Inhibition of Wood-Decay Fungi by Wetwood of White Fir

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ABSTRACT

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Growth assays of wetwood fluid in vitro suggested that wetwood of white fir is inhibitory to *Heterobasidion annosum* (= *Fomes annosus*). Redox potentials and oxygen diffusion rates indicated hypoxic conditions in wetwood, and *H. annosum* and other decay fungi were sensitive in vitro to such oxygen availabilities. Although C_x cellulase of *H. annosum* was unaffected by O_2, laccase activity was highly oxygen-dependent, indicating that wood-decay capability may be even more sensitive to O_2 availability than is growth on laboratory media. In addition, low molecular weight organic acids (acetic, propionic, butyric) were present in wetwood in concentrations that inhibited or prevented fungal growth. The data indicated that properties of intact wetwood may substantially restrict or prevent colonization and decay by *H. annosum* and that individual trees vary greatly in properties influencing fungal growth.

Additional key words: Abies concolor, Coriolus versicolor, Fomes fomentarius, Fomitopsis officinalis, Fomitopsis pinicola, Hirschioporus abietinus, Phaeolus schweinitzii, Phellinus weirii, Poria cocos, Poria placenta.

Heartwood in trees is generally older wood that no longer conducts water and has no living tissue. Thus, it is incapable of active resistance against fungi causing root, butt, and heart rot. Wood may be conditioned for passive resistance to such fungi during parenchyma death as part of normal heartwood formation (20) or response to injury (17).

Extractives are often present in much greater quantities in heartwood than in sapwood and have been implicated in heartwood resistance to several fungi (35). Some members of Pinaceae lack recognized fungistatic phenols and terpenes in heartwood. *Abies* spp. (firs) in particular are generally nonresinous and low in heartwood extractives (9). Although heartwood of *Abies* spp. is considered by some to be among the least resistant of conifer heartwoods in service (8), its resistance in the standing tree may be considerably greater. Etheridge (15) demonstrated the heat-sensitive fungistatic property of heartwood of *Abies grandis*.

Heartwood of *Abies* spp. commonly contains wetwood, in which wood appears to be water-soaked and dark-colored (41,44). In the first description and study of wetwood (in pine), Lagerberg (28) mentioned a forester who considered that "wetwood arises directly in the heart through some kind of fermentation" and is "secreted as a protection against decay." Although Lagerberg dismissed those ideas, recent work suggests: an internal nonpathological etiology for wetwood in *Abies* spp. (12,44,45), the presence of fermentative bacteria in wetwood of all species examined (42), and inhibition of fungal growth at the O_2 concentrations [O_2] found in wetwood of cottonwood (40).

Early papers on O_2 in heartwood of hardwoods reported concentrations of O_2 ([O_2]) in gas extracted from the wood to be commonly $<5\%$ and occasionally $<1\%$ (11,25,39). In wetwood of black cottonwood [O_2] ranges from 0 to 2.5% O_2 (40), while that of elm wetwood reported in an early study was 4.5% (10). Oxygen is apparently absent in wetwood of cottonwoods, which contains methane (47) and obligately anaerobic bacteria.

The wood decay fungi studied thus far apparently require O_2, but show a sharp growth increase at low levels of O_2. Various studies have identified this critical region of O_2 dose-response as 1–2% for *Polystictus* (*Coriolus*) *versicolor* (36) and 0–10% for several fungi, including *P. versicolor* (24). Gundersen (16) reported that *Fomes annosus* (Fr.) Cooke [= *Heterobasidion annosum* (Fr.) Bref.] grew as fast in a "trace" of O_2 as in air, but no growth occurred in 0% O_2. In $<0.1\%$ O_2, the growth of two fungi from cottonwood was greatly reduced and decay was negligible (40). Recent studies with *Phanerochaete chrysosporium* indicate that induction and activity of the ligninolytic system is dependent on [O_2] (3) and that even degradation of free cellulose to substrates for glycolysis may involve enzyme systems directly or indirectly dependent on O_2 (13).

