# Ethanol in Douglas-fir with black-stain root disease (*Leptographium wageneri*)

## Rick G. Kelsey and Gladwin Joseph

**Abstract**: Diseased and healthy Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) were identified at two black-stain root disease centers, caused by *Leptographium wageneri* var. *pseudotsugae* Harrington & Cobb, in the Oregon Coast Range near Coos Bay. Phloem and sapwood near the root collar were sampled monthly for 1 year, whereas roots were sampled in October and November. Ethanol concentrations in sapwood of diseased trees near the root collar were 4–24 times higher than in healthy trees for all months of a year, except January and June. Roots from diseased trees in October had 5 times more ethanol in the phloem and 19 times more ethanol in the sapwood than corresponding tissue from healthy trees. Ethanol concentrations in roots from diseased trees in November were no different from October. Within trees, ethanol concentrations varied substantially among positions around the root collar and among different roots. Ethanol may play an important role in the biology of *L. wageneri* and beetle–pathogen interactions in Douglas-fir. Ethanol also may be useful in detecting stressed or diseased trees.

**Résumé** : Des Douglas taxifoliés (*Pseudotsuga menziesii* (Mirb.) Franco) sains et malades ont été repérés dans deux centres d'infection d'une maladie de racines causée par le *Leptographium wageneri* var. *pseudotsugae* Harrington & Cobb, dans la chaîne côtière de l'Oregon, près de Coss Bay. Le phloème et le bois d'aubier situés près du collet ont été échantillonnés à chaque mois pendant 1 an, tandis que les racines ont été échantillonnées en octobre et en novembre. Chez les arbres malades, les concentrations en éthanol près du collet étaient 4 à 24 fois plus élevées que chez les arbres sains pendant tous les mois de l'année, excepté en janvier et en juin. En octobre, les racines des arbres malades avaient 5 fois plus d'éthanol dans le phloème et 19 fois plus dans le bois d'aubier que dans les tissus correspondants chez les arbres sains. Les concentrations en éthanol dans les racines des arbres malades étaient les mêmes en octobre et en novembre. Chez un arbre, les concentrations en éthanol variaient passablement selon la position autour du collet et entre différentes racines. L'éthanol pourrait jouer un rôle important dans la biologie du *L. wageneri* et les interactions insecte–pathogène chez le Douglas taxifolié. L'éthanol pourrait aussi être utile pour détecter les arbres stressés ou malades.

[Traduit par la Rédaction]

## Introduction

Many plant tissues synthesize ethanol when subjected to hypoxic or anoxic conditions (Kimmerer and MacDonald 1987; Harry and Kimmerer 1991). Ethanol occurs in the phloem and xylem sap of various tree species during the spring (Kimmerer and Stringer 1988; MacDonald and Kimmerer 1991), possibly because of hypoxia in the cambium generated by rapid aerobic respiration rates needed to support growth (Eklund 1990; Harry and Kimmerer 1991). Ethanol synthesis may allow tissues to avoid harmful effects of acidosis, and produce small quantities of ATP to maintain necessary metabolic activities until aerobic conditions are restored (Vartapetian and Jackson 1997). High ethanol concentrations occur in tree tissues under various conditions, including roots and stems of flooded seedlings (Crawford and

Finegan 1989; Joseph and Kelsey 1997), topped or girdled trees (Sjödin et al. 1989), stumps (von Sydow and Birgersson 1997), stems containing heart rot (Gara et al. 1993), and severed stems left on the forest floor through winter (Kelsey 1994*a*, 1994*b*; Kelsey and Joseph 1997).

Ethanol has long been known to attract a variety of scolytid beetles to artificial traps when released alone or in various binary or tertiary combinations with monoterpenes and pheromones, (Moeck 1971; Pitman et al. 1975; Borden et al. 1982; Klimetzek et al. 1986; Phillips et al. 1988; Liu and McLean 1989; Schroeder and Lindelöw 1989). Studies with injured or dying trees have demonstrated that ethanol functions as a key kairomone for ambrosia beetles, *Trypodendron lineatum* (Oliver), *Gnathotrichus retusus* (LeConte), and *G. sulcatus* (LeConte) (Coleoptera: Scolytidae). Their selection and colonization of aged and delimbed logs (Moeck 1970; Kelsey 1994*a*, 1994*b*), injured trees (Sjödin et al. 1989), or certain host species over others (Kelsey and Joseph 1997) is strongly influenced by ethanol.

