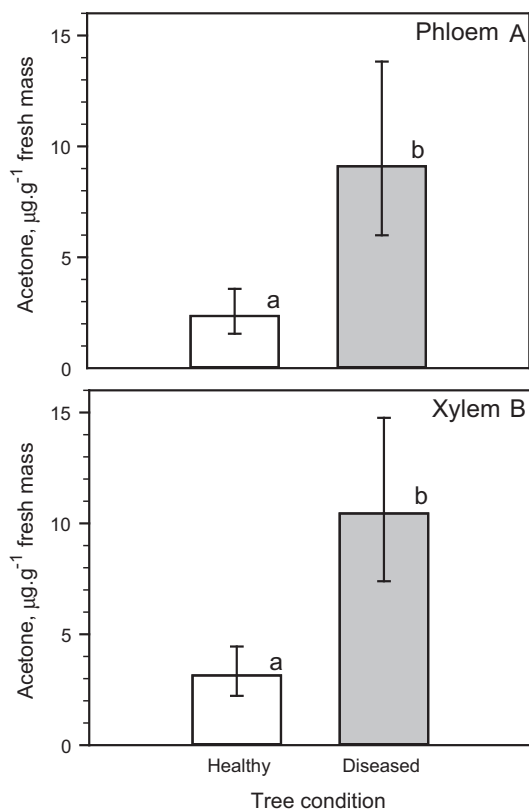


Fig. 2. Sites 1, 2 backtransformed mean acetone concentrations (95% CI) in Douglas-fir root phloem (A) and xylem (B) from diseased and healthy trees. Bars with the same letters are not statistically different, see Table 1 for *P* values.

Xylem ethanol concentrations depended on the tissue distance from the pathogen (Table 2, Fig. 3) with highest amounts in the 0–2 cm zone immediately adjacent to the stained-infected tissue that contained the lowest quantities. Xylem 2–4 cm and 4–6 cm away from the infection boundary had intermediate quantities of ethanol that were not statistically different from one another or the 0–2 cm and infected tissues. Phloem at 0–2 cm from the xylem infection contained over three times the amount of ethanol as phloem at 2–4 cm, but there were no statistical differences among any of the three phloem distances (Table 2, Fig. 3).

Xylem acetone concentrations were also dependent on the tissues distance from the infection (Table 2, Fig. 3), but the opposite of ethanol with quantities in infected tissue statistically greater than those in adjacent healthy xylem 0–2, or 2–4 cm away. Acetone in xylem at 4–6 cm was not statistically different from the amounts in any of the other distance categories. Phloem acetone concentrations also did not differ statistically among samples collected at three increasing distances from the stained xylem (Table 2, Fig. 3).

3.3. Sites 1, 2 pre-dawn water potentials, root tissue water contents, and precipitation

Pre-dawn water potentials of diseased trees were similar to healthy trees indicating that the infected trees were not experiencing additional water stress over healthy trees (Fig. 4A, Table 3). All trees regardless of their condition were experiencing some water stress in September near the end of summer, but it had been eliminated by November with the fall rain (Fig. 4B). There was no tree condition by month interaction (Table 3).

Table 2

Statistical analysis results for ethanol and acetone in transect segments of phloem and xylem at different distances from the stained-infection boundary in diseased roots.

Volatile	Effect	Phloem			Xylem		
		d.f.	F	P	d.f.	F	P ^a
Ethanol	Distance	2,1.41	8.60	0.162	3,10.2	10.2	0.042
Acetone	Distance	2,8.88	1.98	0.195	3,11.7	4.18	0.005

^a Bold *P* values are statistically significant.

Mean phloem water content in roots was dependent on the month sampled only and not the tree condition or their interaction (Appendix A, Table A1, Fig. A1). Phloem water contents were lowest in September, intermediate in November and highest in May with quantities at all dates statistically different from one another (Appendix A, Fig. A1C). Alternatively, the xylem water contents were not different between diseased and healthy trees, among months, or their interaction (Appendix A, Table A1, Fig. A1).

Precipitation measured between the study sites (Corvallis Water Bureau station, 44.508°, –123.458°, elev. 180.4 m) was 3.0 cm from 1 July to 27 September, 43.6 cm from 27 September to 27 November, and 125.3 cm from 27 November to 23 May.