Observations of natural decay columns caused by *H. annosum* white fir [*Abies concolor* (Gord. and Glend.) Lindl.], and circumstantial evidence from two inoculation experiments, suggest that wetwood may be inhibitory to *H. annosum* (45). This paper reports investigations into fungal inhibition by wetwood and several of the properties of wetwood potentially responsible for that phenomenon.

MATERIALS AND METHODS

Oxygen diffusion rate (ODR) and redox potential. Nineteen trees 23–58 cm (average, 33 cm) in diameter at 1.5 m height were sampled in June 1981 in the El Dorado and Lassen National Forests in California. Tapered platinum microelectrodes, which could be driven into wood, were designed and constructed by the manufacturer of the oxygen diffusion ratemeter (Jensen Instruments, Tacoma, WA 98406). These were placed in 3.6-mm pilot holes drilled at least 2 cm past the wetwood border and were implanted carefully with a mallet. The brass anode was placed in a similar hole filled with saturated KCl. Another hole, also filled with KCl solution, was widened at the outer 5 cm to accommodate the reference electrode. Amperage was read after 2 min at 0.65 V. A variety of known anaerobic solutions all showed a current of 0.2 μA, so this was subtracted from all readings before conversion to ODR.

After implanting the electrodes and before making ODR measurements, we monitored redox potential with a millivoltmeter (Meratronik, Poland). Redox potential was recorded when it stabilized (usually within 30 min).

Gas mixtures. High-purity N_2 ([O_2]<1 ppm) and air were purchased from Airco, Inc. (San Leandro, CA 94577). Flow rates

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were adjusted to give the desired mixtures by metering humidified gases through drawn capillaries, with pressure regulated by water column (22). Air was first bubbled through concentrated KOH to remove CO_2. In test chambers, N_2 caused complete decolorization of methylene blue indicator strips within 5 hr, and a gradient of blue color was detectable in $[O_2]$ up to 0.15%.

Fungal isolates. Sources of isolates of *H. annosum* were described previously (46). Other cultures were supplied by T. C. Harrington (University of California, Berkeley).

Enzyme assays. For cellulase production, fungi were grown for 30 days at room temperature in a liquid medium containing 0.5% carboxymethylcellulose, 0.06% glucose, 0.3% NH_4 tartrate, 0.06% KH_2PO_4, 0.03% $MgSO_4 \cdot 7H_2O$, 0.003% $CaCl_2$, and 0.02% yeast extract. Cultures were centrifuged (10,000 *g*, 10 min) and the supernatant dialyzed for 2 hr each in two changes of 2 L of cold distilled H_2O. The dialyzed supernatant was assayed according to the method of Highley (18) with carboxymethylcellulose as substrate, except that the pH was 5.4 and the mixture was bubbled with 10 ml/min N_2, 1.8% O_2, or air in sealed tubes during incubation at room temperature. Boiled dialyzate was used for controls. Boiling had no effect on sugar content.

Laccase production was undependable in several liquid media, but when satisfactory activity was detected in stationary 3% malt extract cultures, laccase was quantitatively assayed by a modification of the method of Kaufmann and Wellendorf (26). Five milliliters of 10 mM guaiacol in 0.1 M citrate-phosphate buffer (pH 5.4) were bubbled for 10 min with gas mixtures containing various $[O_2]$ at 20 ml/min in sealed tubes before 1 ml of culture supernatant was added. The reaction proceeded for 15 min with bubbling before stopping. Stopping the reaction and preserving the product proved difficult because enzyme poisons reversed the reaction when used in excess. The reaction was stopped by adding 250 μl of 6.25% HCl solution; this allowed reading of the A_{480nm} within a reasonable period of time without significant change in absorbance. Boiled supernatant was used for controls.

Growth assays. Wetwood fluid was expressed by squeezing blocks in a laboratory press at 69 MPa (10,000 lb/in^2). The fluid was centrifuged, and filter sterilized or autoclaved (25 min at 121 C). Malt extract broth powder (Difco) was added for a final concentration of 1% before sterilization. Three layers of Whatman No. 1 filter paper were placed in 6-cm-diameter plastic petri dishes, and dishes were sterilized with propylene oxide. Test solution (2 ml) was added, and 4-mm-diameter plugs from margins of actively growing PDA cultures were placed, mycelium down, at the edge of the dish.