In coniferous forests of western North America trees infected with fungal pathogens are known to be selectively attacked and killed by bark beetles (Goheen and Hansen 1993). However, the nature and extent that changes in physiology or biochemistry of diseased trees contribute to their increased susceptibility to bark beetles remains unclear. Low

vigor and diseased lodgepole pine (*Pinus contorta* Dougl. ex. Loud) have a different terpenoid and phenylpropanoid composition than healthy trees (Nebeker et al. 1995) and are often selectively attacked by mountain pine beetle (*Dendroctonus ponderosae* Hopkins). In south-central Oregon, these beetles also prefer to land on lodgepole pine with fire scars and heartwood decay, rather than unscarred trees with no decay (Geiszler et al. 1980; Gara et al. 1984). In a subsequent study in central Oregon, lodgepole pine with heartwood decay were found to emit more ethanol and monoterpenes than healthy neighbors (Gara et al. 1993).

Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) and ponderosa pine (*Pinus ponderosa* Dougl. ex Laws.) with black-stain root disease (BSRD), caused by *Leptographium wageneri* (Kendrick) Wingf. (Goheen and Hansen 1993) are attacked by bark beetle and weevil vectors of *L. wageneri*. These insects are attracted to ethanol released from baited traps (Harrington et al. 1985; Witcosky et al. 1987; Bedard et al. 1990) and to root segments infected with BSRD (Witcosky et al. 1987). To our knowledge no one has reported ethanol in roots or stems of trees infected with BSRD, and we hypothesized they would have higher ethanol concentrations than tissues from healthy trees. We were particularly interested in seasonal and spatial variation, therefore phloem and sapwood collected near the root collar of diseased and healthy Douglas-fir were sampled and analyzed for ethanol each month for a year. Ethanol was also measured from roots of these same trees in October and November.

## Material and Methods

Douglas-fir trees, 35–40 years old, were sampled in two BSRD centers (43°19′43″N; 123°51′52″W for site 1 and about 1 km northeast for site 2) at 427 m elevation along a ridge top in the Oregon Coast Range, Coos County, east of Coos Bay. Dead trees had characteristic sapwood staining at the base of stems (Wagener and Mielke 1961). Typical of BSRD, both disease centers were located where soil displacement and probably compaction had occurred 35–40 years ago during stand harvesting (Hansen 1978; Hansen et al. 1988). At each site, eight symptomatic and eight nonsymptomatic codominant trees were selected and tagged. Symptomatic trees were identified with one or more of the following symptoms: reduced terminal growth, sparse or chlorotic foliage, a distress cone crop, or basal resinosis (Witcosky and Hansen 1985; Hansen et al. 1988). In addition, eight control trees were selected from the forest adjacent to each disease center. These trees were codominant with normal leader growth and dense green foliage. Symptomatic, nonsymptomatic, and control trees had stem diameters (cm) at breast height of 30.8 ± 2.6 (mean ± SE), 31.0 ± 1.4, and 32.2 ± 1.9 at site 1 compared with 28.4 ± 1.9, 29.2 ± 1.3, and 30.1 ± 1.7, respectively, at site 2. There were no significant differences in tree diameters between the two sites.

Trees were sampled once a month beginning in February 1994 and continuing through January 1995. Each month, one increment core (5 mm i.d.) was taken from the stem base, near the root collar. The phloem (with cambium attached) and 1.0 cm of sapwood were separated and sealed in vials (15 × 45 mm o.d.), frozen with dry ice in the field, and stored at –36°C in the laboratory. Core holes were plugged with snug fitting corks. Each month, cores were taken 3–6 cm around the stem circumference from the previous core. To minimize intertree inoculation with BSRD, controls were sampled first, then nonsymptomatic, and finally symptomatic trees. Also, the borer was dipped into a 20% Chlorox®[2] solution for a few minutes after removing each core.

Three or four roots from all trees were sampled in October. Soil was removed and a core taken on top of the root at 30 cm or less from the stem. Holes were plugged and cores processed as described above. Roots with high ethanol concentrations in October were resampled in November after heavy autumn rain to determine whether changes in soil moisture affected ethanol concentration. Roots from 10 symptomatic (four at site 1 and six at site 2), three nonsymptomatic (all at site 1), and six control trees (three at each site) were resampled at the same position as October, but off to the side about 5–8 cm.

