Inhibition of Wood-Rotting Fungi by Stilbenes and Other Polyphenols in Eucalyptus sideroxylon

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Journal Article No. 6555 from the Michigan Agricultural Experiment Station.

This work was mainly done during the visit of J. H. Hart as Overseas Research Fellow at the Forest Products Laboratory, Melbourne.

The assistance of Andrew Rozsa, Forest Products Laboratory, is gratefully acknowledged.

Accepted for publication 18 January 1974.

ABSTRACT

Heartwood of red ironbark (*Eucalyptus sideroxylon*) was not decayed in vitro by *Polyporus versicolor* or *Poria monticola.* Methanol extractives of heartwood diluted × 1,000 in 3% malt extract were toxic to *P. monticola,* but not to *P. versicolor.* Heartwood blocks extracted with methanol retained their decay-resistance. The ether-soluble (rich in stilbenes) and the water-soluble (rich in ellagitannins) fractions were inhibitory to both fungi in 3% malt extract at concns below that for both classes of compounds in the original wood. Impregnation of cottonwood (*Populus deltoides*) blocks with pure resveratrol (3,4′,5-trihydroxystilbene) did not result in decay resistance although relatively high proportions of the methanol extractives, and the water-soluble fraction did so. This study emphasized the difficulties in relating the results of in vitro testing of toxicity of wood extractives and their pure components with the durability of the wood from which the extractives were obtained.

Phytopathology 64:939-948.

The decay resistance of a number of heartwoods has been associated with the presence of different types of polyphenols and other extractives. Some of these compounds have conferred resistance to wood normally susceptible to decay (12,29). The stilbene class of polyphenols has been reported to have significant toxicity to wood-decaying fungi (34). Highly durable woods, notably wandoo (*Eucalyptus wandoo*) and the bloodwood class of eucalypts (13), *Nothofagus fusca* (17, 20), and *Pterocarpus* spp. (4), all of which were durable even in soil-burial tests, contained appreciable amounts of stilbenes. Nevertheless, some eucalypts which contain stilbenes are not more durable than others which lack them (7, 8).

The fungistatic and fungitoxic properties of the stilbenes pinosylvin (PS) and its monomethyl ether (PSME) were shown with a variety of wood decay fungi (26, 27, 28). These compounds are present in pine heartwoods (9) and in lesions where they appear to confine the spread of infection (6, 18, 36). The toxicity of stilbenes is apparently due to the inactivation of fungal enzymes containing -SH groups; the laccase formed by white rot fungi is not inhibited (23). Early bioassays were conducted by incorporating stilbenes in 2% malt extract agar. Sawdust impregnated with stilbenes showed little or no toxicity to several fungi (29). A more detailed examination (22) showed that bioassays on malt extract agar cannot be extrapolated to the natural substrate, and that PS and PSME may not be significant factors in the resistance mechanism of pines to fungal attack.

The heartwood of red ironbark (*Eucalyptus sideroxylon*) is rated as highly durable in soil burial tests (3) and is used widely in southeastern Australia because of these properties. Stilbenes (resveratrol and others) are present in this dense (0.89 g/cc) timber in which the wood is characterized by thick cell walls (19).

Extractives, especially the stilbenes, of red ironbark wood were examined to determine their contribution to the durability of the wood, and to review the efficacy of the bioassay methods available.

MATERIALS AND METHODS.—*Chemical characterization.*—A red ironbark log (*Eucalyptus sideroxylon* A. Cunn ex Woolls) collected at Rushworth, Victoria, Australia, had a diameter inside the bark at 1.4 m above ground of 38 cm, and a sapwood thickness of about 2.5 cm. Fresh sapwood and heartwood (1,400 g, from the bottom 2 m of the tree) were converted to thin longitudinal shavings, extracted with methanol (at 22-24 C) for 3 days, the extract was concd in vacuo at less than 40 C, finally freeze-dried, and stored at −10C. After extraction, the sapwood was cream colored; the heartwood was brownish-red.

The methanol extract (A) of the heartwood was redissolved in a minimum amount of methanol and slowly added to constantly stirred distilled water to produce a suspension which was extracted with ether in a liquid-liquid extractor for 24 h (Fig. 1). The dried ether extract was redissolved in methanol and washed five or six times with heptane. The ether-extracted aqueous material was partly concd in vacuo, extracted with ethyl acetate (six 4-h extractions) in a liquid-liquid extractor, and the insoluble material (D) was separated from the water-soluble residue (C). All fractions were vacuum- or freeze-dried.

A red oil extracted with heptane from the ether extract was not examined further. The ether-soluble fraction (E) was redissolved in ether:methanol (9:1, v/v) and extracted (each four times) in turn with saturated aqueous sodium bicarbonate or with sodium carbonate. The aqueous extracts were washed with ether to remove entrained material and the washings returned to the main ether solution. The aqueous extracts were neutralized (when necessary), extracted with ethyl acetate, and the extracted components which were recovered (92% yield) were recognized chromatographically.

