

Tolerance of *Polyporus amarus* to Extractives from Incense Cedar Heartwood

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ABSTRACT

Polyporus amarus causes a brown pocket-rot in heartwood of living incense cedar (*Libocedrus decurrens*) trees. It and two other brown rot fungi, *Poria monticola* and *Lentinus lepideus*, were grown in liquid culture containing malt extract and various fractions of incense cedar heartwood extractives.

P. amarus had a relatively high tolerance for all fractions. Growth was stimulated by low concentrations of most of the fractions. Such tolerance may be a factor in the unusual ability of *P. amarus* to degrade this highly decay-resistant wood. Phytopathology 60:919-923.

Polyporus amarus Hedgcock causes a brown pocket-rot in the highly decay-resistant heartwood of living incense cedar trees (*Libocedrus decurrens* Torrey) (4, 7). Unlike many decay fungi, *P. amarus* has a high degree of host specificity. It is almost the only fungus that decays the heartwood of living incense cedar (5, 8, 12, 15). Furthermore, heartwood of incense cedar is the only known natural habitat for this fungus (8, 10). The ability of *P. amarus* to decay incense cedar heartwood differentiates it still further from most brown-rot fungi, to which that heartwood is highly decay-resistant (11). The causes of this specificity, and the mechanisms by which the fungus can attack such a highly decay-resistant wood, are unknown.

Considerable work has been done to identify chemical constituents of extractives of incense cedar heartwood (3, 16) and to determine the fungitoxicity of various extractive components (2). None of these tests included *P. amarus* as a test fungus. Bynum (6) reported the effects of the cold water-soluble extractive fraction on the growth of *P. amarus*; however, comparison with effects on growth of other brown-rot fungi were not made. Anderson et al. (2, 3) have shown that the most highly fungitoxic components of the extractives are part of the acetone-soluble fraction. The present experiment was performed to compare the effects of several fractions of incense cedar heartwood extractives on the growth of *P. amarus* and two other brown-rot fungi commonly used to determine the fungitoxicity of extracts.

MATERIALS AND METHODS.—Liquid culture techniques were employed in this experiment as in the work of Bynum (6). Growth was determined by measuring the oven-dry wt of mycelium. Test fungi employed were: *Polyporus amarus* (USDA, Beltsville 94377-Sp), *Poria monticola* (USFPL, Madison 698), and *Lentinus lepideus* (Madison 534). Previous workers proved that *P. monticola* has a relatively high tolerance and *L. lepideus* a relatively low tolerance for extractives of incense cedar heartwood (2).

The test was performed in 250-ml flasks containing 50 ml of broth consisting of 2% malt extract and various

concentrations of heartwood extractives. The extractives were dissolved or diluted in the appropriate solvent: water for the water-soluble fraction; and acetone for the remaining fractions; they were added aseptically (using 2-ml quantities of a series of dilutions) to previously autoclaved flasks of malt-extract broth. The water-soluble extractives were sterilized by filtration and diluted with sterile distilled water; the acetone solutions of extractives were shown to be sterile by culturing before use.

The inoculum consisted of 1-ml quantities of a mycelial homogenate prepared by blending a mycelial mat of each fungus that had been grown in malt-extract broth and washed with three changes of sterile distilled water. Cultures were incubated for 85 days at 19 ± 2 C on a laboratory bench without exclusion of light. Growth of the test fungi was measured by filtering cultures through tared filter paper discs which were air-dried overnight, oven-dried at 103 ± 2 C for 1 hr, cooled over anhydrous calcium sulfate for 10 min, and weighed. Noninoculated controls were prepared for each concentration of each extractive, and were filtered and weighed in the same manner as the inoculated cultures. Data for oven-dry wt of mycelium were corrected for the amount of wt due to residual extractive present, as determined from the controls.

Extracts were prepared as follows from heartwood of a sound, air-dry, butt sample of a single El Dorado County tree approximately 140 years old:

1) *Water-soluble fraction.*—A 2-kg sample of ground heartwood was moistened with distilled water and then extracted with five changes of 8 liters of distilled water for 24 hr in each change. The extractive solution was drawn off, filtered, and concentrated in a circulating evaporator at 40 C or less. Further concentration was accomplished in a rotary evaporator at a maximum temperature of 40 C following removal of the tropolone fraction 2).

2) *Tropolone fraction.*—The partially concentrated water extract was extracted with chloroform until the chloroform-soluble portion no longer produced a color

change with ferric-chloride-indicator paper. The chloroform was evaporated in an air stream.