To confirm the presence of BSRD, a minimum of three roots were partially excavated and examined from each tree in March 1997. Roots were chopped at about 40 cm from the stem base where stain typically was found in diseased trees. If no disease was found the excavation continued outward until it was observed, or further excavation was not practical. Nonsymptomatic and control trees without detectable black-stain or evidence of other root diseases were considered "healthy."

### Headspace analysis

Tissue ethanol concentrations were determined by headspace gas chromatography. Vials were placed on ice for a few minutes, allowing the cores to thaw before weighing them into preweighed headspace vials (22 × 75 mm, 22 mL). Each vial was sealed immediately with a septum and heated at 102°C for 30 min to deactivate enzymes. Vials were placed in a Perkin Elmer HS40 headspace autosampler connected to a Hewlett Packard 5890 gas chromatograph (GC) equipped with a flame ionization detector. The HS40 settings were: 90°C sample; 100°C needle; 60°C transfer line; 4 min GC cycle; 20 min thermostat; 1 min pressurization; 0.04 min injection; 0.2 or 0.1 min withdrawal; and 120 kPa of helium carrier gas. The GC was fitted with a 30 m × 0.32 mm (i.d.) J&W Scientific DBWax column with a 25-$\mu$m film thickness. The column was threaded through the GC injector port and HS40 heated transfer line allowing direct on-column injection. The GC settings were: 50 or 60°C injector; 250°C detector; and 50°C isothermal oven. Each sample was analyzed twice (the HS40 set in the constant mode) with vial venting between runs. Ethanol concentrations were calculated by the multiple headspace extraction technique described by Kolb et al. (1984). The instrument was calibrated with two vials containing 5 $\mu$L of an ethanol–water standard solution for each carousel of 38 samples. After analysis, vials were uncapped and oven-dried at 102°C for 16 h, cooled in a desiccator for 30 min, and weighed to determine tissue water content. Most analyses were completed within a week of collection. The ethanol peak in tissues from one diseased tree was confirmed by GC-mass spectrometry using a Hewlett Packard 5890 Series II GC with a 5970 mass selective detector. Columns used were the DBWax described above, and a J&W Scientific DB-1, 30 m × 0.25 mm (i.d.), 25-$\mu$m film thickness.

### Data analysis

Ethanol concentrations in diseased and healthy trees were subjected to analysis of variance (ANOVA) using mixed linear models with individual trees as experimental units (SAS Proc MIXED; SAS Institute Inc. 1996). For monthly samples, the phloem and sapwood were analyzed separately to ensure adequate degrees of freedom in the models, and the effect of month was tested as a repeated measure. For roots, the effects of tissue type and disease

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[2] The use of trade names is for the information and convenience of the reader and does not constitute official endorsement or approval by the U.S. Department of Agriculture.

**Fig. 1.** Mean (±SE) monthly (Feb. 1994 to Jan. 1995) ethanol concentrations in phloem and sapwood from above the root collar of diseased ($n = 12$) and healthy ($n = 30$) Douglas-fir trees.
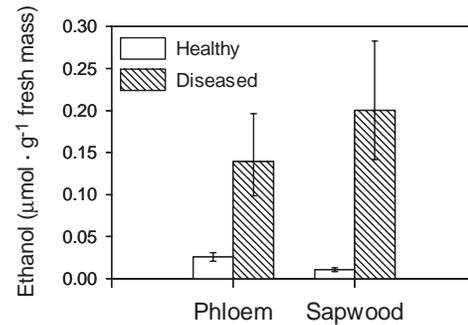