Chromatograms of all fractions and their sub-fractions were made using Whatman No. 1 paper. The principal developing solvents for two-dimensional chromatograms were 1-butanol:27% acetic acid (1:1, v/v) (BAW) in the first direction and 6% acetic acid (6HA) in the second direction. The same solvents were used routinely for one-dimensional chromatograms and cochromatography. Forestal solvent [hydrochloric acid:acetic acid:water (3:30:10, v/v)] was used in one-dimensional chromatograms of acid-treated fractions, and 30% acetic acid or 1-butanol:ethanol:water (4:1:5, v/v) for other fractions. Thin-layer chromatograms of silica gel (G.F. 254:E. Merck Darmstadt) on glass plates using solvent chloroform:ethyl acetate:formic acid (5:4:1, v/v) were also prepared.

Chromatograms were viewed under short (254 nm) and long-wave (365 nm) ultraviolet light, and examined before and after exposure to ammonia vapor. Chromogenic sprays were $FeCl_3$ (1%)-$K_3Fe(CN)_6$ (1%) (1:1, v/v); pNA (diazotised *p*-nitroaniline in 20% sodium acetate); vanillin solution (10% vanillin in ethanol:concd hydrochloric acid 1:1, v/v); and NSSC (15 g Na_2SO_3:3.5 g Na_2CO_3:350 ml water).

The ultraviolet absorption spectra of different components were determined in ethanol solution before and after the addition of dilute aqueous solutions of sodium hydroxide or sodium acetate.

Bioassays.—The white-rot fungus *Polyporus versicolor* L. ex Fr. (Madison 697) and the brown-rot fungus *Poria monticola* Murr (Madison 698) were used in different techniques to test the decay-inhibiting effectiveness of different fractions or substances.

—1) Technique A.—*Polyporus versicolor* was grown in 250-ml Erlenmeyer flasks containing 50 ml of malt extract medium (30 g/liter). The ether (E), ethyl acetate (B) and water-soluble (C) fractions of the methanol extract of the heartwood were dissolved in 1 ml water or 99% ethanol, passed through a Millipore filter (0.5μ pores) and added to each flask which previously had been autoclaved. Concentration of dried fraction was 1 or 10 mg/ml medium. Each