Response of fungi to $[O_2]$ was tested on 1.5% agar with 1.5% Difco malt extract. Plates were cooled, inoculated as above, placed in test chambers, flushed rapidly with N_2 (except the 20.9% O_2 treatment), then treated with desired gas mixtures at 30 ml/min. In various experiments $[O_2]$s were near 0, 0.07, 0.15, 0.55, 0.90, and 20.9% (air).

Response of fungi to organic acids was tested in premixed malt extract agar (Difco). When autoclaved agar cooled to 60 C, acids were added and pH was corrected to 5.4 with 30% KOH, using a pH electrode cleaned by soaking it for ~15 min in 0.1 M HCl. Plates were inoculated as above and incubated in a hood with flowing air to prevent action of volatile acids on neighboring plates.

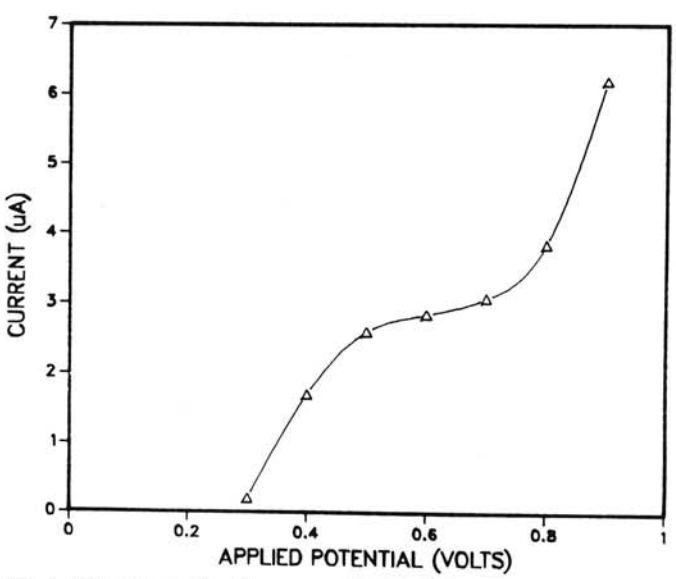

Fig. 1. Current resulting from potentials applied for 2 min to platinum microelectrodes in wetwood of a white fir. Each point represents the mean of three readings at different locations in the wetwood. Similar curves were obtained for other trees.

RESULTS AND DISCUSSION

ODR and redox potential. For measurements of ODR in wood, we first verified the presence of a plateau in the applied voltage-current curve (Fig. 1). This indicates a range of applied voltage at which current is voltage-independent and is instead limited by oxygen diffusion rate (6). We also measured electrical resistances of wetwood samples with an alternating current conductivity bridge and found them to be less than 10^4 ohms per electrode. Kristensen (27) indicates that this is in a favorable range for ODR measurement, especially when current is independent of voltage.

Oxygen diffusion rates in wetwood averaged 35 ng/cm^2/min (range = 12 to 106; $s = 23$), which is similar to values measured in waterlogged soils and sediments (2). The measured redox potentials ($\bar{x}$ = 66 mV; range = −86 to 193; s = 82) also were characteristic of reducing environments (7). If the mean E_h is corrected from pH 5.4 (44) to pH 7, it is reduced by 96 mV (7). Wetwood probably contains low concentrations of active redox couples such as ionic species of Fe, Mn, S, etc. This results in poorly poised potentials (7) and would account for the variability encountered.

The presence of methane (only occasional in fir), obligately and facultatively anaerobic bacteria and organic acids in wetwood of various species, including fir (4,42,44,47), also suggests hypoxic conditions. Jacob (23) found that facultatively anaerobic bacteria in chemostats reduced O_2 partial pressure in solution to 10^{-12} atmosphere (8×10^{-10} torr) before using fermentative pathways. If this is generally true, then the oxygen availability in environments such as that in wetwood must be very low indeed. J. C. Ward and J. G. Zeikus (*personal communication*) state that no O_2 was present in wetwood of white firs, which yielded samples for analysis. Our attempts to sample gases or liquids using high vacuum suggested that only a small proportion of trees could be sampled in this way. Nevertheless, such measurements may be representative. Our ODR and possibly our redox measurements may be overestimates because we could not ensure that air did not enter the wood through small cracks created during entry of the electrode.