**Fig. 2.** Mean (±SE) ethanol concentrations in phloem and sapwood from roots of diseased ($n = 12$) and healthy ($n = 30$) Douglas-fir trees sampled in October 1994.
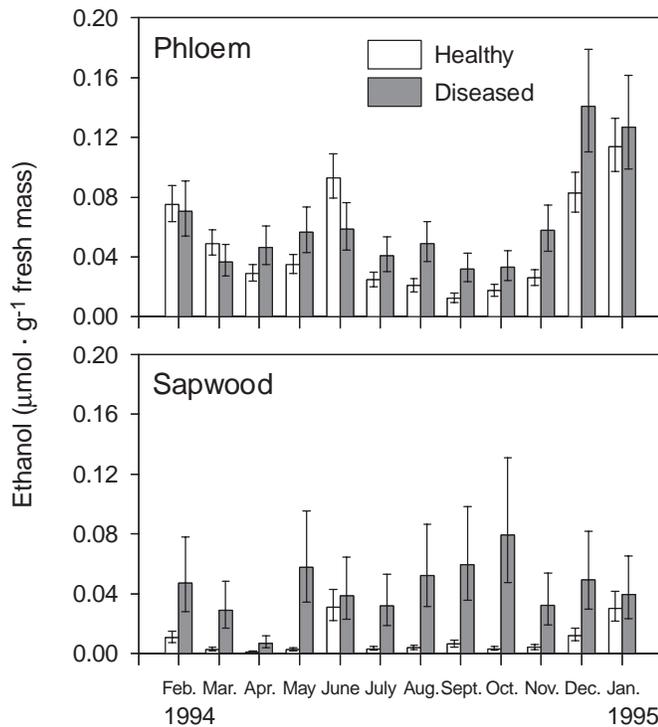


status were tested as a $2 \times 2$ factorial, and the observations for ANOVA were the mean ethanol concentrations from four roots. Significant differences among means were identified using Fisher's Protected least-significant difference at $\alpha = 0.05$. All data were tested for homogeneity and normality before analysis and natural log transformed when necessary. Back-transformed means and standard errors are presented for transformed data.

## Results

Across sites, BSRD was confirmed in 10 symptomatic and two nonsymptomatic trees by partial root excavation. Apparently the amount of disease in the latter two was not sufficient to produce crown symptoms when initially selected. In a related study with ponderosa pine, crown symptoms were not readily apparent until more than one third of the root system was diseased at 30 cm below the root collar (Kelsey et al. 1998a). There was no disease found in the remaining 14 nonsymptomatic or 16 control trees, which were grouped together as "healthy" for analysis. There were six symptomatic trees without detectable BSRD not included in the analysis. One of them was infected with laminated root rot, *Phellinus weirii* (Murr.) Gilb., and another an unknown disease. The remaining four may have been infected with *L. wageneri*, but it was undetected because their entire root systems were not excavated. Alternatively, they were not diseased, but their crowns exhibited diseaselike symptoms, possibly because growing conditions on disturbed soils at the disease centers were less than ideal.

Ethanol concentrations were different in healthy and diseased trees, and the effect of month was significant for both phloem ($P = 0.060$) and sapwood ($P = 0.004$). Ethanol con-

centrations in phloem from diseased trees were significantly higher than in healthy trees during August, September, November, and December (Fig. 1). In sapwood, ethanol concentrations were significantly higher in diseased trees than in healthy trees for all months except June and January. In most months there was one or more diseased tree with an exceptionally high ethanol concentration (above 0.50 $\mu mol \cdot g^{-1}$ fresh mass) in the sapwood. Three diseased trees had exceptionally high ethanol concentrations in May, August, and September, and four trees had high concentrations in December. It was not always the same diseased trees with high ethanol concentrations from month to month. When sapwood from a diseased tree had a high ethanol concentration, the concentrations in adjacent phloem were usually not correspondingly high.

Roots were sampled in October near the end of a dry summer. Total rainfall in the preceding 2-month period was 1.7 cm. Mean ethanol concentrations were dependent on tissue type and disease status ($P = 0.003$). Phloem and sapwood from roots of diseased trees (Fig. 2) contained greater ethanol concentrations than corresponding tissues from healthy trees ($P = 0.001$ for both tissues). In roots of healthy trees, ethanol concentrations in the phloem were greater than in sapwood ($P = 0.001$), whereas in diseased trees there were no differences in ethanol concentrations between tissues ($P = 0.283$). In diseased trees there was high variation in ethanol concentrations among roots from the same tree (Fig. 2). Typically only one or two roots had moderate to high ethanol concentration in their tissues. The highest concentrations of ethanol measured were in the phloem and sapwood from roots. A root from one diseased tree had a high ethanol concentration in October, but sapwood from above the root collar never exhibited a correspondingly high ethanol concentration on any date during the year. Also, two trees had high ethanol concentrations in their sapwood near the root collar on several dates (data not shown), but none of their roots had high ethanol concentrations in October.