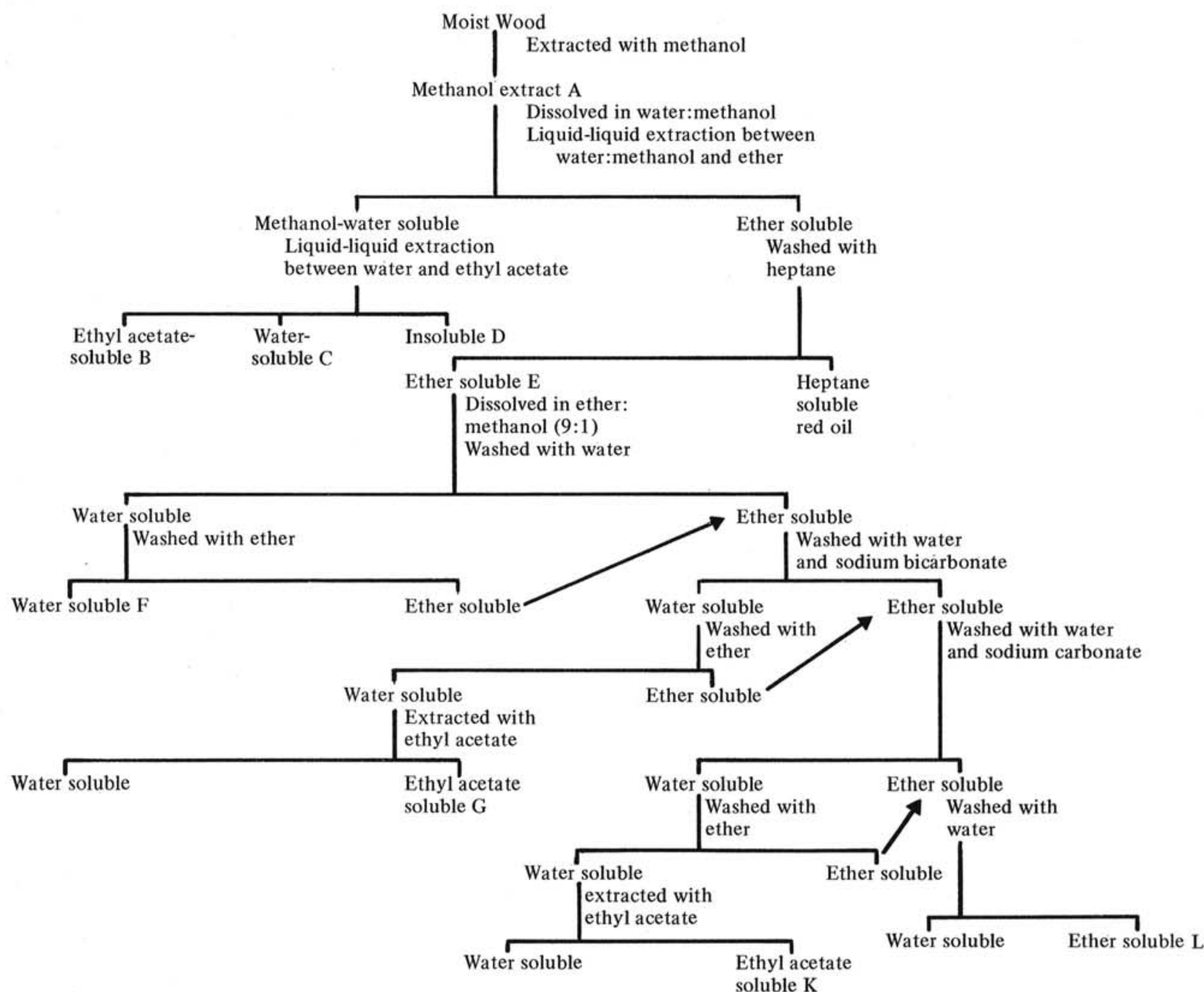

Fig. 1. Separation scheme for heartwood extractives of *Eucalyptus sideroxylon.*

treatment was represented by three flasks and both initial (4.7 - 5.3) and final (4.4 - 4.7) pH of the media were determined. Flasks were placed on a reciprocal shaker in the dark for 2 wk at 20 C. The contents of the flasks were filtered through tared No. 3 Whatman filter paper, dried, and weighed to determine the dry weight of fungal material in each flask.

Both fungi were grown in flasks containing 50 ml malt extract medium and 0.4, 0.2 and 0.1 mg/ml of the L fraction (Fig. 1) of the heartwood extract or authentic resveratrol (3,4′,5-trihydroxystilbene). The dried methanol extracts of the sapwood and heartwood were dissolved in ethanol so that when 1.0 ml was added to the malt extract, the concentration was 1 mg/1 ml medium. The extracts were sterilized through a Millipore filter and the assay was carried out as above.

–2) Technique B.–Fifty μl of ethanol solutions of the L, K, G, and F sub-fractions (Fig. 1) of the ether-soluble material were added to 25 X 125-mm test tubes containing 2.5 ml of 3% malt extract. The tubes contained a final concn of 1 mg of each fraction per ml medium. Only ethanol was added to control tubes. The tubes were kept at 27 C for 48 h to check for contamination and then inoculated with either *P. versicolor* or *P. monticola*. Each fungus was replicated four times with each material and incubated for 10 days.

–3) Technique C.–This procedure was similar to B except that fractions L and K and resveratrol were added to Erlenmeyer flasks, each containing 2 ml of 3% malt extract so that the final concn was 1 mg/ml extractive. Each fungus was replicated three times with each fraction and the flasks examined for growth 4, 7, and 10 days after inoculation. After 10 days, the inoculum was plated on malt extract agar to determine whether the fungus was still alive.

–4) Technique D.–Defect-free blocks of

TABLE I. Fractionation of methanol extract of the oven-dried heartwood of *Eucalyptus sideroxylon*

Fraction	Subfraction	Appearance	Components[a] (in approximate order of amount)	Wt. % of oven-dried heartwood
Ether-soluble	heptane-soluble	red oil		0.95
	heptane-insoluble E	pink powder		0.85
	water-soluble F		S-6,S-13,S-14	0.19
	$NaHCO_3$-soluble G		S-2,S-4,S-14, S-8,S-13	0.27
	Na_2CO_3-soluble K		S-1,S-13,S-2,S-4, S-8,S-3,S-5	0.03
	ether-soluble L		S-1,S-3,S-13,S-5, S-15,	0.36
Ethyl acetate-soluble B		amber powder	S-10,S-11,S-15, S-13,S-14,S-9, S-16,S-12,S-17, S-18,S-23	0.95
Water-soluble C		red-brown gum	S-17,S-18,S-23, S-22,S-10,S-11, S-21,S-20,S-19	7.50
Insoluble D		tan powder	S-15,S-16,S-23	1.15

[a]Number refers to numbered compound in chromatogram Fig. 2 chromatogram.