3.4. Sites 3, 4 root ethanol, acetone, and water contents

The root phloem ethanol contents at sites 3, 4 were similar for diseased and healthy trees (Table 4, Fig. 5A). However, the xylem from diseased trees contained 8.7 µg g⁻¹ fresh mass of ethanol, or 11.8 (95% CI 4.3, 32.4) times more than in the xylem from healthy trees (Table 4, Fig. 5B). Root acetone concentrations in the phloem and xylem of diseased trees were statistically greater than in healthy trees (Table 4, Fig. 6). The 6.3 µg g⁻¹ fresh mass in phloem was 2.1 (95% CI 1.4, 3.2) times greater than in healthy trees, while the 2.3 µg g⁻¹ fresh mass in xylem of diseased trees was 3.1 (95% CI 2.2, 4.4) times greater than in healthy trees. Mean phloem water contents in trees at sites 3, 4 were similar between diseased trees with 1.25 (95% CI 0.91, 1.57) g g⁻¹ dry mass and healthy trees with 1.26 (95% CI 0.72, 1.81) g g⁻¹ dry mass (Table 4). However, the 0.81 g g⁻¹ dry mass of water in xylem from diseased trees was statistically lower (Table 4) than the 0.98 g g⁻¹ dry mass in healthy tree roots.

3.5. Model building with root measurements at sites 1, 2

The xylem volatile concentrations measured in forty trees from sites 1, 2, twenty each presumed healthy and diseased, were used to develop separate logistic regression models by tissue type to predict tree disease condition. Xylem explanatory variables included in the initial univariate models were mean water content and maximum values for acetone, ethanol, and methanol. Purposeful selection of xylem covariates resulted in a final model containing ethanol and acetone concentrations as significant (*P* = 0.076 and *P* = 0.089 respectively) predictors of infection by *P. sulphurascens*. This model was a good fit to the data based on a ROC AUC value of 0.98 (95% CI 0.94, 1.0) and a Hosmer–Lemeshow χ^2_6 = 3.45, *P* = 0.751. In an effort to produce the simplest model possible we also examined the univariate models for both xylem ethanol and acetone. They each had good fit to the data as well, both with ROC AUC values of 0.96 (95% CI 0.91, 1.0) and Hosmer–Lemeshow χ^2_7 = 4.79, *P* = 0.685 and χ^2_7 = 7.25, *P* = 0.404, respectively. These models were then further validated based on their ability to predict diseased and healthy trees with the ethanol and acetone concentrations measured in roots of trees at sites 3, 4. None of the phloem covariates from sites 1, 2 met the thresholds for inclusion in final models

Acetone means comparisons

Means compared ^a	d.f.	t	P
S v 0-2	11.4	4.28	0.001
S v 2-4	12.3	4.18	0.001
S v 4-6	12.4	2.10	0.057
0-2 v 2-4	11.2	0.45	0.664
0-2 v 4-6	12.0	0.64	0.536
2-4 v 4-6	11.7	0.91	0.382

^a S = stained infection, 0-2, 2-4, 4-6 are distance categories in cm from the stain

Ethanol means comparisons

Means compared ^a	d.f.	t	P
S v 0-2	10.1	3.22	0.009
S v 2-4	10.5	1.07	0.311
S v 4-6	10.5	1.05	0.317
0-2 v 2-4	10.1	2.16	0.056
0-2 v 4-6	10.3	1.04	0.321
2-4 v 4-6	10.2	0.32	0.755

^a S = stained infection, 0-2, 2-4, 4-6 are distance categories in cm from the stain