Response for fungi to O_2. To test their response to reduced O_2 availability, fungi were grown on agar under various $[O_2]$. To determine the ODRs that were simulated by a given $[O_2]$, ODR was measured in a rapidly stirred and bubbled solution in equilibrium with the desired $[O_2]$; the values obtained were plotted and generated a curve relating $[O_2]$ to ODR (Fig. 2). Owing to the nonturbulent zone at the electrode surface, the diffusive resistance of the solution cannot be completely eliminated. Thus, we underestimated the ODR to which fungi were exposed in the gas chamber tests, and the amount of growth reduction at a given ODR should be regarded as a lower limit.

H. annosum and other wood decay fungi responded similarly to $[O_2]$ (Fig. 3). The growth response was greatest from 0.1 to 1.0% O_2, which also corresponds with the range of ODRs measured in wetwood (see ODR results and Fig. 2). Even if the measured ODRs are not overestimates, O_2 apparently can be a limiting factor in growth of fungi in intact wetwood.

The tolerance of low $[O_2]$ by wood decay fungi is apparently not unique. Tabak and Cooke (38) concluded that most fungi withstand O_2 deprivation down to 1.3–5% before showing appreciable growth loss, and the results of later studies substantiate

that conclusion (22,30). Early studies (31,32) indicated that *H. annosum* was a "perfect alcoholic fermenter," but anaerobic growth was not adequately demonstrated. This should be reassessed with modern techniques. Occasionally we observed very limited growth (~0.5 mm) in 0% O_2, but this could involve growth prior to the onset of anaerobic conditions. We have obtained growth of *H. annosum* in, and yellowing of, Hugh-Liefson's medium after several months under mineral oil in sealed tubes (*unpublished*), but this is not regarded as conclusive evidence for anaerobic growth.

Bavendamm (5) hypothesized that brown-rot fungi, in contrast to white-rot fungi, are relatively insensitive to lack of O_2. Workers have also suggested that O_2 might exert a selective effect on heart-rot versus slash-rot fungi (29) and that specificity of heart-rot diseases may be determined by differences in O_2 concentrations and tolerances (43). Our data do not support these suggestions. However, since O_2 may have a direct role in the enzymatic degradation of wood (3,13) as well as in growth on simple substrates, there remains a distinct possibility that the different types of decay fungi differ in ability to decay wood under hypoxic conditions.

Enzyme assays. Cellulase activity (C_x sensu Reese et al [34]) was essentially unaffected by O_2 (Table 1). The significant difference for one isolate between activity at 0 and 1.8% is considered anomalous, especially since the activity at 0% was not significantly different from activity at 20.9%.

Laccase activity, however, was highly [O_2]-dependent (Fig. 4). At low [O_2] there was a much greater restriction of laccase activity (Fig. 4) than of growth (Fig. 3). These data roughly correspond with the effect of [O_2] on lignin degradation by *Phanerochaete chrysosporium* (3). This supports recently growing evidence for laccase involvement in lignin degradation (1). Since lignin modification is apparently necessary for cellulose breakdown (19), these data suggest that wood decay by *H. annosum* may be restricted at substantially higher [O_2] than is growth on laboratory