cottonwood (*Populus deltoides* Bartr.) weighing 0.5 to 1.0 g with dimensions of 1.5 cm (radial), 1.0 cm (tangential) and 0.9 to 1.2 cm (vertical), were trimmed to remove loose wood. The blocks were dried for 14 days at 40 C, placed under vacuum for 15 min, various solutions were added and held under vacuum for a further 15 min, after which the blocks were left at atmospheric pressure for an additional 15 min (1). The solutions used, at concns of 0.01, 0.02, and 0.04%, were the methanol extract (A) of heartwood dissolved in acetone:ethanol (9:1, v/v), the water-soluble residues (C) from the methanol extract dissolved in water:ethanol (9:1, v/v), and resveratrol dissolved in acetone. Blocks impregnated with only water or acetone served as controls. The blocks were then steam-sterilized at 99 C for 15 min and placed aseptically onto agar-blocks containing 14-day-old cultures of either *P. versicolor* or *P. monticola* (25). After 4 wk at 27 C, the mycelium was brushed off the blocks, the blocks were dried (48 h at 95 C) and the weight loss determined. Noninoculated blocks which were impregnated with solutions were dried and weighed to determine uptake of extract.

–5) Technique E.–Blocks (ca. 1.6 g each) of sapwood and heartwood of *E. sideroxylon* were autoclaved for 1 or 3 h at 121 C or Soxhlet-extracted for 70 h with ethanol. These blocks were then subjected to the same decay resistance tests described above for Technique D (25).

RESULTS.–*Chemical characterization.*–The total amount of methanol extractives in the heartwood was 13.2% (w/w, oven dry basis), and the different fractions obtained are given in Table 1. The heptane fraction did not contain phenolic compounds detectable by chromatography. Over 22 individual compounds (Fig. 2). were detected on the BAW × 6HA chromatograms of the methanol extract, but only those compounds thought to have some role in wood durability are discussed here. Additional data on the chemistry of the extractives have been published previously (16).

Compound S-I was identified as resveratrol (3, 4′, 5-trihydroxystilbene) after isolation from the L subfraction of the ether extract (Fig. 1) by recrystallization by melting point and mixed melting point with authentic resveratrol, and by gas liquid chromatography of the silylated compound (14), before and after the addition of silylated authentic resveratrol. A smaller peak with a relative retention time of 0.41 of resveratrol was probably that of the cis-isomer of resveratrol (probably compound S-3). Similarly, compound S-8 (from subfraction G) behaved identically with authentic resveratrol β-glucoside; compound S-5 is probably the cis-isomer of S-8.

Compounds S-17 and S-18 in the ethyl acetate extract (B) and the water-soluble residue (C) were shown to be chromatographically identical respectively with authentic ellagitannins D-6 and D-13 in *E. delegatensis* (15,35) which are identical with ellagitannins H-1 and H-3 in *Quercus alba* (12). Identification was by direct chromatographic comparison and cochromatography, and by observing the several color changes after spraying with NSSC. Acid hydrolysis of the fraction yielded ellagic acid and glucose.

The sapwood contained 3.7% (oven-dry basis) methanol-soluble extractives. Chromatographic examination for polyphenols revealed appreciable amounts of gallic acid, polymerized material which was chromatographically irresolvable, and contained leucoanthocyanins, small amounts of ellagitannins and ellagic acid, and very faint traces of materials