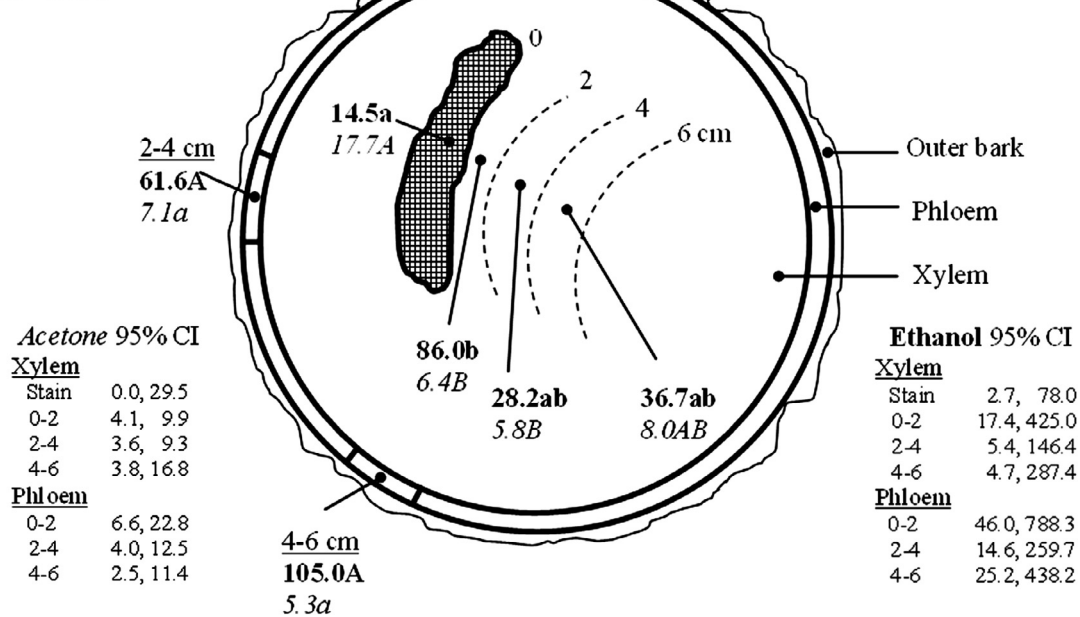


Fig. 3. Backtransformed mean ethanol (**bold**) and mean acetone (*italics*) concentrations ($\mu\text{g g}^{-1}$ dry mass) in xylem and phloem at various distances from *P. sulphurascens* stain-infected xylem in Douglas-fir root cross-sections ($n = 8$) sampled by perpendicular transects through the disks. Within each tissue-compound category those values followed by the same letters are not statistically different. Bold *P* values are statistically significant.

during the purposeful selection stage. To be sure nothing was overlooked we again tested models using sites 1, 2 acetone and ethanol concentrations alone and in combination.

3.6. Model validation and selection with root measurements at sites 3, 4

The xylem models above were independently tested for predicting infection using the 80 trees from sites 3, 4. The univariate model with ethanol concentrations performed the best overall with an ROC AUC of 0.83 (95% CI 0.74, 0.92) indicating excellent fit and a Hosmer–Lemeshow $\chi^2_8 = 10.51$, $P = 0.231$:

$$\text{LOGIT } P(i) = -2.32 + 0.3597 * (\text{root xylem maximum ethanol } \mu\text{g g}^{-1} \text{ fresh mass})$$

$$P(i) = \text{EXP}\{\text{LOGIT}(\pi)\} / [1 + \text{EXP}\{\text{LOGIT}(\pi)\}]$$

Using a 0.40 cut-point for the probability of infection resulted in a 77.5% overall correct classification across all trees and a 68.0% correct prediction for known diseased trees (Table 5). Decreasing the cut-point to 0.20 could increase the correct prediction of diseased trees to 84.0% but the ethanol concentration associated with this cut-point was less distinct than the one for 0.40. Table 6 provides further details regarding predictions with cut-point 0.40 relative to where the stain-infection was observed. There were 10 trees identified as infected without visual confirmation (false positives), but we suspect some portion of them were infected, especially those with the highest ethanol concentrations.

The model with both acetone and ethanol as covariates had an ROC AUC of 0.87 (95% CI 0.78, 0.95) indicating excellent fit and a Hosmer–Lemeshow $\chi^2_8 = 13.06$, $P = 0.110$, but none of the probability cut-points exceeded 53% correct prediction for known diseased trees. The acetone only model had an ROC AUC of 0.84 (95% CI 0.74, 0.95) indicating excellent fit and a Hosmer–Lemeshow $\chi^2_8 = 12.52$, $P = 0.129$, but here again no cut-point exceeded a 40% correct prediction for known diseased trees. Phloem models were also tested with the sites 3, 4 validation data but none of them fit particularly well, with ROC values all <0.72 and overall correct classification rates of <65.0%.

4. Discussion

4.1. Ethanol concentrations

Ethanol concentrations indicate that Douglas-fir roots infected with *P. sulphurascens* experience the most stress in cells nearest the infection zone. Lower ethanol levels further away results from the multiple dissipation processes, or sinks. First, ethanol hydrogen bonds to water (Fileti et al., 2004) and slowly diffuses in all directions into surrounding tissues. Second, it can enter xylem tracheids and be transported more rapidly upward and over greater distances in the transpiration stream as shown by flooding roots (Kreuzwieser et al., 1999; Cojocariu et al., 2004). This likely contributed to the correlation ($r = 0.729$) between concentrations in roots and lower bole in diseased trees at site 1, and to some of

