Phenolic Extractives in Norway Spruce and Their Effects on Fomes annosus

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ABSTRACT

Lignans in sound and *Fomes annosus*-affected tissues of Norway spruce were analysed by gas liquid chromatography. Hydroxymatairesinol, matairesinol, liovil, and conidendrin were identified in sound heartwood, and the *reaction zone* separating sound sapwood from wood incipiently decayed by *F. annosus*. The *reaction zone* contained up to 6% hydroxymatairesinol on a dry weight basis; this was considerably more hydroxymatairesinol than was found in the heartwood. Sound sapwood and wood in advanced stages of decay contained negligible quantities of lignans. Hydroxymatairesinol was significantly more inhibitory to *F. annosus* than was matairesinol or conidendrin in vitro. It is proposed that hydroxymatairesinol in association with alkalinity in the *reaction zone* contributes to the resistance of the sapwood to *F. annosus* in vivo. Phytopathology 61:841-845.

Additional key words: Picea abies, host resistance.

Fomes annosus (Fr.) Karst. causes an extensive heartrot in Norway spruce (*Picea abies* [L.] Karst.). Invasion of living sapwood, however, appears to be limited by the accumulation of inhibitory substances in a *reaction zone* (12). This tissue was produced in sapwood during necrosis of parenchyma as a host response in advance of fungal invasion. *Reaction zone* sap was fungistatic to *F. annosus,* whereas the fungus grew readily on sap expressed from associated tissues. High pH of *reaction zone* sap (ca. pH 8.0) could have contributed to the observed fungistasis. *Reaction zone* sap, however, buffered at pH 5.6 also was fungistatic, which suggested additional causes for fungal inhibition. The *reaction zone* also was significantly more decay resistant than was either heartwood or sapwood (12). An accumulation of phenolic extractives was observed in the *reaction zone.*

The purpose of this investigation was to determine (i) the composition of extractives in sound and affected tissues of Norway spruce; and (ii) if any of the extractives in the *reaction zone* were inhibitory to *F. annosus* at concentrations found in vivo.

MATERIALS AND METHODS.—*Chemical analyses.*—Analyses were conducted on the sapwood and heartwood of one uninfected tree and on the *reaction zone,* usually with associated tissues, of four trees infected by *F. annosus.* All trees originated in the vicinity of Vollebekk, Norway.

Lignans were isolated from methanol extracts of individual tissues by separation on thick layers of Silica Gel-GF 254 using toluene: methyl ethyl ketone (3:2, v/v). They were identified by chromatographic separation with different solvent systems, chromogenic sprays, ultraviolet absorption spectra and, in some cases, mixed mp and infrared spectra. The solvent systems used for thin-layer (Silica Gel-GF 254) chromatography and R_F values of individual lignans were reported previously (8). Butanol:acetic acid:water (6:1:2, v/v) followed by 6% acetic acid were used for developing two-dimensional paper chromatograms. Compounds were detected by spraying chromatograms with diazotized *p*-nitroaniline or sulphanilic acid, or by their appearance under ultraviolet light.

Quantitative estimates of lignans present in individual tissues were made by gas liquid chromatography (8). Tangential sections (30-40 μ thick) weighing ca. 11 mg were placed in small tubes (5 mm × 5 cm) and crushed with a glass rod. After addition of distilled, water-free pyridine (100 μliters), the tubes were tightly corked and stored in a desiccator over P_2O_5 with occasional shaking for 60-120 hr. Ten μliters of TMS mixture [hexamethyldisilazane: trimethylchlorosilane: pyridine (2:1:10, v/v)] and 10 μliters of BSA [N,O-bis (tri-methylsilyl) acetamide] were successively added to 10 μliters of thoroughly mixed pyridine extract of spruce tissue. After 15 min, 5 μliters of this mixture was injected into a Varian 2100 gas chromatograph with flame ionization detectors. The glass, U-shaped column packed with 2.0% Apiezon L on 80-100 mesh DMCS chromosorb-W was 2 m long, with an inside diameter of 3 mm. The oven temperature was 220 C, and detector and injection temperatures were 250 C. Flow rates of carrier gas (helium), hydrogen, and air were 40, 25, and 250 ml/min, respectively. Calibration curves for hydroxymatairesinol indicated a linear response over the range of this compound detected in wood. Retention times of purified silylated lignans were used as markers for relative retention times (RRT) of lignans relative to hydroxymatairesinol. Two or more analyses were made of each sample. Results of five analyses on the same sample over a 3-month period varied within 24% of the mean. This accuracy was considered acceptable in view of losses expected from sectioning and natural variability of the small samples used.