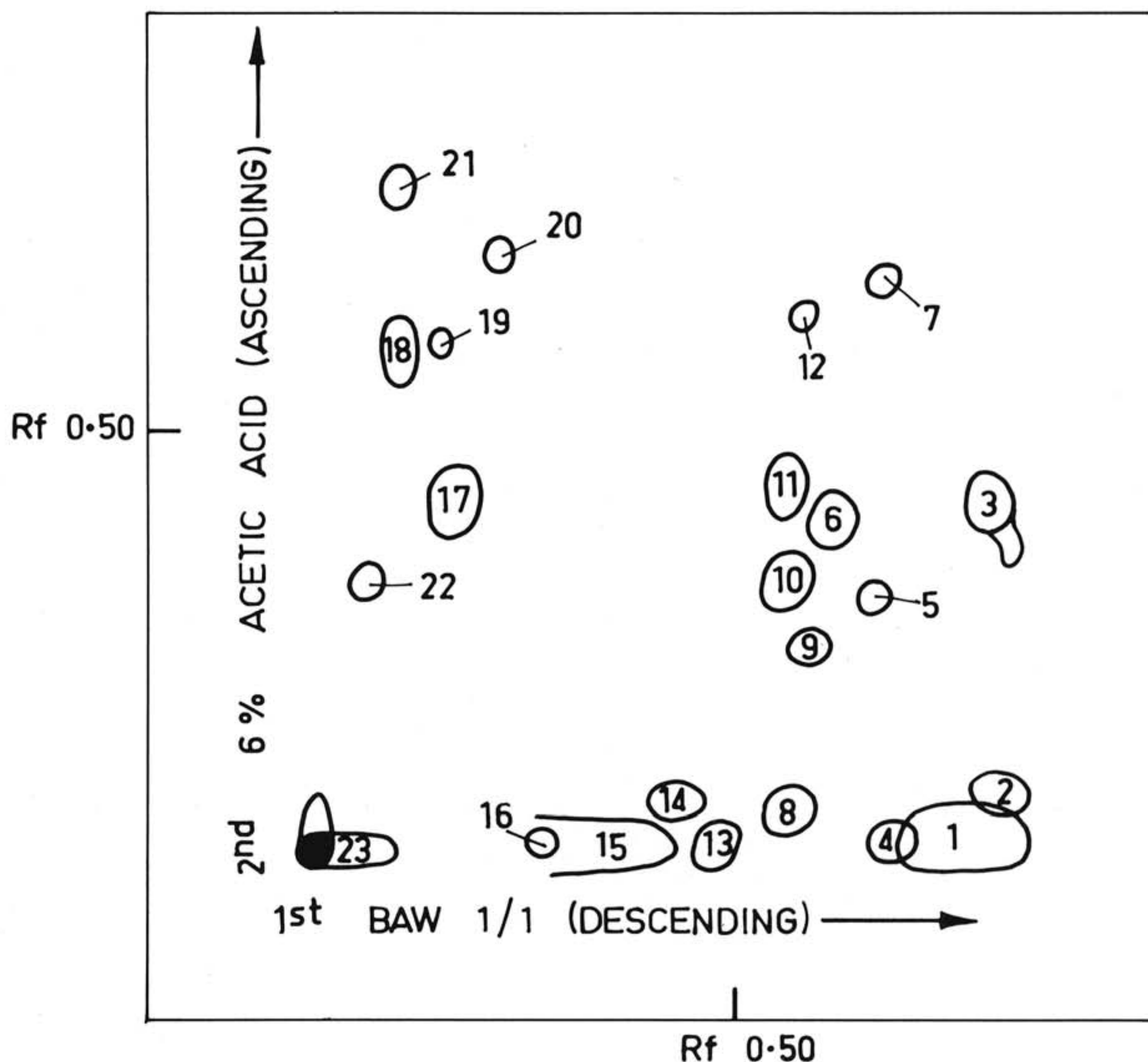

Fig. 2. Diagrammatic representation of the two-dimensional paper chromatographic separation of a methanol extract from the heartwood of *Eucalyptus sideroxylon.*

which could be stilbenes or methyl ellagic acids.

Bioassays.—In vitro tests with cultures of *P. versicolor* and *P. monticola* on blocks of *E. sideroxylon* heartwood showed they were resistant to decay by both fungi (Table 2) and that autoclaving and ethanol extraction of the blocks before testing had no effect on decay resistance. *Eucalyptus sideroxylon* sapwood blocks were decayed by both fungi and most quickly by *P. versicolor*, but autoclaving or extraction of the blocks increased their susceptibility only to *P. monticola* (Table 2). As it is known that commercial steaming of eucalypt boards results in little hydrolysis of ellagitannins, the increase in susceptibility of the sapwood to decay by *P. monticola* is not likely to be due to hydrolysis.

The addition of 0.1% of dried methanol extracts of both sapwood and heartwood to 3% malt extract solution (Technique A) caused inhibition to *P.*

TABLE 2. Weight loss of blocks of *Eucalyptus sideroxylon* sapwood and heartwood exposed to *Polyporus versicolor* and *Poria monticola* for 8 wk at 27 C

		Weight loss[y] (% dry wt)	
Sample	Treatment	*P. versicolor*	*P. monticola*
Sapwood	untreated	15.0 a	12.0 a
Sapwood	autoclaved 1 h	18.0 a	27.0 b
Sapwood	autoclaved 3 h	17.0 a	29.0 b
Sapwood	extracted with ethanol	17.0 a [z]	28.0 b
Heartwood	untreated	0.9 b	0.4 c
Heartwood	autoclaved 1 h	0.9 b	0.4 c
Heartwood	autoclaved 3 h	0.6 b	0.3 c
Heartwood	extracted with ethanol	0.2 b[z]	0.0 c

[y]Each value is the average of five blocks. Any two values in a single column not followed by the same letter are significantly different ($P = 0.01$, by Student's t test).

[z]Exposed for 6 wk.