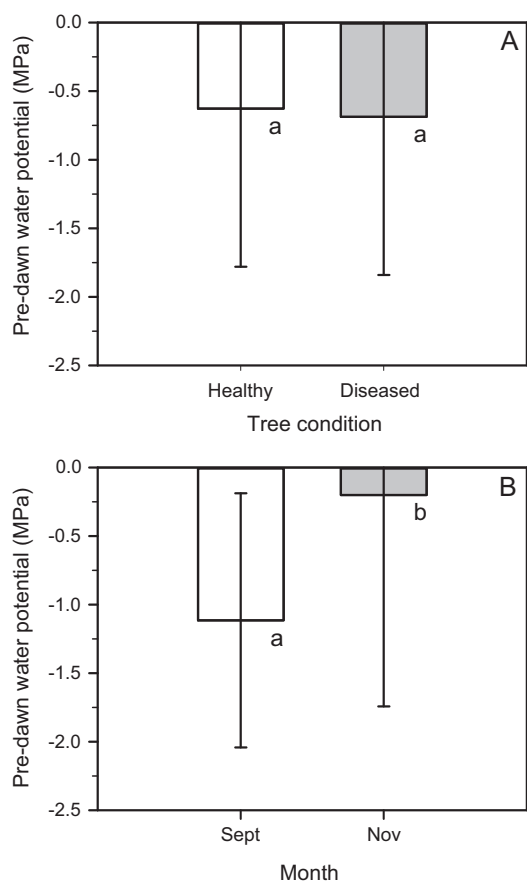


Fig. 4. Sites 1, 2 predawn (95% CI) water potentials in Douglas-fir for the main effects of tree condition (A), month sampled (B). Bars with the same letters are not statistically different, see Table 3 for *P* values.

the seasonal decline in xylem ethanol of diseased tree roots at sites 1, 2, because Douglas-fir sap flow and transpiration are sensitive to soil moisture and vapor pressure deficit (Link et al., 2014). High September ethanol concentrations coincided with dry soils that received only 3.0 cm of rainfall the previous three months. By November, sap flow would have increased in response to 43.6 cm of rainfall the previous month, causing ethanol concentrations to decrease. The subsequent 125.3 cm of winter and spring precipitation allowed extended periods of high sap flow further reducing ethanol to the low May quantities. Metabolism into other cellular constituents (MacDonald and Kimmerer, 1993) or acetaldehyde (Kreuzwieser et al., 1999; Cojocariu et al., 2004; Tohmuram et al., 2012) upon entering healthy live cells is the third major sink. Finally, low ethanol concentrations in the infected tissue may result in part from metabolism by the pathogen, as demonstrated for another tree pathogen, *Armillaria mellea* (Weinhold and Garraway, 1966).

4.2. Acetone and other headspace volatile concentrations

Acetone concentrations were the opposite of ethanol, with highest quantities within the infected-stained area. Thus, samples must contain infected tissue for acetone to be a useful disease indicator. Furthermore, the magnitudes of differences between diseased and healthy tissue were much smaller than ethanol and therefore a less sensitive biomarker. Acetone is generated from tissue components during sample heating (Kelsey et al., 1998b) and is not subject to concentration changes from diffusion or transport in water. Although acetaldehyde, methanol, and propanol concentrations were higher in one or more tissues of diseased trees than

Table 3

Sites 1, 2 statistical analysis results for pre-dawn water potentials in diseased and healthy trees.

Effect ^a	d.f.	<i>F</i>	<i>P</i> ^b
Tc	1,45.9	1.18	0.282
M	1,45.9	276.99	<0.001
Tc × M	1,45.9	2.33	0.134

^a Tc = tree condition, M = month.

^b Bold *P* values are statistically significant.

Table 4

Sites 3, 4 statistical analysis results for ethanol, acetone, and water contents in root phloem and xylem from diseased and healthy trees.

Volatile	Effect ^a	Phloem			Xylem		
		d.f.	<i>F</i>	<i>P</i> ^b	d.f.	<i>F</i>	<i>P</i> ^b
Ethanol	Tc	1,78.0	2.54	0.115	1,78.0	23.84	<0.001
Acetone	Tc	1,78.0	13.61	<0.001	1,73.1	41.17	<0.001
Water	Tc	1,77.8	0.25	0.617	1,78.0	18.95	<0.001

^a Tc = tree condition.

^b Bold *P* values are statistically significant.

healthy trees at sites 1, 2 for one or more sampling dates, none were selected as model parameters and therefore not presented for trees at sites 3, 4, or the root transects.

