Symposium on Wood Decay

Role of Stilbenes in Resistance of Wood to Decay

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Many mechanisms for wood decay resistance have evolved in trees; one of these is the production of inhibitory compounds produced either before infection when sapwood is transformed to heartwood or in response to injury or pathogen invasion. A role in desease resistance for many pre- or postinfectional antimicrobial substances has been suggested but there are few substantiated examples. In most cases inhibitory properties have been inferred from in vitro bioassays in which in vivo factors were not duplicated.

Stilbenes long have been considered important in heartwood resistance to fungal decay (29) and also have been implicated in the resistance of sapwood to its pathogens (24,28,44). Stilbenes are produced either during heartwood formation or as an active response to infection or injury (phytoalexins). Wood containing stilbenes frequently decompose slowly when exposed to various rot fungi. Over 20 species of the Polyporaceae and several species of the Thelephoraceae and Agaricaceae have been tested on a nutrient agar-stilbene substrate. Such tests consistently have provided evidence that stilbenes possess high fungistatic or fungitoxic properties. However, the fungitoxity of stilbenes bioassayed in a woody substrate, is reduced 90–99%. Which system more accurately represents the nature of stilbenes in vivo?

Stilbenes are diphenylethylenes (Fig. 1) which occur in a number of plant families (9,16,19) and tissue types but are most commonly found in wood, bark, and leaves of forest trees. Almost all stilbenes which occur in wood have a resorcinol-A ring (3,5-dihydroxyl). Substitution of additional hydroxy or methoxy groups commonly have been reported, but glycosides are uncommon.

The objectives of this paper are to evaluate selected methods of bioassaying stilbenes and to review the data for decay prevention activity of three heartwood-stilbene systems (pine-pinosylvin, eucalypt-resveratrol, and osage orange-oxyresveratrol). Emphasis will be given to new data which may explain the conflicting results previously reported.

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Decay of stilbene-containing wood. Before decay resistance can reasonably be attributed to a particular substance, tissue containing the material should be significantly resistant to decay. Removal of the substance(s) from the durable wood should render it highly susceptible to decay. A number of tests have been developed to determine the rate at which certain fungi are capable of decaying wood. Either wood blocks or wood meal may be used; the latter is sometimes mixed with agar or a nutrient solution. Amounts of decay are measured by determining the loss in weight, by the quantity of glucosamine formed, or by respirometry (oxygen uptake or CO_2 evolution). Good correlations have been obtained between field resistance and resistance measured in the laboratory by these methods.

Pinosylvin (PS) and its monomethyl ether (PSME) are frequently postulated to be responsible for the durability of pine heartwood, implying that pine heartwood is durable, which it is not (37). In fact, pine generally is grouped with those woods classed as slightly or unresistant to decay (34,43). Nevertheless, the heartwood is significantly more resistant to decay than is the sapwood (Table 1). The natural concentration of PS plus PSME in pine heartwood varied from approximately 0.001 to 0.02 g/g oven-dry wt (ODW) (10,22,44), although trees with 0.04 g/g ODW have been reported (44).

Within a given pine tree, the levels of naturally occurring PS-PSME correlated reasonably well with decay rates (11). However, exceptions also were reported: When sapwood, outer heartwood, and central heartwood were exposed to *Lentinus lepideus,* all three tissues were attacked equally. *Coniophora puteana* attacked the sapwood and central heartwood equally, but caused significantly less decay of the outer heartwood. *Polyporus vaporarius* and *Merulius lachrymans* caused considerable decay of the sapwood, moderate decay of the central heartwood, and slight decay of the outer heartwood. Outer heartwood contained five to six times more PS-PSME than did the central heartwood. The growth of all four fungi was inhibited by similar concentrations of PS-PSME in an agar medium.