Bioassays of spruce lignans.—Three lignans (hydroxymatairesinol, matairesinol, and conidendrin) found in heartwood and *reaction zone* were investigated

to determine their effects on linear growth of *F. annosus*. Autoclaved malt extract (1.25%) agar (1%) was cooled to 50 C, then added to a mixture of lignan and absolute ethanol. Three concentrations (0.1, 0.2, 0.4%, w/v) of each lignan in malt extract agar containing a final concentration of 1% ethanol were prepared. The control medium consisted of malt extract agar containing 1% ethanol. All mixtures were dispersed by sonication (Biosonic III, Bronwill Scientific) for 15 sec with 300 w at 20,000 cycle/sec. Limited quantities of some lignans necessitated the development of a bioassay utilizing small amounts of these compounds. Accordingly, 1.5 ml of medium were pipetted aseptically into sterilized growth tubes prepared from 15- × 1.5-cm test tubes. Tubes were inoculated with discs (5 mm in diam) of mycelium and malt extract agar taken from the periphery of 8-day-old colonies of *F. annosus* isolates 10257 (from *Pinus caribaea* Morelet) and 67/11 (from *P. abies*). Two replicate tubes of each lignan-containing medium and three replicate tubes of the control medium were inoculated with each fungus isolate. Inoculated tubes were incubated in the dark at 25 ± 1 C, and measurements of linear growth of mycelium in each tube were made at periodic intervals up to 7 days. An analysis of variance was calculated for the resulting data. Initial and final pH values of media were determined (E.I.L. pH meter).

RESULTS.—*Chemical analyses.*—Qualitative comparisons of extracts from various host tissues by paper and thin-layer chromatography were substantiated and quantified by gas liquid chromatography (Fig. 1, Table 1). Of the phenolic compounds previously identified in the heartwood of Norway spruce (2), matairesinol (Fig. 2-A), hydroxymatairesinol (Fig. 2-B), conidendrin (Fig. 2-C), and liovil (Fig. 2-D) and, possibly, oxomatairesinol were identified in the present study. Although the *reaction zone* and heartwood were similar qualitatively in lignan content, the former contained from 4 to 28 times more hydroxymatairesinol than did the latter. A blue-fluorescent compound appeared to be more prevalent in the *reaction zone* than elsewhere. This compound had an ultraviolet absorption spectrum typical of spruce lignans; it did not react with either 2% $KMnO_4$ solution or dinitrophenylhydrazine; it was unstable in the presence of hydrochloric acid; it had an R_F value 0.05 lower than hydroxymatairesinol in 6% acetic acid, but in contrast to the latter it was pink when sprayed with diazotized sulphanilic acid. These results suggest that the compound is oxomatairesinol, which is an auto-oxidation product of hydroxymatairesinol (3). Oxomatairesinol and conidendrin (both with RRT = 1.66), liovil (RRT = 0.69), matairesinol (RRT = 1.26), and a lignan (RRT = 0.81) were detectable in all *reaction zones* examined; the last lignan was a substantial component (1.14% of dry wt) of the *reaction zone* of tree C (Table 1).

Lignans in sapwood were fewer in number and quantity than were those in *reaction zone* or heartwood (Fig. 1). The narrow transition zone between sapwood and *reaction zone* had a lignan composition more similar to the latter. Lignans were almost entirely absent

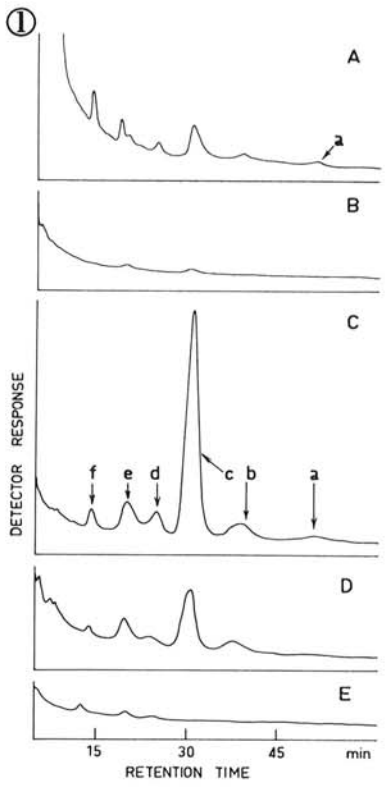

Fig. 1-2. **1)** Gas liquid chromatographs of sound and *Fomes annosus*-affected tissues of Norway spruce. **A)** Sound heartwood from tree E. **B)** Incipiently decayed wood from tree B. **C)** *Reaction zone* (middle portion) from tree B. **D)** Transition zone from tree B. **E)** Sapwood from tree B. Peak a = conidendrin and oxomatairesinol; b = matairesinol; c = hydroxymatairesinol; e = liovil; d and f are unknown. **2)** Structural formulae of lignans. **A)** matairesinol, **B)** hydroxymatairesinol, **C)** conidendrin, **D)** liovil.