4.3. Water and crown symptoms

Water measurements are not useful for detecting trees infected with this pathogen. Height growth of Douglas-fir and other conifers is more sensitive and impaired earlier than radial growth when stressed for water (Rais et al., 2014; Klein et al., 2011), so reduced height growth would be expected if *P. sulphurascens* infections decrease crown water supplies sufficiently. But this appears only in the most advanced disease stages (Bloomberg and Wallis, 1979; Thies, 1983), with no external signs of loss in vigor until after the fungus grows through the bole sapwood (Buckland et al., 1954). Adequate crown water supply is also indicated by trees without crown symptoms blown down during storms although they have extensive root decay. Thies (1983) report no correlation between impaired tree growth and the number, size, or percentage of infected roots (Thies, 1983).

4.4. Model prediction of trees with laminated root rot

The model using xylem ethanol concentrations performed best for predicting trees with infection observed in roots and the root collar because they have a larger infected area with high ethanol. The models limited ability to detect trees with stain only at the root collar (stump surface) most likely resulted because their infected roots were deeper in the soil or beneath the root collar and not sampled. In some trees the lower bole heartwood is decayed, or rotted away, but otherwise surrounded by healthy sapwood with little or no infection in lateral roots (Thies and Westlind, 2005). Root cores would not detect this type of infection. Overall the model performed well given the three dimensional root structures (McMinn, 1963; Mauer and Palátová, 2012) and the limited size and number of samples per tree.

High ethanol outside the stained-infection area allows diseased root detection without sampling infected tissue, so some of the false positive trees were likely diseased. Although the model was created with measurements from 1 cm depth xylem cores, it identified diseased trees at sites 3, 4 using 6 cm depth cores suggesting that core depth in the trees sampled here was not influencing tissue ethanol concentration enough to strongly reduce its value for predicting disease. Core depth, root size, and infection position

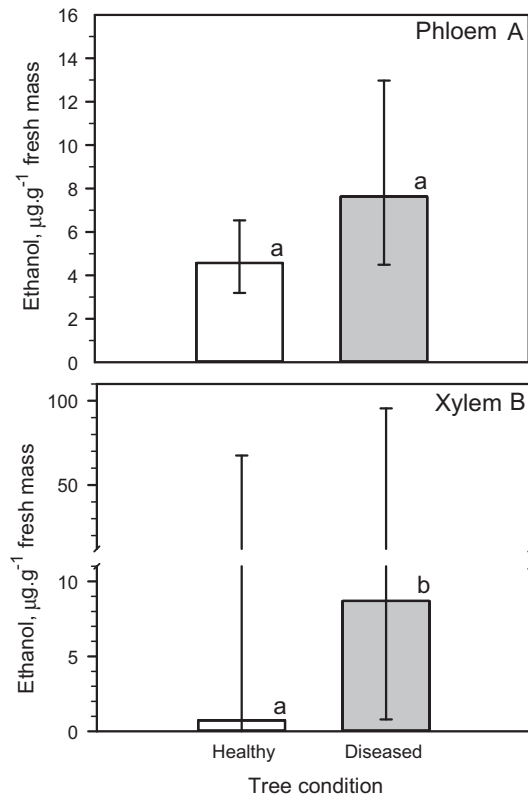


Fig. 5. Sites 3, 4 backtransformed mean ethanol (95% CI) concentrations in Douglas-fir root phloem (A) and xylem (B) of diseased and healthy trees. Bars with the same letters are not statistically different. See Table 4 for *P* values. Note the different *Y* scales and break in xylem graph B.

all interact to influence the measured ethanol concentrations. For example, deep cores from large roots increase the likelihood of reaching tissues with higher ethanol concentrations, but may increase healthy tissue proportions with lower ethanol, causing a dilution effect.

4.5. Ethanol use for hazard tree identification and with extensive ground surveys

Ethanol analysis as done in this study is a useful research tool, but would have limited use for improving hazard tree assessments since elevated concentrations typically occur close to infections. Root coring and visual inspection for stain or decay is adequate for identifying hazard trees with this disease. Decaying roots with soft tissue are easily recognized when the increment borer spins or stops moving forward. Roots here were sampled 5–30 cm beyond their entry point into the litter layer, but for hazard trees a set distance may be better. Sampling closer to the root collar can decrease root excavation time, but would miss some roots with less advanced infections. Battery powered drills quickly extract cores and need to penetrate the entire root diameter to avoid missing any infection. The number of roots sampled per tree can be adjusted to match the potential damages should the tree fall, but four major roots; one per cardinal direction seems a minimum. Sides with no accessible roots can be cored near the root collar into the bole center as a substitute. Drilling slightly above the root collar minimizes twisted cores sticking in the borer, and will help detect trees with decay restricted in their centers. The number, size, and location of roots with decay determine whether the tree is sufficiently hazardous to warrant immediate removal.