Roots were sampled again in November after substantial rainfall. Twenty-two centimetres fell between 8 October and the 16 November sampling date. Ethanol concentrations in the phloem and sapwood of diseased trees did not differ significantly between November and October. In roots of healthy trees, the phloem ethanol concentrations from November were lower ($P = 0.049$) than October, with no differences for their sapwood. Phloem and sapwood from roots of

healthy trees had an 18–31% higher water content than corresponding tissues in diseased trees, irrespective of site or date. A lower water content in diseased roots is consistent with reduced water conductivity in diseased roots of ponderosa pine (Joseph et al. 1998).

## Discussion

Our results demonstrate that sapwood near the root collar of Douglas-fir with BSRD often contains substantially higher concentrations of ethanol than healthy trees. Anaerobic respiration in response to hypoxia or anoxia is the source of ethanol in plants (Harry and Kimmerer 1991). Consequently, tissues in diseased trees had been exposed to more hypoxia than in healthy trees. High ethanol concentrations were probably produced by tree tissues since they maintain constitutive levels of anaerobic enzymes (Kimmerer and Stringer 1988; MacDonald and Kimmerer 1991; Kelsey et al. 1998b). The pathogen could synthesize some ethanol, but this remains to be demonstrated. Hypoxia may have been generated by rapid aerobic respiration in tree tissues associated with their defense response to the infection and by respiration of *L. wageneri*.

Sapwood ethanol concentrations varied tremendously within individual trees at a single point in time as demonstrated by roots of diseased trees in October (Fig. 2). Similar spatial variation in ethanol concentrations was observed among cardinal positions on the root collar of ponderosa pine trees infected with multiple pathogens, including BSRD (Kelsey et al. 1998a). This position effect probably contributed substantially to the high variation in monthly ethanol concentrations near the root collar, because the sampling position changed slightly each month. Positional variation probably results from a sporadic occurrence of the pathogen, and various physiological factors influencing the synthesis and metabolism of ethanol. Infections can be highly variable in the degree of staining and sapwood colonization by hyphae, ranging from near complete colonization of all xylem tracheids in an area to a diffuse, sparse colonization pattern with limited staining (Joseph et al. 1998; Kelsey et al. 1998a), and there can be multiple infection sites along a single root.

The quantity of ethanol a tissue can synthesize will depend on the length of hypoxia, supply of carbohydrate substrate, and amount of nitrogen available for production of anaerobic enzymes regulating the synthesis (Kelsey et al. 1998b). Ethanol is nonionic and readily passes through cell membranes and cell walls, so after synthesis it can diffuse from the source. If it enters the transpirational stream it can move rapidly with xylem sap (Crawford and Finegan 1989; MacDonald and Kimmerer 1991; Joseph and Kelsey 1997), and finally it can be metabolized if it enters tissues with an adequate supply of $O_2$ (MacDonald and Kimmerer 1993). Low ethanol concentrations in Douglas-fir phloem adjacent to sapwood with high concentrations could have resulted from metabolism in the cambium and phloem (MacDonald and Kimmerer 1993) and loss to the atmosphere by diffusion through the bark (Gara et al. 1993). Heavy rainfall and subsequent increase in soil moisture did not affect ethanol concentrations in roots of diseased trees between October and November. This change in soil environment did not appear to have much influence on ethanol concentrations. Flooding, however, will cause roots to synthesize ethanol that will be transported with xylem sap up the stem (Crawford and Finegan 1989; MacDonald and Kimmerer 1991; Joseph and Kelsey 1997).

In spite of the variation in ethanol, high concentrations in sapwood near the root collar at some time during the year was strongly associated with BSRD. In a related study, about half of the ponderosa pine sampled with multiple root pathogens, including BSRD, contained high ethanol concentrations at the root collar (Kelsey et al. 1998a). Therefore, ethanol may be useful in detecting BSRD and possibly other root diseases. However, not all diseased trees showed high ethanol concentrations at the root collar, so its absence does not indicate that roots are healthy. High ethanol concentrations might have been detected in a greater number of diseased trees had we sampled the root collars more intensively on any given date.