TABLE 3. Growth of *Polyporus versicolor* and *Poria. monticola* in cultures containing various extracts of *Eucalyptus sideroxylon*

Sample	Concn of extractive in medium	Growth (% of control) of	
		P. versicolor	*P. monticola*
Bioassay I			
Sapwood methanol extract	0.1%	114	25[a]
Heartwood methanol extract	0.1%	120	2[a]
Bioassay II			
Heartwood methanol extract			
Ether-soluble fraction E[e]	0.1%	0[ab]	nt[c]
Ethylacetate-soluble fraction B	0.1%	104	nt
Water-soluble residue C	0.1%	180[a]	nt
Water-soluble residue C	1.0%	0[ad]	nt

[a]Differed significantly ($P = 0.05$) from the growth of the control.
[b]*P. versicolor* failed to grow from inoculum blocks when they were transferred to malt extract agar.
[c]Not tested.
[d]*P. versicolor* grew from inoculum blocks when they were transferred to malt extract agar.
[e]Letters refer to subfractions in Fig. 1 separation scheme.

monticola but slightly stimulated growth of *P. versicolor* (Table 3). The heartwood water-soluble residue (C) (remaining after ether and ethyl acetate extractions and containing ellagitannins, polymerized leucoanthocyanins and other materials) was also stimulatory at 0.1% concn but strongly inhibitory at 1.0% to *P. versicolor* (Table 3). The latter concn was fungistatic: *P. versicolor* grew from the inoculum blocks when they were transferred to fresh malt extract agar. This water-soluble fraction occurred in the heartwood at a concn of about 7.5%. The ether-soluble fraction (E) of the heartwood inhibited

TABLE 4. Growth of *Polyporus versicolor* and *Poria monticola* in cultures containing resveratrol (sub-fraction L, see Fig. 1 Chromatogram) isolated from the heartwood extractives of *Eucalyptus sideroxylon* or authentic resveratrol

Material	Concn in medium (%)	Growth (% of control) of	
		P. versicolor	*P. monticola*
Sub-fraction L[a]	0.01	106	83
	0.02	90	14[b]
	0.04	0[bc]	0[bc]
Authentic resveratrol	0.01	90	78
	0.02	0[bc]	4[b]
	0.04	0[bc]	0[bc]

[a]Chemical analysis showed this material to be nearly pure resveratrol.

[b]Differs significnatly ($P = 0.01$) from the growth of the control.

[c]Fungus failed to grow from inoculum blocks when they were transferred to malt extract agar.

the growth of *P. versicolor* at 0.1%, and was fungicidal since the fungus failed to grow from inoculum blocks transferred to fresh malt extract agar. The ether-soluble fraction existed in the heartwood at about a concn of 0.85%. Therefore these results indicate that both the ether- and water-soluble fractions are toxic in vitro at concns much lower than those in heartwood.

The different fractions of the ether extract from heartwood, mixed at the rate of 1 mg/ml of 3% malt extract solution, and inoculated with either *P. versicolor* or *P. monticola* (Technique B), had different effects. Subfraction F (Table 1) did not affect growth, subfractions L and G reduced growth, and subfraction K was shown to be fungicidal: the fungus failed to grow from inoculum blocks transferred to fresh malt extract agar. The examination of subfractions L and K was repeated with Technique C, and they were found to be fungicidal. Subfractions L and K contained large proportions of resveratrol and the slight differences in toxicity are probably due to impurities.

Using Technique A, the L subfraction (which was present at about 0.32% concn in heartwood) was fungicidal to both fungi in vitro at 0.04% concn (Table 4). However *P. monticola* was inhibited by 0.1% crude heartwood extract (Table 3) which contained a subtoxic 0.003% of fraction L (resveratrol), suggesting that some other factor(s) inhibited the growth of this fungus.

The decay-susceptible sapwood of cottonwood impregnated with appropriate extracts and with resveratrol gave results (Table 5) which differed from those expected from the above observations. As expected, blocks impregnated with the methanol extract (A) or with the water-soluble fraction (C) became more resistant to both fungi. However, blocks impregnated with a 0.4% solution of resveratrol (a fungicidal dose in in vitro tests) decayed at a rate similar to that of the blocks impregnated with

TABLE 5. Weight loss of cottonwood blocks impregnated with various extractives of *Eucalyptus sideroxylon* heartwood exposed to *Polyporus versicolor* or *Poria monticola* for 4 wk at 27 C

Extract	Concn of impregnating solution (%)	Uptake (%)	Weight loss[y] (% dry wt)	
			P. versicolor	*P. monticola*
Methanol extract A[z]	16	17.5	0 a	0 a
	4	7.7	6 b	14 b
	1	2.7	19 c	27 cd
Acetone alone			24 c	29 cd
Resveratrol L	0.4	1.0	20 c	25 c
	0.1	1.1	23 c	29 cd
	0.025	1.1	17 c	27 c
Water-soluble residues	8	11.0	4 b	1 a
	2	3.9	26 c	24 c
	0.5	1.9	21 c	30 cd
	0.1	1.1	27 c	33 de
Water alone			24 c	37 e

[y]Each value is the average of five blocks. Any two values in a single column followed by the same letter are not significantly different ($P = 0.05$ by Duncan's multiple range test).