Incorporating ethanol analysis as conducted here into extensive ground surveys has two major limitations; (1) the need for

multiple samples per tree and (2) laboratory analysis with expensive instruments, or a considerable time requirement. As discussed later, we propose that ethanol release to the atmosphere contributes to bark beetle host selection in trees with advanced disease. Whether trees with less infection release sufficient ethanol to confirm the disease presence remains to be determined. If so, noninvasive field sensors could have value detecting them if they provide rapid, accurate detection and are easily portable under the harsh and variable conditions of natural environments. There are various technologies with potential to meet these criteria with further research and development, including proton transfer reaction mass spectrometry (PTR-MS; Ellis and Mayhew, 2014; Holzinger et al., 2000; Rottenberger et al., 2008; Kaser et al., 2013), intelligent electronic nose systems (Baietto et al., 2010; Naher et al., 2013), wireless smart phone sensors (Azzarelli et al., 2014), and handheld analyzers used to measure ethanol in human breath (Workman, 2012; Andrews, 2013). Dogs are also a possibility as they can detect rot in chemically treated utility poles (Davner, 1986).

4.6. Ethanol's potential role in primary beetle attraction and host selection

Standing Douglas-fir infected with *P. sulphurascens* are more vulnerable to Douglas-fir beetle, *Dendroctonus pseudosugae* Hopkins, attack than adjacent healthy trees (Goheen and Hansen, 1993), but not until the fungus has grown through the lower bole sapwood (Buckland et al., 1954). Diseased true firs, *Abies* spp., are typically colonized by the fir engraver, *Scolytus ventralis* LeConte, and sometime *Dryocetes confusus* Swaine, but only after extensive root crown infection (Lane and Goheen, 1979). This late bark beetle

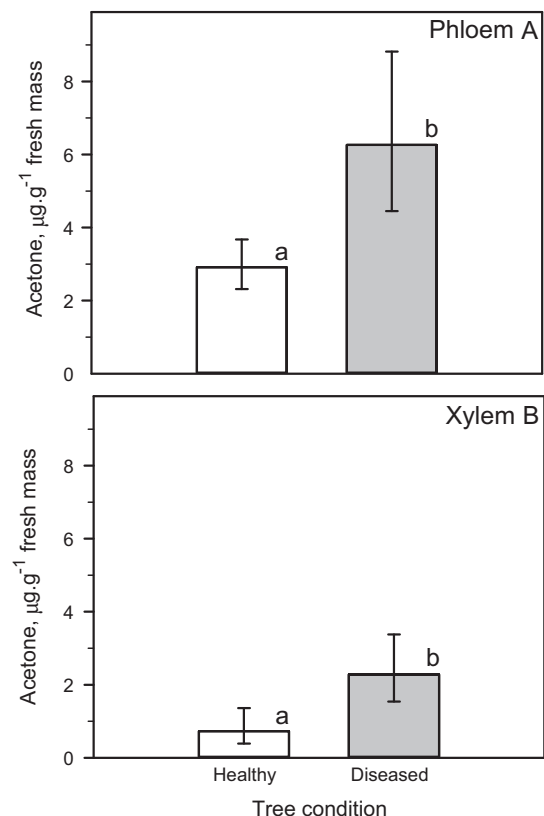


Fig. 6. Sites 3, 4 backtransformed mean acetone (95% CI) concentrations in Douglas-fir phloem (A) and xylem (B) of diseased and healthy trees. Bars with the same letters are not statistically different. See Table 4 for *P* values.

Table 5

Classification table based on the ethanol logistic regression model using a cut-point probability of 0.40. The overall rate of correct classification for both uninfected and infected trees = $(45 + 17)/80 \times 100 = 77.5\%$; overall rate of correctly predicting the infected trees only = $(17/25) \times 100 = 68.0\%$.