Between-tree comparisons (33) were more variable, some tissues with similar concentrations of PS-PSME had significantly different rates of decay; in other cases, tissues from trees with low levels of PS-PSME were more resistant to decay than were those of other trees that had higher concentrations of PS-PSME. For example, the outer heartwood of one tree (0.021 g PS-PSME per gram ODW) lost 0–5% weight, while the central heartwood (0.011 g PS-PSME per gram ODW) had a 40–55% weight loss when exposed to *Coniophora puteana, Merulius lachrymans,* and *Polyporus vaporarius.* Other trees with lower concentrations of PS-PSME (0.008–0.009 g per gram ODW) in the heartwood were more resistant to these fungi (1–30% weight loss). A similar phenomenon was reported for several blue stain fungi, which caused less discoloration of heartwood containing a lower concentration of PS-PSME than did impregnated sapwood, indicating that other factors in the heartwood must play an important role (32).

The heartwood of many species of *Eucalyptus* is exceedingly resistant to most species of decay fungi under a variety of test conditions (5,14,40,42). Typically, the heartwood lost 1–5% of its weight during 2- to 5-mo exposure periods. Similar treatment of the sapwood caused a 15–60% loss in weight. Resveratrol (3,5,4′-trihydroxystilbene) and trace amounts of 3,5,3′4′-tetrahydroxystilbene and their glucosides occur in the heartwood of a number of eucalypts (17,21,23). The heartwood of one species, *E. sideroxylon,* contained 0.004 g of resveratrol per gram ODW (14).

The heartwood of osage orange (*Maclura pomifera*) is exceptionally durable as shown by its extremely long service life under conditions favorable to decay. Osage orange heartwood exposed to decay fungi under controlled environmental conditions conducive to rot, was very durable (15,45) (Table 2). Barnes and Gerber (3) reported that the heartwood contained approximately 0.01 g/g (ODW) of 3,5,2′4′-tetrahydroxystilbene (oxyresveratrol). Wang (45) found oxyresveratrol and had evidence for the presence of tri- and penta-hydroxystilbenes.

Decay of stilbene-free wood. Blocks of *Pinus taeda* sapwood were extracted with acetone for 115 hr, and exposed for 14 wk to *Fomes annosus* (44); the weight of the blocks decreased 17% compared to 15% for extracted control blocks. In similar tests, the weight of extracted heartwood of *P. taeda* decreased 11% compared to 4% for nonextracted controls. The heartwood contained 4,100 μg PS-PSME per gram ODW before extraction. Extraction removed 6% of the ODW of the wood, but no data were reported for residual quantities of stilbenes. No phenols were detected in the sapwood before or after extraction.

Extraction of blocks of heartwood of *Eucalyptus sideroxylon* with ethanol for 70 hr did not increase susceptibility to decay (14), but the resistance of heartwood sawdust from nine eucalypt species was markedly reduced by ether-methanol extraction (42). Subsequent extraction with alkali caused a further reduction in resistance, but the heartwood sawdust of most species still had significant resistance to decay following extraction.

The decay resistance of osage orange heartwood was greatly reduced by prolonged extraction with various alcohols or similar solvents, although it never became as susceptible to decay as the sapwood (Table 2). Even after extensive extraction with a solvent that removes stilbenes, extracted wood still retained a significant amount of residual stilbene.

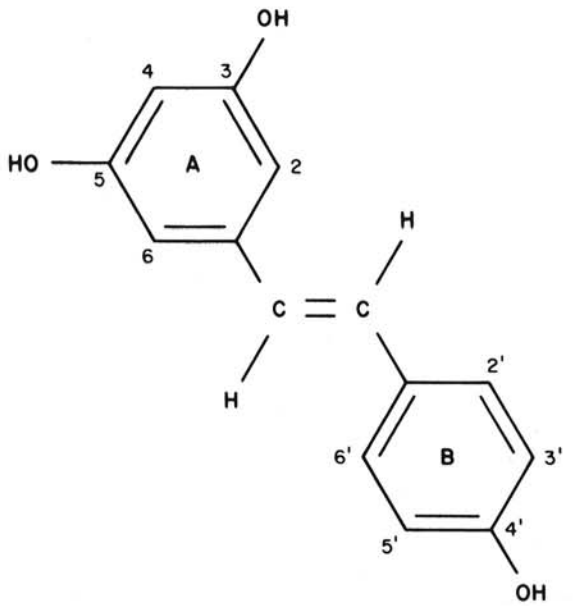

Fig. 1. Chemical structure of resveratrol.