3) *Steam-volatile fraction*.—A second 2-kg sample of heartwood chips, prepared as in 1) above, was extracted with five changes of 8 liters of acetone for 24 hr in each change. The extract solution was drawn off, filtered through cheesecloth and filter paper, and concentrated in a rotary evaporator at a maximum temperature of 40 C. The concentrated solution was extracted with chloroform. This produced a dark-brown precipitate which was washed with eight 100-ml changes of chloroform and used in the production of the phlobaphene fraction 5). The supernatant liquid and washings were filtered and concentrated in a rotary evaporator at 40 C or less until the odor of chloroform had disappeared. Approximately 400 ml distilled water was added and the mixture was distilled at 100 C for a total of about 18 hr.

4) *Steam-nonvolatile fraction*.—Residue from the distillation under 3) was dissolved in chloroform, transferred to a storage container, and the chloroform was removed in an air stream.

5) *Phlobaphene fraction*.—The washed, chloroform-insoluble material in 3) above was dried until all odor of chloroform had disappeared, and then was ground to a fine powder.

All extracts were stored under nitrogen until used.

The work of Anderson and others (3, 16, Anderson, *personal communication*) has demonstrated the composition of the extractive fractions used in the present study. The water-soluble fraction consists primarily of carbohydrates, but may include also a small amount of low-molecular-weight tannins. A small quantity of the tropolones may appear in a water extract, but in this experiment they were removed as a distinct fraction by chloroform extraction. The steam-volatile fraction should contain all of the significantly fungitoxic components of the heartwood extractives, including the tropolones and monomeric phenols; this fraction also would contain the terpenes. The steam-nonvolatile fraction contains the dimers and a trimer of the monomeric phenols of the steam-volatile fraction, along with some of the phlobaphenes and tannins. The phlobaphene fraction contains the majority of the polyphenolic phlobaphenes.

RESULTS AND DISCUSSION.—The quantities of extractives obtained from 2 kg of air-dry heartwood chips are shown in Table 1. Results of the cultural experiments are shown in Fig. 1. In general, growth of *P. monticola* equaled or exceeded that of *L. lepidus* at all concentrations of each fraction, while growth of *P. amarus* was greater than that of the other test fungi. In all fractions except the water-soluble fraction, low concentrations of each fraction stimulated growth of *P. amarus* and, to a slight degree, that of *P. monticola*. With the exception of *L. lepidus*, growth was increased by addition of the carbohydrate-containing, water-soluble fraction to the cultures in a manner

TABLE 1. Quantities of extractives obtained from 2 kg of air-dry incense cedar heartwood

Fraction	Quantity of extractive	% Original air-dry wt of wood	Estimated concn ^a
	g	%	mg/cc of heartwood
Water soluble	15.09	0.8	2.79
Tropolone	13.17	0.7	2.44
Steam volatile	55.62	2.8	10.29
Steam nonvolatile	129.66	6.5	23.99
Phlobaphene	28.30	1.4	5.24
Total	241.84	12.2	44.75

^a Assuming a sp gr 0.37 (14).

roughly proportional to the amount of extract added.

The most highly fungitoxic individual extractive components in incense cedar are the tropolones, primarily γ -thujaplicin (2, 3, 11). The monomeric phenols contained in the steam-volatile fraction of the present study were strongly fungicidal when tested in the combination normally found in incense cedar heartwood, although they exhibited a low degree of fungitoxicity when tested separately (1). In previous work the remaining fractions have been shown to have little or no fungitoxicity (1, 2, 3, Anderson, *personal communication*).

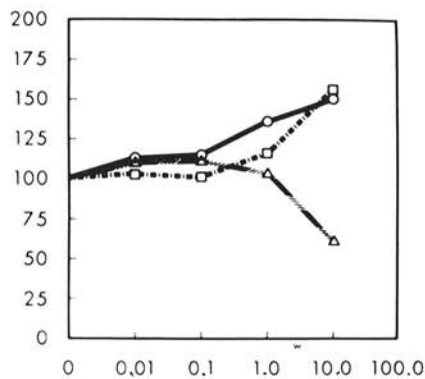
With all fractions, including the tropolone and steam-volatile fractions, the growth of *P. amarus* was greater at a given concentration than that of the other two fungi, except for the highest concentrations where total inhibition occurred. This is true in spite of the fact that *P. monticola* has the highest tolerance for these extractives of any fungus previously tested (2). Except for the water-soluble fraction, the lower concentrations of each fraction had a stimulatory effect on the growth of *P. amarus* and, to a lesser degree, on that of *P. monticola*. This stimulation may be similar to the stimulation of growth by low concentrations of toxicants observed and reviewed by Schultz-Dewitz (13) and Lyr (9). The concentrations producing stimulation in the work of Schultz-Dewitz were comparable to those of the present study, while those reported by Lyr were lower than the lowest concentration used in this study. The stimulatory effect was much more prevalent with *P. amarus* than with the other two fungi. These results suggest that *P. amarus* possesses a relatively high degree of tolerance for the fungitoxic extractives in incense cedar heartwood, and that this tolerance may be a factor in its ability to attack this highly decay-resistant wood. Anderson et al. (1, 3) found, from a comparative analysis of the residue within decay pockets and the sound heartwood surrounding them, that the concentrations of the highly fungitoxic tropolones and monomeric phenols were considerably lower within the decayed zone than outside it, while the concentration of the dimer libocedrol was