TABLE 1. Gas liquid chromatography of extractives from sound and *Fomes annosus*-affected tissues of Norway spruce

Tree	Tissue	Relative retention time[a]								
		0.58	0.61	0.69	0.81	0.96	**1.00**	1.16	1.26	1.66
		%[b]	%	%	%	%	%	%	%	%
A	Sapwood									
	Outer Late wood	0.01	0.02	*	Tr[c]	0.01	*	*	*	*
	Early wood	*	*	*	0.01	0.03	*	Tr	*	*
	Middle Late wood	0.01	0.01	*	0.01	0.05	*	Tr	*	*
	Early wood	0.07	0.07	*	0.04	0.13	*	0.07	*	*
	Inner Late wood	*	*	*	0.06	0.05	*	*	*	*
	Reaction zone									
	Outer Late wood	*	0.08	0.18	0.07	*	**2.21**	*	0.04	Tr
	Early wood	*	0.34	0.54	0.27	*	**5.99**	*	0.30	Tr
	Middle Late wood	*	0.14	*	0.08	*	**0.75**	*	0.02	Tr
	Early wood	*	0.12	0.21	0.12	*	**2.61**	*	0.02	Tr
	Inner Late wood	*	*	*	0.11	*	**3.91**	*	*	Tr
	Early wood	*	*	*	*	*	**4.79**	*	*	Tr
	Incipiently decayed wood									
	Early wood	*	*	*	0.03	*	**0.02**	*	0.01	*
B	Sapwood									
	Outer	*	*	0.04	0.01	*	*	*	*	*
	Middle	*	*	0.04	0.01	*	*	*	*	*
	Inner	*	*	0.04	0.02	*	*	*	*	*
	Transition zone	*	*	0.12	0.04	*	**0.35**	*	0.08	*
	Reaction zone									
	Outer	*	*	0.21	0.13	*	**2.00**	*	0.15	Tr
	Middle	*	*	0.21	0.13	*	**1.48**	*	0.15	Tr
	Inner	*	*	0.25	0.22	*	**1.66**	*	0.13	Tr
	Decayed wood									
	Outer	*	*	0.04	*	*	**0.03**	*	*	*
	Middle	*	*	0.05	0.03	*	*	*	0.02	*
	Inner	*	*	0.01	0.01	*	**0.01**	*	0.01	*
C	Sapwood	*	*	*	0.01	*	*	*	*	*
	Transition zone	0.26	*	*	*	*	**0.45**	*	*	*
	Reaction zone									
	Outer	0.17	*	0.28	0.17	*	**1.42**	*	0.08	*
	Middle	*	*	0.67	1.14	*	**1.45**	*	0.24	0.39
	Inner	*	*	*	*	*	**1.34**	*	0.21	0.41
	Decayed wood	*	*	0.01	*	*	**0.03**	*	*	*
D	*Reaction zone*	*	*	0.11	0.12	*	**2.94**	*	0.07	0.03
E	Sapwood	*	*	0.01	0.01	*	*	*	*	*
	Heartwood	*	0.07	0.03	0.04	*	**0.21**	*	0.02	Tr

[a] Relative retention time: 0.69 represents liovil; **1.00,** hydroxymatairesinol; 1.26, matairesinol; and 1.66, conidendrin and possibly oxomatairesinol; others are unknown.
[b] Average dry wt for 2-5 determinations; * = not detectable with the methods used.
[c] Trace.

from the extractives of decayed wood. Traces of hydroxymatairesinol were detected in incipiently decayed wood, but this compound was not detectable in wood where decay was more advanced.

Early and late wood of the same tissue had a similar lignan composition, but early wood contained more lignans on a dry wt basis. This can partially be explained by the higher specific gravity of late wood, which contains up to 3 times as much wood substance/unit volume than does early wood (7).

Bioassays of spruce lignans.—Bioassay tests using *F. annosus* isolate 67/11 are summarized in Table 2. Conidendrin was not inhibitory at any of the concentrations tested, whereas matairesinol and hydroxymatairesinol significantly inhibited mycelial growth. The effect of concentration was not significant for matairesinol, but was highly significant (1% level) for hydroxymatairesinol, which was more inhibitory (1% level) than the former at all concentrations tested. These results were further supported by preliminary tests. Similar results were obtained with *F. annosus* isolate 10257; however, greater variability occurred within treatments and controls with this isolate. Even so, analysis of pooled data for both isolates yielded the same statistical result. Owing to shortage of some lignans, further testing was not possible. Initial and final pH values of all media (Table 2) were within the range suitable for growth of the fungus (9).