[z]Letters refer to subfractions in Fig. 1 extraction scheme.

acetone alone. Postexperimental methanol extraction of resveratrol-impregnated blocks not exposed to decay fungi, but otherwise treated similarly, revealed that the test procedure itself had not significantly altered the amount of the resveratrol. The authors have no explanation for the high retention values which resulted when the more dilute impregnating solutions were used.

DISCUSSION.–The results from the in vitro tests using *P. versicolor* and *P. monticola* on *E. sideroxylon* heartwood blocks (Table 2) were characteristic of the natural behavior of this durable wood. The sapwood blocks were considerably less durable.

The retention of durability by the red-colored heartwood blocks after ethanol extraction (Table 2) was probably not due to density (33) but rather to toxic components retained, possibly combined with the cell wall. These compounds could be leucoanthocyanins, ellagitannins, or stilbenes. The resistance to decay of the light-colored sapwood was reduced by extraction, particularly to *P. monticola*. This could have been due to the partial removal of ellagitannins which were detected in the extractives (together with polymerized leucoanthocyanins) but not stilbenes, because these compounds were more toxic to *P. monticola* than to *P. versicolor* (12).

Differences in the growth behavior of the two fungi was shown also by the cultures containing wood extractives (Table 3). The heartwood of *E. sideroxylon* contains at least two groups of compounds (ellagitannins and stilbenes) which show some toxicity to *P. versicolor* and *P. monticola*. The ellagitannins of white oak (*Quercus alba*) have been shown previously to be inhibitory to *P. monticola* (in bioassay tests with or without a wood substrate), and to be stimulatory to *P. versicolor* at a concn of 0.1% (12). Ellagitannins of *E. sideroxylon* (some of which were the same ellagitannins which occur in white oak) had similar properties whether bioassayed with or without a woody substrate (Tables 3 and 5). If the ellagitannin concn was increased enough (e.g., to the 11% level in Table 5), these compounds imparted to the wood resistance to decay by *P. versicolor* and more so to *P. monticola*. As the water-soluble fraction constituted 7.5% of the heartwood of *E. sideroxylon*, sufficient ellagitannins were probably present to inhibit both fungi.

The stilbenes have also been credited as being responsible for natural wood durability (26, 27, 28), although this hypothesis has been questioned (22, 31). When resveratrol was added to 3% malt extract solution at a concn of 0.04% (Table 4), the material was fungicidal. However, when the concn of resveratrol was increased 10-fold to 0.4% (which was approximately equal to its natural concentration in *E. sideroxylon* heartwood) and impregnated into decay-susceptible wood, the durability of the wood was not changed (Table 5). This raised the question of the true role of resveratrol (and possibly other stilbenes) in the durability of wood. Does resveratrol have a significant effect in reducing decay in the tree when bioassay systems using a woody substrate failed to demonstrate this, or were toxicity data obtained in malt extract solutions more indicative of its real role in the tree? Several possible causes are discussed below.

Choice of assay fungi.–*P. versicolor* and *P. monticola* used in the present work can differ in their effect in wood decay. These representatives of white rot (*P. versicolor*) and brown rot (*P. monticola*) fungi produce extracellular enzymes which differ in their mode of action and molecular size and may be stereospecific (39).

The aromatic moieties in lignin and the fungal toxic components differ from carbohydrates in their response to polyphenol oxidase enzyme systems such

as laccase, which is very strongly present in *P. versicolor* but not in *P. monticola* (12). Other enzyme systems, such as detoxification enzymes (24), present or induced, could be different in other microorganisms so that the choice of test fungi could affect the value of in vitro tests in the assessment of durability (32). Also, with in vitro testing the succession of organisms which occurs in the natural decay process does not occur (38), so that results could differ widely from natural behavior.

The use of malt extract as substrate for bioassays.—This type of bioassay has provided evidence that PS and PSME possess fungistatic and fungitoxic properties when a variety of wood decay fungi are used as test organisms (6,11,26,27,28). This toxicity was apparently due to the inactivation of fungal enzymes containing -SH groups in their active sites (23). White rot fungi which synthesized laccase were not inhibited by PSME at high concns (23) whereas nonlaccase-producing brown rot fungi were affected both in malt agar substrate, and in impregnated wood blocks (21).