The bark beetle *Hylastes nigrinus* (Mannerheim) and the weevil *Steremnius carinatus* (Mannerheim) are vectors of *L. wageneri* in Douglas-fir (Harrington et al. 1985; Witcosky et al. 1986a, 1986b). They are attracted to pitfall traps baited with ethanol, α-pinene, or their combination (Harrington et al. 1985; Witcosky et al. 1987; Bedard et al. 1990). Both insects respond more strongly to Douglas-fir roots with BSRD than healthy roots (Witcosky et al. 1987), probably in part because diseased roots contain higher ethanol concentrations. However, diseased roots may also release higher quantities of α-pinene and other monoterpenes from localized patches of resin soaked bark or wood (Witcosky and Hansen 1985; Hessburg and Hansen 1987).

High ethanol concentrations in trees with BSRD may contribute to the development of existing disease centers (Hansen and Goheen 1988) and initiation of new centers on adjacent disturbed sites. After the first tree at a new disease center becomes infected with BSRD, increases in tissue ethanol concentrations enhance the likelihood of additional attacks. Subsequently, some *H. nigrinus* offspring emerging from this infected tree are likely to carry spores of *L. wageneri* to roots of adjacent healthy trees where they feed to complete maturation (Zethner-Møller and Rudinsky 1967) and may expand the disease center, or they may be attracted to disturbances at new sites that were previously disease free. Healthy Douglas-fir stands may be at risk of attracting insect vectors after precommercial thinning (Harrington et al. 1985; Witcosky et al. 1986a, 1986b) because stumps release monoterpenes and ethanol (R.G. Kelsey and G. Joseph, unpublished data). Roots of live trees adjacent to such stumps are often wounded by *H. nigrinus* and could be infected with BSRD if inoculum is present (Harrington et al. 1985; Witcosky et al. 1986a).

Ethanol in roots also might enhance the growth of *L. wageneri* by serving as a carbon and energy source. *Leptographium wageneri* cannot penetrate cell walls of healthy live cells because it does not produce cellulolytic or pectolytic enzymes (Smith 1967, 1969; Hessburg and Hansen 1987). This restricts hyphal growth to the lumen of tracheids, limiting its nutritional supply to substances "leaked" from parenchyma into the tracheids, to cytoplasmic constituents released during autolysis of maturing tracheids (Hessburg and Hansen 1987), or to materials carried in

xylem sap which are low in nutritional quality. Ongoing experiments have confirmed that *L. wageneri* can grow when ethanol is the only source of carbon and energy available (unpublished data). *Armillaria mellea* (Vahl. ex Fr.) Kummer can also metabolize ethanol for growth (Weinhold and Garraway 1966). In addition, ethanol allows *A. mellea* to enhance its use of glucose, fructose, and sucrose for growth of mycelia and rhizomorphs. Ethanol synthesis and diffusion through cell membranes and cell walls may provide *L. wageneri* with an important nutrient source derived from the host.

Ethanol in diseased trees may be a key factor in beetle–pathogen–tree interactions. Although the role of kairomones in host selection by aggressive bark beetles is not yet clear (Raffa et al. 1993), there is evidence that certain aggressive species such as *D. ponderosae* Hopkins and *D. brevicomis* LeConte selectively colonize pine trees infected with pathogens (Goheen and Cobb 1980; Gara et al. 1984; Goheen and Hansen 1993). Like Douglas-fir, diseased lodgepole and ponderosa pine also contain higher ethanol concentrations than healthy trees (Gara et al. 1993; Kelsey et al. 1998*a*). The accumulation or release of ethanol in response to physiological stress in a tree may provide various beetle species with a chemical signal identifying it as a potentially weakened individual. Constitutive and inducible defense mechanisms in stressed trees may be weaker than in healthy trees (Nebeker et al. 1993), so successful colonization by a small number of beetles may be easier in stressed trees. Diseased trees may be important in maintaining endemic populations and the buildup of epidemic populations of aggressive beetles (Geiszler et al. 1980).

In conclusion, Douglas-fir infected with BSRD often have high ethanol concentrations in their roots and stems near the root collar. Since ethanol alone can attract beetles, or enhance beetle attraction to monoterpenes and pheromones, trees containing high ethanol concentrations are at risk of attack and colonization. A better understanding of ethanol synthesis in diseased trees might contribute to development of new alternatives for managing endemic beetle populations and help mitigate the spread of pathogens, such as *L. wageneri*, vectored by insects.

## Acknowledgments

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