Fig. 1. Growth of test fungi in liquid media containing malt extract and various concentrations of incense cedar heartwood extractives. "W" indicates an estimate of average original concentration of each extractive fraction in the wood.

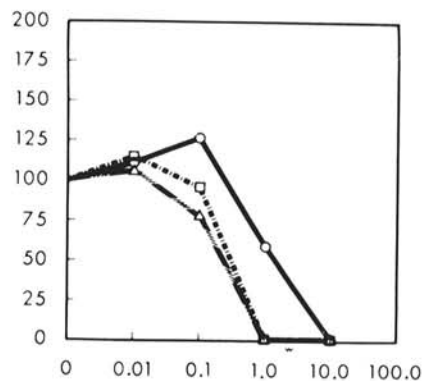
OVEN-DRY WEIGHT OF MYCELIUM

(% OF CONTROL)

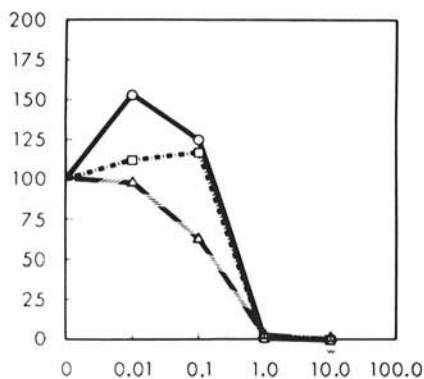
WATER-SOLUBLE FRACTION



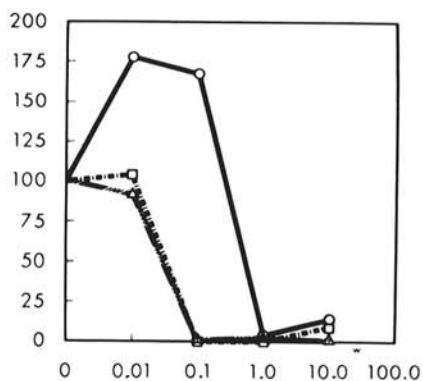
TROPOLONE FRACTION



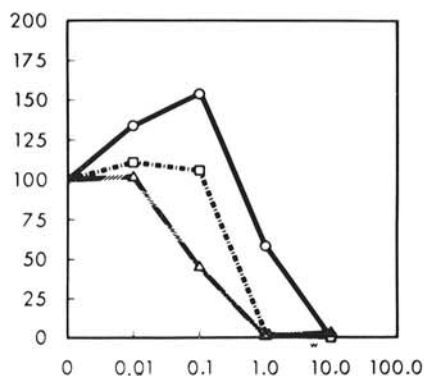
STEAM-VOLATILE FRACTION



STEAM-NONVOLATILE FRACTION



PHLOBAPHENE FRACTION



- = P. AMARUS
- = P. MONTICOLA
- △--- = L. LEPIDEUS

EXTRACTIVE CONCENTRATION

(MILLIGRAMS/MILLILITER)

higher in the decay residue than in sound wood. In light of the results of the present study, it is possible that *P. amarus* may be able to metabolize the monomeric compounds or to participate in their polymerization to less fungitoxic compounds.

The water-soluble fraction produced an increase in growth with increasing extract concentration for *P. amarus* and *P. monticola*. There is no explanation apparent for decrease in growth of *L. lepideus* at the high concentrations, unless it is that this fungus was sensitive to low-molecular-weight tannins while the other two were not.

According to Anderson et al. (2, 3), components of the steam-novolatile and phlobaphene fractions should have little or no fungitoxicity. Their experiments were performed at an extractive concentration of 1.2%, based on the weight of wood treated with the extract. This is roughly equivalent to a concentration between the 1.0 and 10.0 mg/ml concentrations of the present study (disregarding any loss of effectiveness due to adsorption in the wood or precipitation from the aqueous medium), at which level there was a considerable fungitoxic effect apparent. Furthermore, in the present study the toxic effect of the steam-novolatile fraction on *P. monticola* and *L. lepideus* was even greater than that of the tropolone and steam-volatile fractions. These results differ from the expectation suggested by the work of Anderson et al.