	Observed		Total
	Uninfected 55	Infected 25	
<i>Model predictions</i>			
Uninfected $P(i) < 0.4^a$	45	8	53
Infected $P(i) > 0.4$	10	17	27

^a Probability of infection.

arrival may result from their inability to detect trees with less severe disease, a slow weakening of the tree's chemical defenses, or some combination. Using the new ethanol information here with previous literature reports for ethanol, pathogen biology, and Douglas-fir beetle primary attractants allows us to propose a mechanism of how stress induced ethanol may play a central role in bark beetle primary host selection of trees infected with this pathogen.

Release of bole generated primary attractants is consistent with the absence of beetle attacks on wind thrown trees with severe root infection. The pathogen's infection behavior is an important factor because the mycelium grows upward through root xylem but initially enters only the lower bole heartwood near the sapwood border. It spreads along this boundary fusing with adjacent mycelium to form an arc shaped band, or circle of infection (Buckland et al., 1954; Thies and Sturrock, 1995). From this interior position the pathogen slowly grows vertically and radially through the bole sapwood, but with minimal impact on sap flow as shown with water potential measurements. When the lower bole sapwood becomes thoroughly infected vigor starts to decline, crown symptoms appear (Bloomberg and Wallis, 1979) and eventually bark beetles attack (Buckland et al., 1954).

As the infection progresses from roots to lower bole there are two sources contributing to elevated ethanol in the bole tissues. The first is ethanol synthesized in stressed roots and transported by sap flow before the pathogen enters the root collar. This would contribute to the strong correlation observed between lower bole and root concentrations in sapwood ($r = 0.729$) of diseased trees at site 1. But the absence of beetle attacks before bole infection suggests this ethanol supply is not adequate for their detection, probably because of the multiple internal sinks limiting accumulation. A portion of the ethanol entering the lower bole by sap flow is transient and will continue moving upward while another portion will diffuse into surrounding tissue diluting the concentration, including some being hydrogen bound in cellulose and other cell wall polymer (Stamm and Tarkow, 1950; Mantanis et al., 1995)

temporarily holding it in place. Any diffusing radially outward will enter healthy cambium and phloem where it can be metabolized (MacDonald and Kimmerer, 1993). These sinks limit the quantity escaping through the outer bark. Low ethanol levels in Douglas-fir boles has been proposed as an explanation for the absence of Douglas-fir beetle attacks on trees severely stressed by foliar infection of Swiss needle cast (Kelsey and Manter, 2004). Roots with advanced infection may also release some ethanol into surrounding soils but what portion can escape above ground is unknown. It is likely minimal from roots beneath the bole or deeply buried because of soil water absorption and uptake by healthy tree roots (Joseph and Kelsey, 2000) or other plant roots, hydrogen bonding to any cellulosic matter or charcoal (Stamm and Tarkow, 1950; Mantanis et al., 1995; Kelsey et al., 2013), or a portion might be metabolized by microbes, as in live tree cells (MacDonald and Kimmerer, 1993).

A second source of bole ethanol can be initiated after the pathogen begins stressing live xylem cells in the inner most sapwood. However, there is no immediate change in ethanol release at the bark surface for two reasons. First, the inner bole xylem has a limited live cell volume (Stockfors and Linder, 1998) and produces much lower quantities of ethanol than the roots and phloem (Kelsey et al., 1998a) or cambium (Kimmerer and Stringer, 1988). Second, its accumulation there will be strongly influenced by the three sinks mentioned above. The abundance of surrounding tissue allows dilution by diffusion in any direction, movement radially across the sapwood is vulnerable to unhindered sap flow transport upward, and any entering healthy cambium and phloem is at risk of metabolism. However, these factors all change radically once the pathogen spreads through the sapwood and begins stressing cambium and phloem. First, ethanol synthesis increases because both tissues have greater live cell volume (Stockfors and Linder, 1998) and can produce more ethanol than sapwood (Kelsey et al., 1998a; Kimmerer and Stringer, 1988). Second, the radial distance from site of synthesis to the atmosphere is shorter and the accompanying sink strengths decline. While diffusion can still occur in all directions, it no longer passes through sapwood, eliminating the sap flow sink. Also, as the pathogen penetrates cambium and phloem they most likely lose some ability to metabolize ethanol and this sink also declines. At some point ethanol release to the atmosphere increases as demonstrated by trapping it on the bark surface after infusing sapwood in bole segments (Kelsey et al., 2013), from boles with flooded roots (Ranger et al., 2013), or lodgepole pine stems with active heartwood decay fungi (Gara et al., 1993).