TABLE 1. Decay resistance of three *Pinus* spp.

Fungus	Time of exposure to fungus (wk)	Weight loss of pine wood blocks inoculated with: *P. sylvestris*[a] Sap-wood (%)	*P. sylvestris*[a] Heart-wood (%)	*P. taeda*[b] Sap-wood (%)	*P. taeda*[b] Heart-wood (%)	*P. contorta*[c] Sap-wood (%)	*P. contorta*[c] Heart-wood (%)
Coniophora puteana		68	26				
Lentinus lepideus		42	31				
Poria vaporaria		50	34				
Fomes annosus	14-12			14.5	4	12	5
Cryptoporus volvatus	8					22	6
Fomes pini	12					5	1
Coriolus versicolor	8					22	11
Poria placenta	6					4	4
Europhium clavigerum	12					0.9	0.4

[a] Information from Rennerfelt (34).
[b] Information from Shain (44).
[c] Information from Hart, Shrimpton, and Campana (*unpublished*).

Bioassay of stilbenes in non-woody substrates. An extractable antimicrobial substance should be inhibitory in agar or nutrient solution and the degree of inhibition should be quantitatively related to the concentration of the substance. Measurements of radial growth of mycelium on a solid medium or of fungal dry matter in liquid cultures are commonly used. Such assays are quick and simple but, more important, they permit the bioassaying of very small quantities of the test compound. The preparation of pure stilbenes is expensive. It is, therefore, necessary to use them sparingly. Nutrient solution or agar bioassays are particularly valuable for comparative studies (12).

Some authors have emphasized the dangers of basing conclusions regarding the causes of decay resistance on in vitro tests utilizing agar or nutrient solutions (1,14,26,37,43). Results of tests on wood substrates do not always agree with those obtained from tests on nutrient media.

An additional difficulty affecting the use of stilbenes in malt agar or nutrient solutions is their low water solubility. It is impossible to dissolve stilbenes directly into an aqueous medium. Therefore they are dissolved in absolute alcohol and the ethanolic solution is added to the agar or nutrient solution. This may cause the medium to become turbid but good distribution generally is achieved. An equal concentration of alcohol must be added to the control substrate because 1% ethanol may influence fungal growth (4,31).

Another problem that is unique to assays on agar is that the mycelium frequently grows superficially on the agar surface with minimal contact with the test chemical. Hence, higher amounts (generally twice) of stilbenes are required in agar tests to acheive the same degree of inhibition obtained in nutrient solutions (31).

There is abundant evidence that PS and PSME are fungicidal to a number of wood decay fungi in malt agar or in nutrient solutions (2,4,11,12,26,29,30,31,35,44) (Fig. 2). Normally, PSME is less toxic than PS and the dimethyl ether is essentially nontoxic. Fungal growth was inversely correlated with PS concentration but not with PSME concentration. The amount varied somewhat, depending upon the test method and fungus but, generally, 50 μg PS per milliliter resulted in total fungistasis.

The methanol extractives from the heartwood of several eucalypts are inhibitory to decay fungi when bioassayed in malt agar (14,42). If the extract is fractionated, most of that inhibition can be attributed to the ellagitannins or to resveratrol (14,36,40). Resveratrol is lethal in nutrient solutions at a concentration of 400 μg/ml (14). Pure oxyresveratrol has not been tested against decay fungi in agar or nutrient solution bioassays.