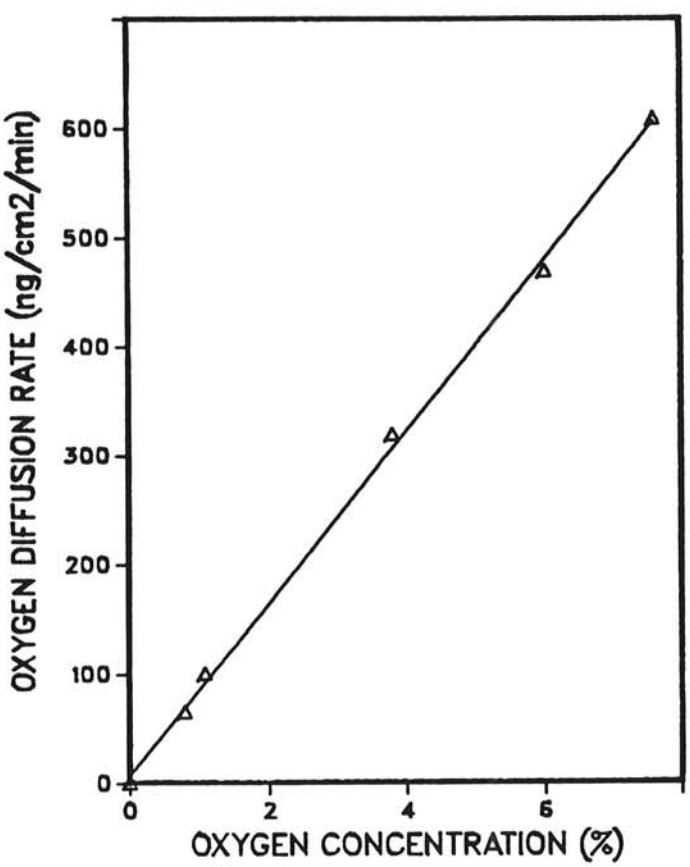

Fig. 2. Relationship between [O_2] in gas mixtures and oxygen diffusion rate in a stirred solution of 1.5% malt extract in equilibrium with the gas.

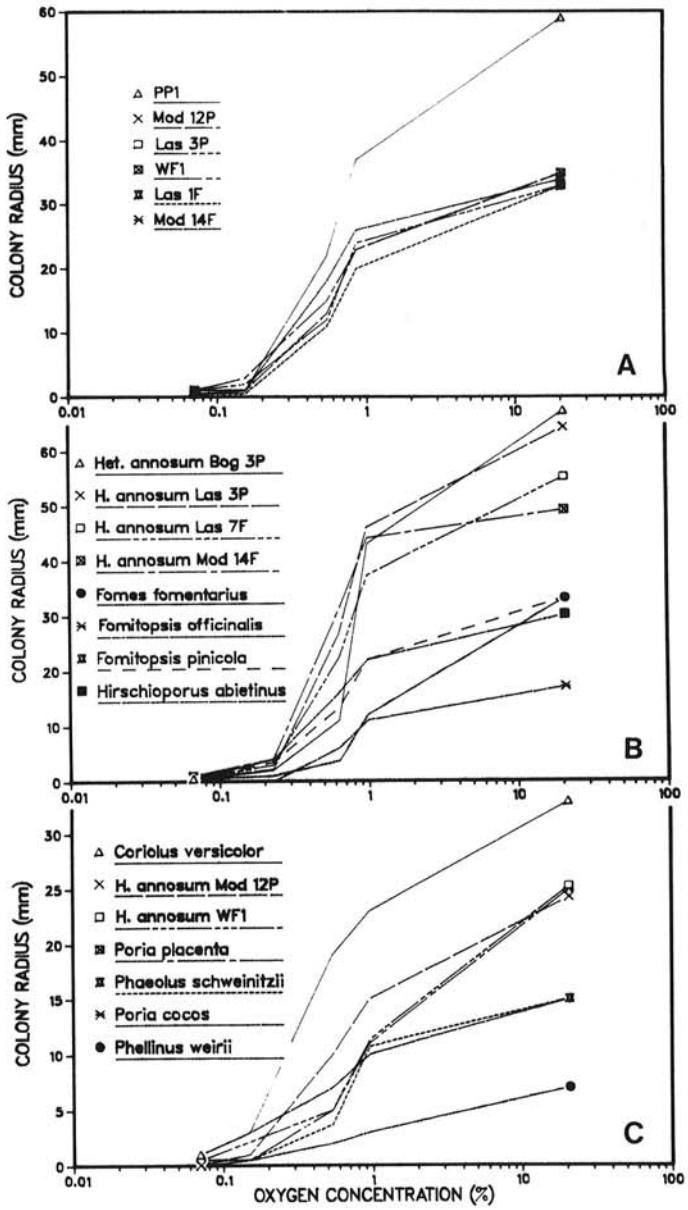

Fig. 3. Radial growth of *Heterobasidion annosum* and other wood decay fungi on malt agar under varying [O_2] in three representative experiments. **A,** Isolates of *H. annosum* after 8 days. Isolates with designations containing the letter P are from pine; those with the letter F are from fir. **B,** Wood decay fungi after 10 days. **C,** Wood decay fungi after 5 days.