DISCUSSION.—The most conspicuous feature of the lignan content of host tissues is the striking increase of hydroxymatairesinol in the *reaction zone* (Fig. 2). It is perhaps very significant that this compound also was the most inhibitory of the lignans bioassayed against *F. annosus*. The dosage-response relationship (40% reduction in growth at a concentration of 0.4% suspended in an agar medium) was not particularly impressive by antibiotic standards. The concentration of hydroxymatairesinol in an early wood band of a *reac-*

TABLE 2. Inhibition of linear growth of *Fomes annosus* isolate 67/11 by different concentrations of lignans and pH of media before and after 7 days of growth

Lignan	Concn	Mycelial growth	Inhibition	pH of media	
				Initial	Final
	%	*mm*	%		
Conidendrin	0.1	55.0	−2	6.5	5.9
	0.2	53.5	1	6.0	5.9
	0.4	54.0	0	5.2	5.9
Matairesinol	0.1	49.0**[b]	9	6.0	5.8
	0.2	51.5*	4	5.8	5.8
	0.4	50.0**	7	5.4	5.8
Hydroxymatairesinol	0.1	40.5**	25	[a]	[a]
	0.2	38.0**	30	6.3	5.6
	0.4	32.0**	40	5.8	5.5
	Control	54.0	0	6.1	5.3

[a] Data not available.

[b] * = Growth mean significantly different from that of controls at 5% probability level. ** = Growth mean significantly different from that of controls at 1% probability level.

tion zone, however, was up to 15 times greater than that in this bioassay. Discrete bands containing high concentration of this lignan may play an important role in impeding fungal invasion. These bands may only be revealed by intraincrement analyses rather than by averaging larger samples. Furthermore, hydroxymatairesinol is more soluble in aqueous media than is either conidendrin or matairesinol, and hence could be present in *reaction zone* sap in quantities sufficient to impede fungal growth. Indeed, ultraviolet absorption spectra and thin-layer chromatograms indicated that filter-sterilized *reaction zone* sap [which was fungistatic (12)] contained as much as 0.5% soluble lignan (w/v), most of which was hydroxymatairesinol. From the foregoing, it seems likely that hydroxymatairesinol in association with alkalinity (12) plays an active role in the development of disease resistance in vivo.

Solubility in aqueous media is a factor which has received little attention in evaluating fungal inhibitors for disease resistance. It follows that a compound with relatively low aqueous solubility (e.g., pinosylvin) will have to be active at lower concentrations than one with relatively high aqueous solubility (e.g., hydroxymatairesinol). In addition, greater aqueous solubility usually would ensure a more uniform distribution of inhibitor in a protective tissue. Other factors which should be taken into account are the absolute amounts of inhibitor present and its rate of destruction by the pathogen. The last two could give an indication of the rate of spread of nonobligate parasites through necrotic *reaction zones.*

Matairesinol, which may convey some natural durability to heartwood of *Podocarpus* sp. (10), probably is not important in the resistance of spruce to *F. annosus.* This is because inhibition of the fungus, even though statistically significant in the bioassay, was only slight at levels present in the *reaction zone.*

In contrast to the *reaction zone,* where normal heartwood lignans accumulate, a different mixture of lignans was found in wound resin after cambial injury. This mixture included (+)-pinoresinol, (+)-epipinoresinol, (+)-isolariciresinol, and secoisolariciresinol (13). It would be of interest to determine if the last three lignans, which are not found in heartwood, are antifungal. The contrast in lignans produced in response to injury by differentiated parenchyma in a *reaction zone* and differentiating tissue in the cambial region probably reflects the greater metabolic versatility of the latter.

The *reaction zone* of *Pinus taeda* also was characterized by the accumulation of normal heartwood phenols. In the early stages of *F. annosus* infection, the proportion of the most fungitoxic of these extractives, pinosylvin, was about 3 times that found in heartwood (11). Pinosylvin also accumulated in *Pinus radiata* around lesions caused by the *Sirex-Amylostereum* complex (5). The pattern of phenol accumulation during the response of sapwood of pine and spruce, therefore, appears to be a disproportionate mobilization of a normal, antifungal heartwood constituent.

Phenols not normally present in heartwood sometimes accumulate in the sapwood of other tree species in response to infection. For example, the lignan, isoolivil, and the coumarin, scopoletin, accumulated in sapwood of *Prunus jamasakura* and *Prunus domestica* (4, 6), respectively, after fungal attack. Neither of these compounds was detectable in sound heartwood. The production of new antifungal compounds by herbaceous angiosperms in response to infection is well documented (1).

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