Bioassay of stilbenes in woody substrates. Clearly, if the contribution of one component of the wood to the overall decay resistance is being tested, the preferred substrate is wood (37). When incorporated into a decay-susceptible woody substrate at a concentration equal to that in the resistant wood, it should impart an equivalent level of decay resistance. Subjection of stilbene-impregnated nondurable wood to test fungi is considered to give a more reliable indication of in situ behavior than the use of malt extract, which contains nutrients that may not exist in the natural substrate.

As a test substrate, wood has a number of significant drawbacks. No data exist which show that naturally and artificially impregnated woods contain stilbenes in the same location or in the same chemical form (14). Binding between woody components and stilbenes may differ in naturally and artificially impregnated wood. The amount of stilbene required to impregnate wood blocks is large, although the sawdust-dish technique (6,41) and the wood meal-respirometry technique (45) require much smaller amounts. Finally, a uniform concentration is hard to achieve, at least in solid wood blocks.

The relative amounts of stilbenes needed to inhibit the growth of fungi on agar or in nutrient solutions may differ considerably from the amounts needed to protect wood from the same fungi (14,26,29,38). When either PS or PSME is impregnated into solid wood or sawdust, its inhibition is reduced approximately 50- to

TABLE 2. Weight losses caused in blocks of sapwood, heartwood, and exhaustively extracted wood of *Maclura pomifera* by four wood decay fungi

Fungus	Weight loss[a] (%) caused in blocks of: Heartwood[b]	Sapwood[b]	Extracted[c] heartwood	Extracted[c] sapwood
Poria placenta	0.4	39	16	49
Gleophyllum trabeum	0.2	32	2.5	24
Coriolus versicolor	0.6	35	37	78
Lentinus lepideus	0.4	38	7	56

[a] Information from Wang (45).
[b] Exposed to the fungus for 8 wk.
[c] Exposed to the fungus for 9–16 wk.

TABLE 3. Weight loss of white pine blocks impregnated with an acetone solution of resveratrol and exposed to *Coriolus veriscolor, Poria placenta,* or *Fomes annosus* for 6, 4, or 12 wk, respectively, at 27 C

Treatment	Rate	Weight loss (% dry weight) caused by: *C. versicolor*	*P. placenta*	*F. annosus*
None	···	18.0[a]	26.6	10.1
Acetone alone	···	13.2	26.2	16.4
Resveratrol	250 μg/ml	13.0	23.3	10.4
	1,000 μg/ml	16.1	23.7	11.3
	4,000 μg/ml	13.2	23.8	9.5

[a] Each value is the average of five blocks; means for each fungus do not differ significantly, $P = 0.05$.

TABLE 4. Oxygen consumption by *Coriolus versicolor* or *Gloeophyllum trabea* after 3 wk of incubation in aspen sawdust impregnated with resveratrol in acetone

Treatment	Rate (μg/g)	Oxygen consumption (μl/hr) *C. versicolor*	*G. trabea*
None	···	40[a]	91
Acetone alone	···	44	91
Resveratrol	4	43	57
	8	46	56

[a] Each value is the mean of six determinations.