TABLE 1. Effect of oxygen concentration [O_2] on cellulase (C_x) activity[a] of three isolates of *Heterobasidion annosum*

Isolate	C_x activity at [O_2] of:[b] 0%	1.8%	20.9%
Las 21F	0.23	0.21	0.21
Las 7F	0.24	0.19	0.18
Las 1F	0.15*	0.22*	0.20

[a] Milligrams of reducing sugar (as glucose) produced from 10 mg of substrate. Gas mixture with specified percent O_2 was bubbled through the reaction mixture at 10 ml/min in sealed tubes. Each reaction mixture contained 1 ml of dialyzed culture supernatant, 1 ml 0.1 M citrate-phosphate buffer (pH 5.4), and 10 mg of substrate, and was incubated for 16 hr at room temperature (~21–23 C). Each figure is the mean of three replicate tubes.

[b] * Means within a row followed by an asterisk are significantly different according to Student's *t*-test ($0.05 > P > 0.01$).

media.

Response of fungi to organic acids and wetwood fluid. Acetic, propionic, and butyric acids have been detected in wetwood of *Abies concolor* (44), *A. alba* (4), and two hardwoods (37). These volatile acids, assayed in vitro at the in vivo pH, were inhibitory at concentrations found in firs to isolates of *H. annosum* from pine and fir and to two other wood decay fungi (Fig. 5). Acetic acid was the least inhibitory. The mean concentration of acetic acid in white fir wetwood was about 18 mM (44), suggesting that in vivo concentrations were generally not very inhibitory and possibly may be stimulatory. However, individual trees had as much as 38 mM acetic acid, which may reduce growth by half. Propionic acid was substantially more inhibitory. Its mean concentration in wetwood was about 28 mM, with individual trees ranging to 55 mM. Thus, propionic acid is probably a very effective fungal inhibitor in wetwood of many trees, and some trees may have concentrations that prevent growth altogether. Butyric acid was even more inhibitory, but the mean concentration in wetwood was low (~9 mM). At this concentration, slight inhibition may occur, but again some trees had more inhibitory concentrations.

Antifungal properties of organic acids, especially propionic acid, and their salts have long been recognized and have been studied for preservation of food, wood chip piles (14), and silage (29), and for use in selective media (33). Hintikka (21) studied the response of 125 basidiomycetes to acetic acid and found that wood decay fungi are tolerant of high concentrations. *Fomitopsis annosa* (= *H. annosum*) did not grow in concentrations ≥0.075% (0.075% v/v = 13 mM). Sensitivity may have been greater in his study because he allowed the pH to drop with acid addition (to pH 4.4 at 0.075%).