Bole released ethanol mixed with Douglas-fir monoterpenes, or resin will be a strong primary attractant for *D. pseudosugae* (Jantz and Rudinsky, 1966; Rudinsky, 1966; Pitman et al., 1975). Fresh Douglas-fir monoterpenes or oleoresins released alone function as primary attractants for this beetle (Rudinsky, 1966; Johnson

Table 6

Ethanol logistic regression model predictions at cut-point probability of 0.40 sorted by observed stain locations.

Model predictions:	Observed stain location	No. trees (observed)	%
<i>Infected:</i>		(25)	
Correct	Stump surface	1/7	14.3
Incorrect, false negative	Stump surface	6/7	85.7
Correct	Core sample	5/7	71.4
Incorrect, false negative	Core sample	2/7	28.5
Correct	Stump and core	11/11	100.0
Incorrect, false negative	Stump and core	0/11	0
<i>Not infected:</i>		(55)	
Correct	No stain	45/55	81.8
Incorrect, false positive? ^a	No stain	10/55	18.2

^a These may or may not be false positive trees as one of their root cores may have been near infected tissue that was not observed. Additional root sampling or excavation would be needed to verify infection.

and Belluschi, 1969; Pureswaran and Borden, 2005), but sufficient quantities may not be released alone from *P. sulphurascens* infected Douglas-fir as there is no external resin leakage or resinosis prior to beetle attacks. Ethanol alone can also function as a primary attractant for *D. pseudosugae* (Stoszek, 1973; Pitman et al., 1975), but host monoterpenes are always present and their release rates may increase with ethanol release rates, as demonstrated for healthy and diseased lodgepole pine (Gara et al., 1993). Pioneering *D. pseudosugae* attacks need not start at the root collar given their preference for positions higher on the bole (Furniss and Kegley, 2014) and their unfocused response to attractants that results in attacks on unbaited trees 15 m or more from baited trees (Rudinsky, 1966; Johnson and Belluschi, 1969; Thier and Patterson, 1997). Any successful attacks by pioneering beetles will quickly initiate stronger secondary attraction when monoterpene and ethanol vapors mix with pheromones (Pitman et al., 1975; Ross and Daterman, 1995). We suspect ethanol mixed with monoterpenes also attract *S. ventralis* to trees infected with *P. sulphurascens* given the absence of known pheromone-mediated secondary attraction for this bark beetle (Macías-Sáman et al., 1998). Additional experiments on ethanol and monoterpene emissions from *P. sulphurascens* infected trees are needed to further validate this proposed mechanism.

4.7. Potential changes in chemical defense against bark beetles

When the first pioneering *D. pseudosugae* beetle(s) initiate their attack on *P. sulphurascens* stressed Douglas-fir the quality and quantity of constitutive oleoresin, followed by induced oleoresin flow, function as the critical chemical defense (Jantz and Rudinsky, 1966; Rudinsky, 1966), as for other resinous conifers (Franceschi et al., 2005). In general, a reduction in tree vigor is accompanied by reduced resin flow (Fettig et al., 2007). In south-western ponderosa pine, resin flow declines in conjunction with reduced basal area increment growth (BAI) (McDowell et al., 2007), because the number of resin ducts correlate positively with BAI growth (Kane and Kolb, 2010). The latter investigators found trees surviving drought-associated bark beetle attacks had larger resin ducts, at higher densities and a greater proportion of the annual growth than in dead trees. Douglas-fir resin flow also declines when BAI decreases in response to needle loss and infection from the Swiss needle cast fungus, *Phaeocryptopus gaeumannii* (Rhode) (Kelsey and Manter, 2004). However, since Douglas-fir infected with *P. sulphurascens* do not show changes in radial growth or water status, both factors that can directly influence resin flow for short periods (Lorio, 1994; Fettig et al., 2007), until after bole sapwood is thoroughly infected, we suspect minimal changes in constitutive or induced resin flow chemical defense until the most advanced stages of infection. Pioneering Douglas-fir beetles will attack healthy trees with unimpaired resin flow defenses when host primary attractants are fresh enough (Johnson and Belluschi, 1969). We suspect enhanced release of ethanol and monoterpenes may stimulate them to attack *P. sulphurascens* prior to substantial reductions in constitutive or induced resin flow defense, but such attacks may not be successful until there is sufficient loss of tree vigor to further impair these defenses. Even if these defenses do mitigate successful tree colonization, it is highly likely that ethanol release in conjunction with host monoterpenes drive the initial host finding and selection of *P. sulphurascens* stressed trees by pioneering beetles.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.foreco.2015.10.039>.

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