A possible explanation for such discrepancies could be a difference in the effective concentration of extractive available for interaction with the fungus. When originally present in the wood, and presumably when returned to wood, extractives may be present largely within cell walls of a porous material whose volume is primarily free space. There is considerable void volume available for the fungus to occupy in the wood without contacting significant quantities of extractive. In a liquid culture, however, the fungal hyphae would be in continuous contact with the extractives. Even at high concentrations of acetone-soluble extractives (which precipitated to the bottom of the culture flasks when they were added), an equilibrium would be established which would maintain a saturated solution of the material available for continuous fungal contact. It is possible that a portion of the extractive material was, when added to wood, adsorbed or bound into the wood in such a manner that it was unavailable for interaction with the fungi. Therefore, the extractives may express toxicity at a lower concentration in liquid culture than when present in wood. The concentration of most of the extractive fractions, as originally present in incense cedar heartwood, also lies in the 1.0 to 10.0 mg/ml range, as can be seen in Table 1.

The results of the present study differ somewhat from those of Bynum (6). Using a cold-water extract, he found that the extract had an inhibitory effect on growth when it was added to a 1% malt-extract culture medium, but that it stimulated growth, up to a concentration of 2.27 mg/ml, when it was the sole source of nutriment. Above a concentration of 2.27 mg/ml, the extract alone also had a decidedly fungitoxic effect. Since Bynum's extract was not treated with any solvent

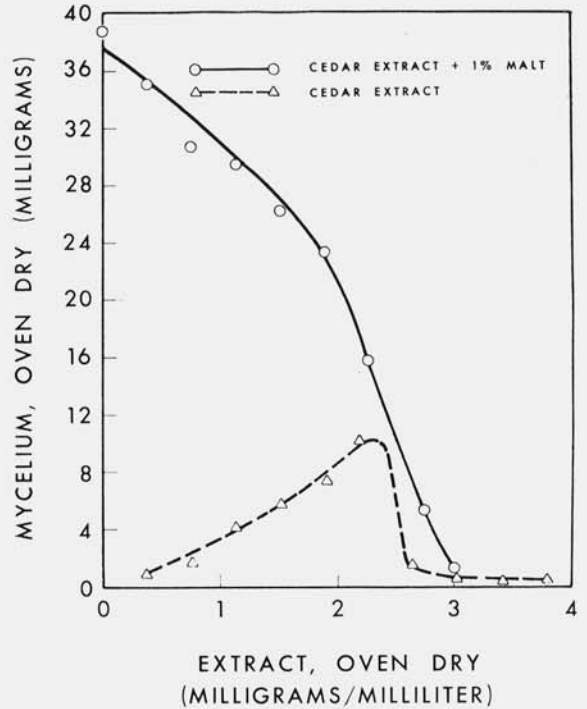


Fig. 2. Growth of *Polyporus amarus* on various concentrations of a water extract of incense cedar heartwood, with and without an additional carbon source. Redrawn from data of Bynum (6).

other than water, presumably it would have contained a portion of the highly fungitoxic tropolones which, in the present experiment, were removed by chloroform extraction. Therefore, the extract would be expected to suppress growth except perhaps at very low concentrations. Such a conclusion is supported by Bynum's data when the two curves are plotted on the same axes (Fig. 2). The zone of strong growth suppression occurred at approximately the same concentration for both culture conditions. Presumably the growth increase shown when the extract was the sole source of nutrients was due to increasing, but suboptimal, levels of an available carbon source below the concentration where expression of the toxic action of the fungicidal components was possible. Assuming that the tropolone fraction of the present study most closely represents the fungitoxic component of Bynum's culture media, the concentration at which fungitoxicity was expressed is similar in the two studies. The stimulation of growth, apparent at low concentrations of the tropolone fraction in the present study, occurred at concentrations lower than those used in Bynum's study; this may account for the fact that he did not observe this phenomenon in the media containing 1% malt extract.

Polyporus amarus appears to have a relatively high tolerance for all fractions of the extractives in incense cedar heartwood. This tolerance may play a role in the unusual ability of the fungus to degrade this highly decay-resistant wood. There is evidence from the work of Anderson et al. (1, 2) that the concentration of

highly fungitoxic tropolones and monomeric phenols decreases, while that of a nonfungitoxic dimeric phenol increases as a result of decay by this fungus. This suggests that *P. amarus* may be capable of metabolizing or detoxifying the extractives primarily responsible for the high degree of decay resistance of incense cedar heartwood. Individual extractive compounds must now be exposed to the action of *P. amarus* under controlled conditions in order to determine the type of action involved.

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