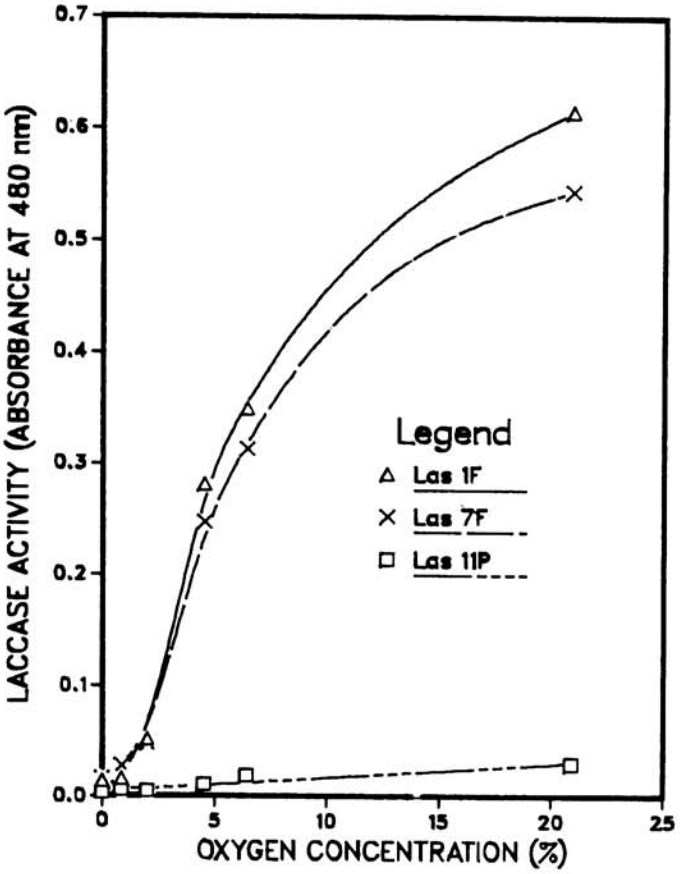

Fig. 4. Effect of oxygen on activity of laccase from three isolates of *Heterobasidion annosum*. Gas mixtures containing various [O_2]s was bubbled for 10 min through tubes containing 5 ml 10 mM guaiacol in 0.1 M citrate-phosphate buffer (pH 5.4). One milliliter of culture supernatant was added, bubbling was continued 15 min, the reaction was stopped by adding 250 μl of 6.25% HCl solution and absorbance was measured immediately. Readings for controls with boiled culture supernatant were subtracted. Each point is the mean of three replicates.

Several studies have shown that organic acids are more inhibitory at lower pH (29).

Further evidence of the fungistatic properties of wetwood is the observation that *Trichoderma* sp. and other fungi were unable to grow when spray inoculated on wetwood-affected cut disks, although the fungi grew extensively in the transition zone and fairly well in the sapwood (Fig. 6). This general phenomenon was first

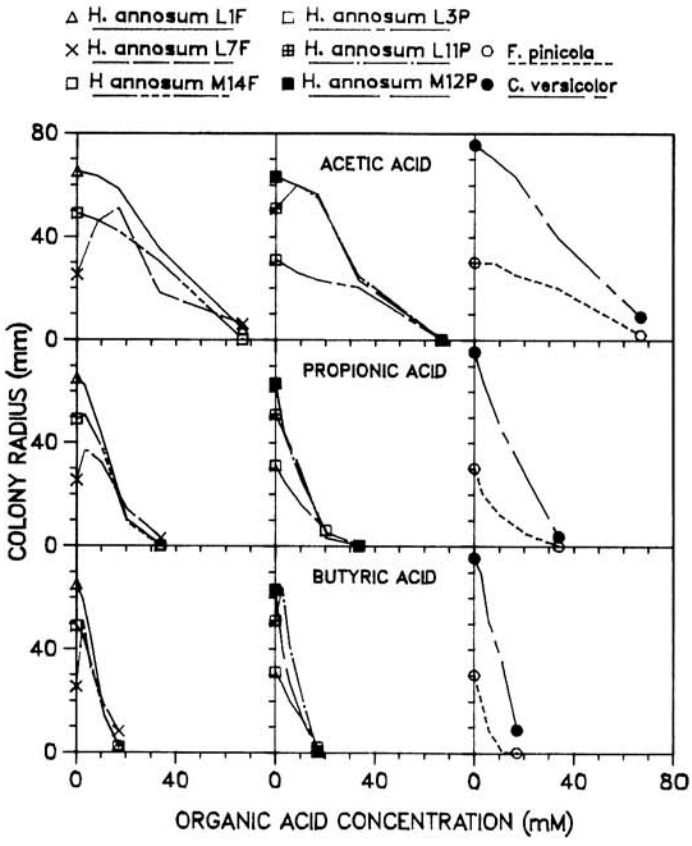

Fig. 5. Effect of acetic (first row), propionic (second row), and butyric acids on growth of *Heterobasidion annosum* isolates from fir (first column), isolates of *H. annosum* from pine (second column), and *Fomitopsis pinicola* and *Coriolus (Polystictus) versicolor*. Acids were added to molten agar, pH was corrected to 5.4, and fungi were grown on plates for 10 days. Each point is the mean of three replications.