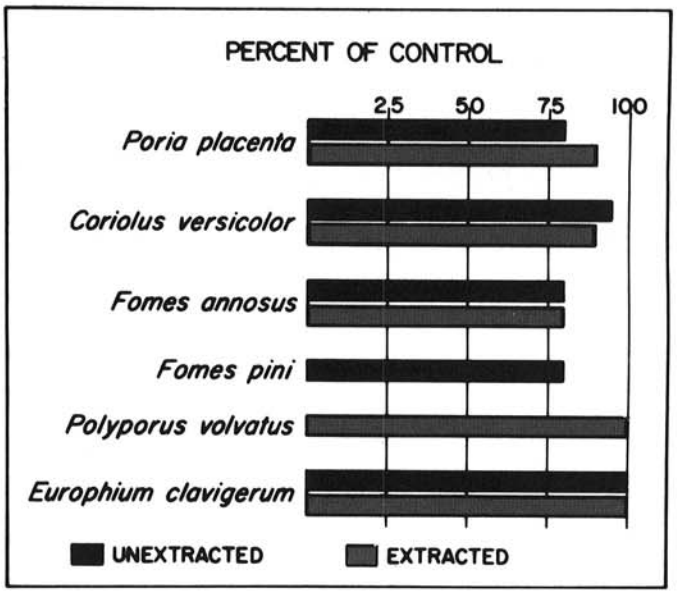

Fig. 2. Radial growth of wood decay fungi on malt extract agar supplemented with lodgepole pine heartwood (HW), pinosylvin (PS), or both substances.

100-fold. If enough (usually over 0.01 g PS-PSME per gram) is impregnated into the wood, significant decreases in decay occur (33).

Coniophora olivacea decayed wood meal treated with 0.0011 or 0.0033 g PSME per gram at the same rate as untreated controls, but treatment with 0.01 g/g prevented decay (39). *C. puteana* was killed by 50 μg PSME per milliliter in malt extract agar (35), but caused a 9.2% weight loss in 4 mo in pine sapwood blocks impregnated with 0.028 g PSME per gram (33). *Lentinus lepideus* failed to grow on malt extract agar containing 0.00005 g PSME per milliliter (35), but decayed wood meal impregnated with 0.0033 g/ml at the same rate as untreated meal (39). Blocks of pine sapwood infiltrated with 0.012 g PSME per gram or 0.03 g PSME per gram and exposed to *L. lepideus* had weight losses of 14.1 or 6.8%, respectively (33). Untreated sapwood lost 45% of its weight in the same experiment. Acetone-extracted pine heartwood lost 40% of its weight after exposure to *L. lepideus* for 3 mo (29). The same tissue impregnated to contain 0.0075 g PSME per gram lost 14.2% of its weight. However, *Fomes annosus* caused similar weight losses of both substrates.

The ameliorating effect of wood on the fungitoxic property of PS and PSME also has been demonstrated in malt extract agar (26, Fig. 2) or in nutrient solutions (Hart, Shrimpton, and Campana *unpublished*). Ten percent extracted or nonextracted heartwood reduced by approximately tenfold the fungitoxicity of PS or of an acetone extract of *P. contorta* heartwood. Even as little as 1% unextracted pine heartwood essentially negated the toxicity of PS in nutrient solutions. The addition of 1% cellulose failed to reduce the toxicity of 50 μg PS per milliliter to *Coriolus versicolor* in malt extract broth.

While 400 μg of resveratrol per milliliter of nutrient solution is lethal, blocks of cottonwood (14) or of white pine (Table 3) impregnated with up to 4,000 μg resveratrol per gram were not protected from three decay fungi. By using oxygen consumption as a measure of fungal growth in impregnated wood meal, 8,000 μg resveratrol per milliliter did not inhibit *Coriolus versicolor,* but significantly reduced oxygen uptake by *Gloeophyllum trabea* (Table 4). However, colonization of the treated samples by both fungi was identical to untreated samples as measured by visual examination.

Few tests on the toxicity of pure oxyresveratrol impregnated into woody substrate have been conducted because the compound has not been available. The available data indicate that concentrations similar to those reported in osage orange heartwood will allow fungal growth much in excess of that observed in heartwood. Oxyresveratrol (0.01 g/ml) failed to inhibit four wood decay fungi when incorporated into sawdust of *Eucalyptus regnans* (38). Growth of three decay fungi on cellulose filter paper treated to retain 0.024 g/g of a stilbene fraction obtained from osage orange heartwood was one-fourth that on untreated paper (45).