The most common method of evaluating the toxicity of extractives to wood-destroying fungi has been to determine the effect of the extractive on the development of pure cultures growing in or on a nutrient solution or agar. Such assays are quick and simple but most importantly they allow for the bioassaying of very small quantities of the extractives. However, the dangers of basing conclusions regarding the causes of decay resistance on tests utilizing agar or nutrient solutions, have been stated (2, 10, 22, 30, 34). Comparative tests on wood do not always agree with results obtained from nutrient media. The induction and synthesis of cellulolytic and lignolytic enzymes by wood-decaying fungi differ depending on the substrate. The use of a substrate containing a directly available carbon source (such as agar) reduces or delays the production of cellulase and laccase (22). An additional drawback to the use of nutrient media is that the extractives must possess some water solubility.

The use of impregnated wood as a substrate.—The assay technique which involves the subjection of a nondurable wood impregnated with extractives to test fungi is considered to give a more reliable indication of in vivo behavior than the use of malt extract which contains nutrients which may not exist in the natural substrate. However consideration has not previously been given to the differences between naturally and artificially impregnated wood cell wall.

When heartwood is formed, the vacuoles containing polyphenols and cytoplasmic compounds rupture and come into contact with the cell wall consisting of cellulose microfibrils, lignin, and a gelatinous matrix of the pectinaceous nature and small capillaries. The vacuolar components diffuse into the intrafibrillar spaces, possibly displace moisture and combine with the gelatinous material. Under natural conditions, the extractives could (for example) be partly enzymically changed, by the oxidative enzymes known to be present (37). Methanol extraction does not completely remove the toxic material from *E. sideroxylon* heartwood (Table 2) or other extractives from a number of undried woods (Hillis, *unpublished*). Also, fungal activity appears initially to release the bond PS and flavonoids in the tracheids in the heartwood of *Pinus contorta* var. *latifolia* (21). Recent evidence (5) suggests that dissolution of the S-1 and S-2 layer carbohydrates is caused by diffusion of enzymes from microhyphae in the bore holes in the cell wall and not from hyphae in the lumen, as has generally been assumed.

When wood dries, the colloidal nature of the gelatinous matrix changes irreversibly, and with the loss of water the cell wall shrinks and the capillaries or pore spaces disappear. Soaking wood in swelling solvents such as water or methanol results in partial return to the original dimensions. It appears unlikely that a nondurable dried wood could be made as completely durable as a durable wood by the artificial impregnation of the toxic extractives. The dried wood would not return to its original form, the diffusion of an aqueous extract would differ from that of a methanol extract because the gelatinous matrix is more likely to swell partially in an aqueous medium than it would in an alcoholic medium. A more intimate contact with the carbohydrate substrate would reduce fungal degradation. That ellagitannins convey greater durability to cottonwood blocks than stilbenes, may be due to the much greater water solubility of the former class of compounds, so that it can partly combine with the gelatinous matrix and confer durability.

More significant than the above aspects of the problem is the fact that the reputedly highly toxic stilbene resveratrol conveyed little durability when impregnated into cottonwood blocks (Table 5). Considering the large number of compounds claimed to be toxic to wood-destroying fungi, few attempts have been made to bioassay wood extractives impregnated back into wood (2, 10, 12, 22, 26, 29, 30, 31). Decay-susceptible wood blocks impregnated with 0.7-0.8% PSME decayed at the same rate as unimpregnated blocks by *Fomes annosus* whereas decay by *Lentinus squamosus* was reduced in the treated wood as compared to the untreated (26). Sawdust impregnated with other stilbenes was slightly- or nondurable under attack by several fungi (31). Loman (22) concluded that PS and PSME are probably insignificant causes of toxicity when wood meal is incorporated into the culture medium. Hence four groups (22, 26, 31 and Table 5) reported no (or greatly reduced) toxicity to wood-rotting fungi when various stilbenes were impregnated into some type of woody substrate. Ellagitannins (12), tropolones (2, 30) and some alcohols (10) are inhibitory both in vitro and when impregnated into decay-susceptible wood. However, certain amines (10) gave very different results when tested in agar and in wood.

The circumstantial evidence for stilbenes being responsible for wood durability is so strong that their failure to be toxic when wood is impregnated with them requires close study. The aspects mentioned above may be responsible for the different behavior of stilbenes from the ellagitannins and the tropolones

when impregnated into wooden blocks.

Obviously there are a number of uncertainties in the existing in vitro bioassay techniques. In future work, more attention needs to be given not only to the composition of the medium, but [in view of Loman's work (22)] to the addition of wood meal to the bioassay media. However, because natural wood decay results from sequential, stepwise degradations mediated by a succession of primary microorganisms (38), the use of unaffected wood has its limitations. Also, because wood extractives are usually a complex mixture, the interactions of the components, both toxic and nontoxic, with each other and with fungi during decay need consideration. Additional work on the relative rates at which decay fungi colonize media containing (or not containing) toxic extractives is needed. Colonization will occur as the microorganisms detoxify these compounds and the threshold concentrations at which these compounds begin to influence the rate of colonization will need to be specified.

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