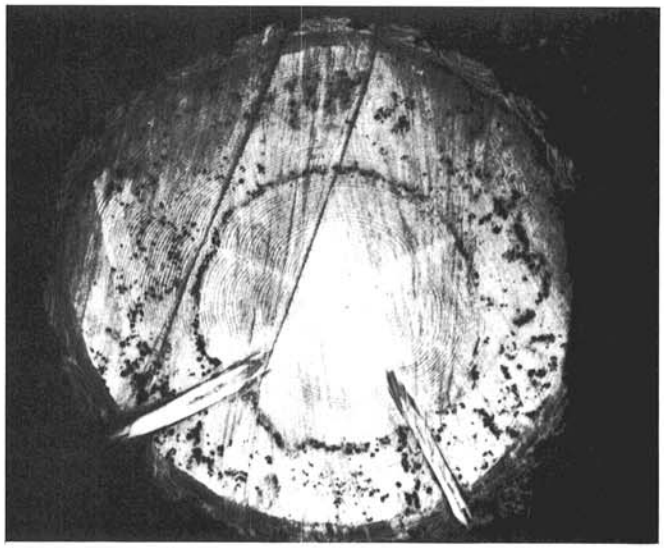

Fig. 6. Growth of *Trichoderma* sp. on a disk of white fir incubated in a moist chamber for 10 days.

TABLE 2. Inhibition of three isolates of *Heterobasidion annosum* by autoclaved or filter-sterilized fluid from wetwood of four *Abies concolor* trees[w]

Sterilization technique	Tree no.	Concentration (%)[x]	Growth (mm) of isolate: WF1	Las 1F	Las 11P
Autoclave	control	0	19 d [y]	23 c	33 b
	1	50	4 c	7 b	0 a
	2	50	0 a	1 ab	0 a
Filter	1	50	2[z]	6 ab	0[z]
	2	50	1 ab	2[z]	0 a
	3	50	21 d	19 c	18 b
	4	50	2 bc	0 a	0 a
	1	100	0 a	0 a	0 a
	2	100	0 a	0[z]	0 a

[w] Two milliliters of test solution were added to sterile 6-cm-diameter plastic petri dishes containing three 5.5-cm diameter Whatman No. 1 filter papers. Plates were inoculated and incubated for 8 days.

[x] Concentration of wetwood fluid, diluted with water where necessary. All treatments contained 1% (final concentration) malt extract broth powder.

[y] Means in a column followed by the same letter are not significantly different according to Duncan's multiple range test ($P = 0.05$).

[z] Insufficient number of replicates for inclusion in analyses. All other figures are means of three replicates.

noted by Etheridge (15), although the wetwood was not identified as such, and suggests that the inhibition is a function of more than lack of O_2.

Wetwood fluid itself inhibited the growth of *Heterobasidion annosum* in vitro (Table 2). Growth was prevented or greatly reduced in 50% wetwood fluid from three trees, while fluid from another tree had no significant effect on growth. This corresponds with the known variability in organic acid content among trees. Autoclaving had no significant effect on inhibitory properties of fluid from two trees tested. Etheridge (15) showed that the inhibitory property of balsam fir heartwood was heat-sensitive. This discrepancy is unexplained, but he autoclaved wood disks, which may allow more volatilization of inhibitory materials than autoclaving of fluid in flasks.

Summary. Inoculations, patterns of decay in naturally infected trees (45), and growth assays indicated that wetwood of white fir is inhibitory to *H. annosum* and other fungi. Organic acids and low availability of O_2 probably directly limit growth of *H. annosum* and other fungi in wetwood. Fungal growth may be further limited indirectly by the effect of low [O_2] on activity of laccase or similar oxidative enzymes necessary for wood decay. Other materials present in wetwood of fir, such as phenols (4,44), may also have a fungistatic effect.

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