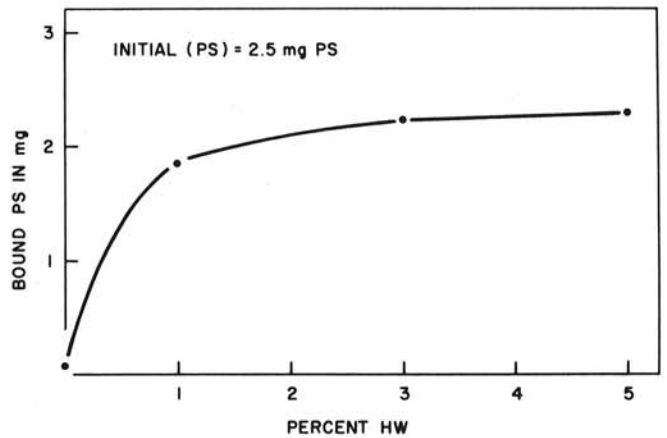

Fig. 3. Binding of pinosylvin (PS) by lodgepole pine sawdust after 16 hr in malt extract broth.

Binding of stilbenes to woody substrates. The previous sections have shown that the relative amounts of stilbenes needed to inhibit the growth of fungi on agar or in nutrient solutions are much lower than those needed to keep the same fungi from attacking decay-susceptible woody substrates into which stilbenes are impregnated. Why is the toxicity of stilbenes so markedly affected by the type of bioassay system utilized? We have information that may help to answer this question.

First, a brief review of biosynthesis of stilbenes is needed. Evidence of several types supports the conclusion that heartwood stilbenes (as well as other secondary metabolites) are formed in situ in dying parenchyma cells at the heartwood-sapwood boundary (20). The location of the stilbenes after formation is unknown; they may be in the cell lumens or in cell walls but probably do not occur as free compounds. Most likely they enter the wall of the cell following death and combine with other wall components (eg lignin). Stilbene monomers are known to be minor constituents of some condensed tannins (13,18) and hence may be bound to the wall components. The evidence supporting this hypothesis is that stilbenes which are soluble in ether cannot be extracted from wood with ether (7,8,18), nor can they be completely removed from finely ground wood following extensive extraction with methanol or acetone (14,42,45). These are significant differences between stilbenes and most other wood extractives. Knowledge of the precise location of stilbenes in natural and in impregnated wood would help to understand their role in decay and disease resistance.

In agar or nutrient broth, it is reasonable to assume that the stilbenes are not bound to other constituents in the medium and hence are free to interact with the fungus. As mentioned, the addition of heartwood or extractive-free heartwood, but not cellulose or the extractives themselves, to nutrient broth containing PS greatly reduced its toxicity to a number of wood decay fungi. The possibility that the PS binds with the wood under assay conditions and is thereby inactivated was studied with the aid of ^{14}C-PS specifically labeled at one of the bridge carbon atoms. Heartwood, even when it comprised only 1% of the medium, effectively bound most of the available PS (Fig. 3). The wood-PS linkages effectively remove much of the PS from the broth and thus lower the observed toxicity. Cellulose was ineffective as a binding agent. Preliminary experiments showed that lignin will bind PS, but not as effectively as does heartwood (Hart, Shrimpton, and Campana, *unpublished*).

We have additional information pertinent to this hypothesis gained by calculating the amount of unbound ^{14}C-PS in the broth medium when different concentrations of heartwood were added.

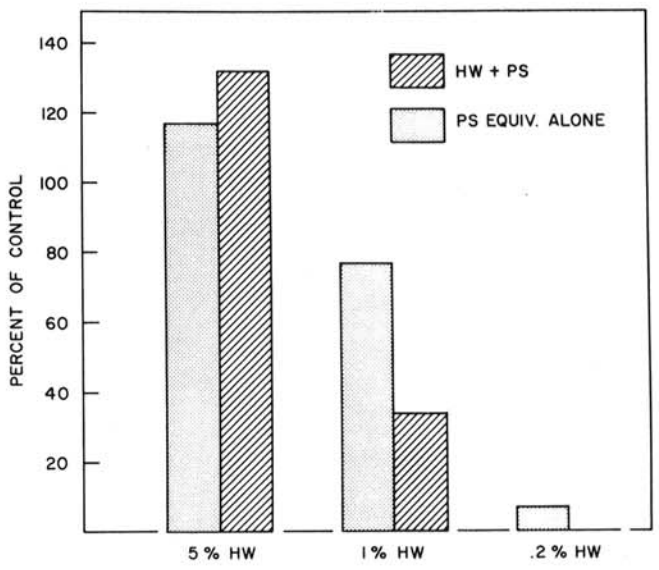

Fig. 4. Growth of *Coriolus versicolor* in malt extract broth containing pinosylvin (PS) or pinosylvin plus lodgepole pine heartwood (HW).

C. versicolor was then grown in broth culture containing PS and varying amounts (0.002–0.05 g) of heartwood per milliliter, or in cultures containing PS at concentrations equivalent to the values calculated for the unbound PS at each heartwood concentration. The results (Fig. 4) suggest that similar concentrations of unbound PS result in similar growth rates of *C. versicolor,* but additional data are necessary with other fungi to confirm this observation.

While it appears that PS-wood linkages may play a major role in reducing the toxicity of PS in various woody substrates, other possibilities have been suggested. It may be that in a nutrient-rich medium stilbenes acquire toxic properties that are not possessed by them in wood. In broth culture, decay fungi may produce enzymes, that are capable of converting stilbenes to transient fungitoxic compounds or to polymers. These oxidation products, which may be more toxic than the stilbenes themselves, could be responsible for the observed inhibition (27). In wood, such a phenomenon may not occur because of low oxygen tension in the heartwood, or enzymes may be induced which metabolize stilbenes to nontoxic compounds. Both the time interval for enzyme production and the amount of extra-cellular enzymes produced are substrate-dependent.

CONCLUSIONS

A correlation exists between the presence of stilbenes in wood and high decay resistance. The hypothesis that the stilbenes, which are known to be antibiotics, may be the cause of such decay resistance cannot be discarded lightly. What is not known is whether the results obtained from bioassays with or without woody substrates are more indicative of the role of naturally occurring stilbenes in wood. Since results obtained with woody substrates show much reduced fungitoxicity in comparison to tests in nutrient media and generally are in closer agreement with the in situ behavior of the compound, the role for stilbenes in decay resistance is still in doubt.

The likelihood of in situ interaction between decay fungi and stilbenes can be questioned, primarily because the compounds are insoluble in aqueous media, and also may be compartmentalized ("membrane effect") or chemically bound to other wood components. That stilbene-containing wood often has a rich microflora, (even of stilbene-sensitive species) strengthens this contention. However, certain fungi can cause increases in the concentration of PS after incubation in heartwood-nutrient broth cultures, while other fungi can cause a rapid decrease in PS concentration (25). These observations suggest that wood-inhabiting fungi and stilbenes do come into direct contact. Nevertheless, the residual stilbene in wood following exposure to decay fungi to determine if the compounds are no longer present in substantial amounts.

Wood extract is a complex mixture; the interaction of the components, both toxic and nontoxic, with each other and with fungi during decay, needs consideration. Evidence is accumulating that decay resistance is a multifunctional phenomenon and it may be impossible to confer on one substance out of the entire heartwood sole responsibility for resistance. Very likely the compounds act synergistically. It is axiomatic that all in situ events cannot be duplicated by any bioassay either with or without a woody substrate. Testing compounds singly, even in wood, may yield very misleading data.

Really critical experimentation has yet to be conducted. One important, and as yet unanswered, question is whether stilbene-containing woods retain resistance if exposed to decay fungi totally insensitive to stilbenes. Or, to ask the question another way, would the heartwood of a mutant osage orange whose heartwood failed to contain stilbenes decay rapidly? Until additional information becomes available, caution is urged in assigning a major role to stilbenes in wood decay resistance.

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